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KINETIC MODELS FOR PHYCOCYANIN PRODUCTION BY FED-BATCH CULTIVATION OF THE *SPIRULINA PLATENSIS*

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ABSTRACT: C-Phycocyanin (CPC) is high-value bioproduct, generated in blue-green algae *Spirulina platensis*. Its wide application in different industries, widely used in food pharmaceuticals and cosmetics. There are many factors that influence the yields of *Spirulina platensis* cultivation, such as temperature, light intensity, pH, nutrient etc. Nitrogen is one of the most important factors in cell growth and pigment productivity. The fed-batch process is a strategy to control the growth and enhance CPC accumulation. In this study, *Spirulina platensis* was cultured in batch and fed-batch modes to investigate the CPC production. Kinetic model of growth and production on nitrate concentration so as CPC accumulation. On batch, cultivation found that increasing nitrogen source concentration led to increased CPC accumulation. The best CPC concentration (2.258 $mg \cdot mL^{-1}$) occurred when $NaNO_3$ was 3.5 $g \cdot L^{-1}$. The fed-batch cultivation proved to be an effective strategy to further enhance the CPC production of Spirulina platensis. The results indicate that the maximum CPC production (4.354 $g \cdot L^{-1}$) and productivity (97.53 $mg \cdot L^{-1} \cdot d^{-1}$) was obtained when using fed-batch with the NH_4HCO_3 3.0 mM. A kinetic model to describe the *Spirulina platensis* culture system including cell growth, CPC formation, as well as nitrogen consumption was proposed. The data fitted the model well. This was in good agreement with the experimental results and could be employed to predict the production of biomass, phycocyanin and the consumption of nitrogen in culture.

Keywords: *Spirulina platensis*, Phycocyanin, Fed-batch cultivation, Kinetic models

1. INTRODUCTION

Spirulina platensis (*Arthospira platensis*) is one of the widely cultured commercial microalgae that can provide raw materials for food, pharmaceuticals, animal feed, bioenergy. The chemical composition of *Spirulina platensis* indicated that it has high nutritional value due to its content of a wide range of essential nutrients, such as provitamins, minerals, proteins and polyunsaturated fatty acids such as gamma-linolenic acid (GLA) [1]. It is known as a "super food". The United States Food and Drug Administration confirmed in 1981 that spirulina is a source of protein and contains various vitamins and minerals, moreover, it may be legally marketed as a food supplement. Many countries have set up food quality and safety standards for spirulina.

There are many factors that influence the yields of *Spirulina platensis* cultivation, such as temperature, light intensity, pH, nutrient etc. Nitrogen is one of the most important factors in cell growth and pigment productivity. Colla *et al.*, (2007) [2] reported nitrogen source important for growth and accumulation of nutrients in the cells of *Spirulina platensis*. Nitrogen is involved in the formation process of essential components such as amino acids, chlorophyll, nucleic acid amylase, especially protein with up to 60-70% of dry weight. Protein in Spirulina contains mainly two phycobiliproteins namely C-phycocyanin (CPC) and allophycocyanin (APC) approximately a ratio of 10:1 [3]. The supply of nitrogen source in the medium is a fundamental requisite to cultivate *Spirulina platensis* and ammonium salt is also shown to be particularly effective not only to produce biomass but also to exalt its CPC content.

Nutrient-rich medium is used to ensure high growth rate of the microalgae cells, by switching to a different composition to induce the nutrient stress. This can be achieved by the fed-batch techniques. The fed-batch process is an important strategy because it makes possible to control the growth and the CPC accumulation phased by modifying the feed throughout the cultivation process. The current study aimed to investigate the CPC production by comparing between culturing in batch and fed-batch and study kinetic model of growth and production on nitrate concentration as CPC accumulation was also established in this work.

2. MATERIALS AND METHODS

2.1 Operation of Batch Cultivation

The batch cultivation was conducted in 2 L of the bottle. The microalgae were pre-cultured and

inoculated in the bottle, the medium used for strain culture was adapted from Zarrouk medium consisting of (per liter): 16 g $NaHCO_3$, 2.5 g $NaNO_3$, 1 g NaCl , 0.5 g K_2HPO_4 , 0.2 g $MgSO_4$ and study difference four level of sodium nitrate (1.5 2.5 3.5 and 4.5 g·L^{-1}). The initial biomass was maintained as 0.37 g·L^{-1}. The culture was controlled at 28 - 30 ºC, pH 9-10 (Algae Connect, Model ALS-SPARC-2A, USA), and LED illumination (BASTVA, Model GW-AQM55W, China) with the ratio of red and blue as 3:1 with lighting period of 16 hours per day at 350 µmol·m^{-2}·s^{-1} [4]. During the cultivation, the CO_2 either pure from the tank was bubbled into the bottle to control the pH at 9.0-9.5 as the carbon source. (Fig 1). During cultivation liquid samples were collected at set time intervals to determine the cell concentration, CPC concentration and residual nitrogen source concentration.

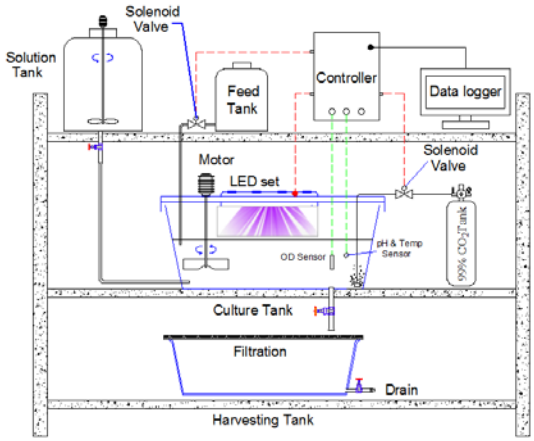

Fig 1. Operation of batch and fed-batch cultivation

2.2 Operation of Fed-batch Cultivation

Three type ammonium salts were selected for nitrogen sources in *Spirulina platensis* cultivation. Three levels in each salt are containing the range of high and low medium (1.0 2.0 and 3.0 mM). By mean of 1.0 mM is minimum concentration which *Spirulina platensis* use for living and 3.0 mM is the concentration at toxicity phenomena in cultivation [5]. All treatment was carried out in 2 L of the bottle which was controlled the medium of culture (per liter) that include: 16 g $NaHCO_3$, 2.5 g $NaNO_3$, 1 g NaCl , 0.5 g K_2HPO_4 , 0.2 g $MgSO_4$. The initial biomass was maintained as 0.37 g·L^{-1}. The culture condition was controlled at 28 - 30 ºC, pH 9-10 and LED illumination with the ratio of red and blue as 3:1 with lighting period of 16 hours per day at 350 µmol·m^{-2}·s^{-1}. For fed-batch cultivation, time regulator was set up for pulse-feeding nitrogen source during the experiment. Liquid samples were collected at set time intervals to determine the cell concentration, CPC concentration and residual nitrogen source concentration**.**

The nitrite concentration was measured by the chronotropic acid method [6]. The biomass concentration was determined by measuring the optical density of the sample at a wavelength of 680 nm (denoted as OD_{680}) using a UV/Vis spectrophotometer (Model SPECTROSC, USA). The OD_{680} values were converted to wet biomass concentration (the moisture content in the wet basis is 83.59 ±0.66 %) via appropriate calibration between OD_{680} and cell weight.

$$W=5.8667\times OD_{680}-2.5563 \quad (1)$$

The specific growth rate of Spirulina culture was obtained by the following calculation.

$$\mu=\frac{\ln(W/W_0)}{t} \quad (2)$$

Where W and W_0 indicate the biomass concentration (g·L^{-1}) at initial and cultivation time (days:d), respectively. The biomass productivity during the culture period was calculated from the Eq. (3).

$$P_w=\frac{\Delta W}{\Delta t} \quad (3)$$

2.3 Determination of C-phycocyanin Content

A fixed amount of the biomass (5 g) was mixed with 25 ml of 0.1 M sodium phosphate buffer (pH = 7.0), then keep in freezing condition (-10 ºC) for overnight and thawing next in room temperature. After that, the ultrasound-assisted extractions based on research of Ruangyot *et al.* (2016) [7] was used as the extraction method. The cell debris was removed by centrifugation at 3500 rpm for 30 minutes, and the supernatant (blue color) was collected to CPC analysis. The supernatant (crude extract) was measured the absorbance by using UV/Vis spectrophotometer at the wavelengths of 620 nm and 652 nm for calculating the concentration of CPC (g·L^{-1}) according to the following Eq. (4) [8]. The content of CPC (%) was calculated according to Boussiba and Richmond (1979) [9] following Eq. (5). The yield (mg·g^{-1}) and productivity of CPC (g·L^{-1}·d^{-1}) according to the following Eq. (6) and (7)

$$C_{CPC}=\frac{A_{620}-0.474A_{652}}{5.34} \quad (4)$$

$$\%CPC=\frac{A_{620}\times V\times 100}{3.39\times W\times \%DW} \quad (5)$$

$$Y_{CPC}=\frac{CPC\times V}{D} \quad (6)$$

$$P_{CPC}=\mu\times W\times \%CPC \quad (7)$$

2.4 Kinetic Model Development

Monod model and Haldane model are widely used for describing the effect of substrate concentration (C_s) on specific growth rate (μ), Eq. (8) and Eq. (9).

$$\mu=\mu_m\left(\frac{C_S}{C_N+C_S}\right) \quad (8)$$

$$\mu=\mu_m\left(\frac{C_S}{C_N+C_S+\frac{C_S^2}{C_I}}\right) \quad (9)$$

Where C_N and C_I are optimal substrate concentration and inhibit concentration at the maximum specific growth rate. In this study, the kinetic model is modified from the Monod model and the Haldane model for simulating the kinetic parameters of *Spirulina platensis* cultivation namely specific growth rate (μ) and CPC productivity (P_{CPC}). In these equations, the maximum growth rate constant (μ_m) and maximum CPC productivity constant ($P_{CPC,m}$) are assumed to be a function of nitrate concentration (C_{S1}) and ammonium concentration (C_{S2}) as shown in Eq. (10) and Eq. (11). Furthermore, in fed-batch cultivation, the kinetic model would be considered to account the substrate inhibition of growth at higher substrate concentrations as shown in Eq. (12) and Eq. (13).

$$\mu=\mu_m\left(\frac{C_{S1}}{C_{N1}+C_{S1}}\right)\left(\frac{C_{S2}}{C_{N2}+C_{S2}}\right) \quad (10)$$

$$P_{CPC}=P_{CPC,m}\left(\frac{C_{S1}}{C_{N1}+C_{S1}}\right)\left(\frac{C_{S2}}{C_{N2}+C_{S2}}\right) \quad (11)$$

$$\mu=\mu_m\left(\frac{C_{S1}}{C_{N1}+C_{S1}+\frac{C_{S1}^2}{C_{I1}}}\right)\left(\frac{C_{S2}}{C_{N2}+C_{S2}+\frac{C_{S2}^2}{C_{I2}}}\right) \quad (12)$$

$$\mu_{CPC}=P_{CPC,m}\left(\frac{C_{S1}}{C_{N1}+C_{S1}+\frac{C_{S1}^2}{C_{I1}}}\right)\left(\frac{C_{S2}}{C_{N2}+C_{S2}+\frac{C_{S2}^2}{C_{I2}}}\right) \quad (13)$$

3. RESULTS AND DISCUSSION

3.1 Effect of Sodium Nitrate on Cell Growth and CPC Production

Nitrogen source is an important factor that affects the viability and productivity of microalgae in batch cultivation. The final *Spirulina platensis* biomass in different sodium nitrate concentration was given in Fig. 2. The result shows that a rapid increase on biomass production was observed in medium containing 3.5 g·L^{-1} sodium nitrate that was presented the maximum biomass concentration (4.859 g·L^{-1}). At 3.5 g·L^{-1} sodium nitrate was presented the maximum biomass concentration, both CPC productivity and significantly increased when the concentration of sodium nitrate was increased from 1.5-3.5 g·L^{-1} (Table 1). However, a sharp decrease was observed when the concentration of sodium nitrate reached 4.5 g·L^{-1}, which is this level fall inhibition region. This could be directly supported by the observation of changing cells color from green to white during cultivation interval the first five days. The growth rate began to decline in the third day. Thus, 3.5 g·L^{-1} sodium nitrate seemed to be the optimal concentration of sodium nitrate for the growth and CPC accumulation of spirulina platensis, with the maximum biomass productivity of 0.314 g·L^{-1}·d^{-1}, the specific growth rate of 0.169 d^{-1}, CPC productivity of 44.59 mg·L^{-1}·d^{-1} and 14.20% of yield.

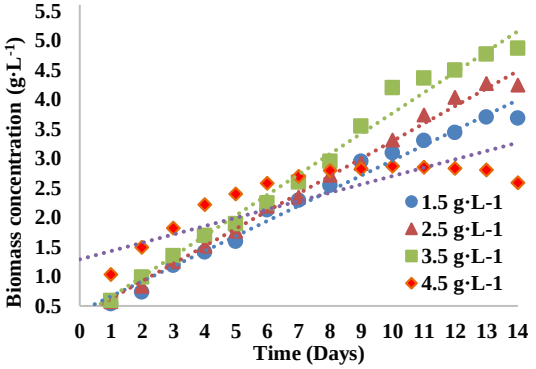

Fig 2. The effect of sodium nitrate concentration on cell growth

3.2 Time Course Performance on Cell Growth and CPC Production

The cellular component of microalgae usually varies with the cell growth phase. In this work, spirulina was cultivated in a batch culture around 14 days to investigate variation in CPC production (Fig 3). The CPC concentration increased simultaneously along with nitrogen consumption and the maximum values (2.840 mg·mL^{-1}) was obtained at the beginning of nitrogen depletion. This trend is well in agreement with the report of Chen *et al.* (2013) [10]. It has been suggested that CPC belongs to a family of phycobiliproteins which have obtained a secondary role as intracellular nitrogen storage compounds and mobilized for other purposes in times of nitrogen shortage. Therefore, in order to attain the maximum CPC concentration, the beginning of nitrogen depletion period should be the optimal time for adding a nitrogen source to cultivate and enhance the cell growth rate and CPC concentration. Thus, it is necessary to develop an effective strategy that could enhance biomass production and achieve the high CPC concentration simultaneously.

Table 1. The effect of sodium nitrate concentration on kinetic parameters of *Spirulina platensis* cultivation.

Nitrate concentration ($g \cdot L^{-1}$)	W ($g \cdot L^{-1}$)	P_X ($g \cdot L^{-1} \cdot d^{-1}$)	μ (d^{-1})	P_{CPC} ($mg \cdot L^{-1} \cdot d^{-1}$)	% CPC
1.5	3.692	0.231	0.151	25.55	11.06
2.5	4.237	0.272	0.163	30.71	11.29
3.5	4.859	0.314	0.169	44.59	14.20
4.5	2.865	0.154	0.128	8.79	5.71

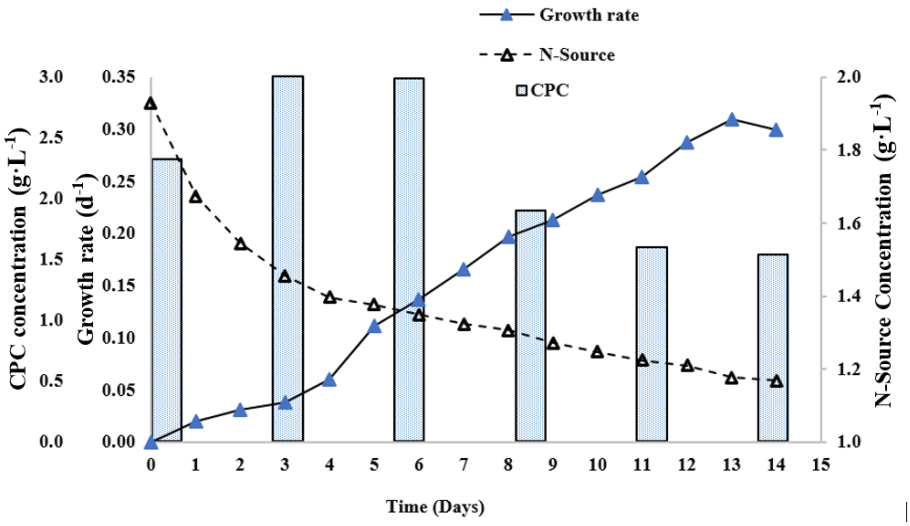

Fig.3 Time-course profiles of growth rate (empty symbol), nitrate concentration (full symbol) and CPC concentration (column) during the batch cultivation of *Spirulina platensis*.

3.3 Improvement of CPC Production of *Spirulina Platensis* by Using Fed-Batch Operation

On the basis of batch culture results, different pulse-feeding fed-batch protocols were investigated with the aim of increasing the total availability of the supplied nitrogen source (N-source) as well as avoiding the above inhibitory level. All experiment was carried out using $NaNO_3$ because it was particularly important as nitrogen source at the beginning of the cultivation. Then, the concentrated ammonium solution was used as N-source pulse-feeding every day to reach a concentration in the medium including 1, 2 and 3 mM. Ammonia is preferentially assimilating over nitrate because the above mention is described it is favorable nutrient of *Spirulina platensis* in term of the energy situation. The results of the fed-batch process were shown in Fig. 4 and Table 2.

Spirulina platensis responses were varied with different ammonium solution. The biomass production and productivity of *Spirulina platensis* were increased significantly when increasing the NH_4HCO_3 concentration. Since nitrogen was required for synthesis of the amino acid, which was used to making proteins up. Additionally, increasing of nitrogen concentration might because of an increase in protein biosynthesis. The maximum biomass concentration and growth rate were obtained in the culture of pulse-fed with 3 mM NH_4HCO_3, but a decrease in both was observed when increasing the NH_4Cl and $(NH_4)_2SO_4$ concentration (2 and 3 mM) indicating that the inhibition phenomena of growth were appeared by the high level of ammonium.

Moreover, to investigate the performance of the fed-batch operation, four strategies were defined for reducing times of feeding and increase the concentration of a solution. For the time-course profiles of growth rate, four strategies were defined namely: pulse-fed with 3 NH_4HCO_3 every day; 3 NH_4HCO_3 every two days; 6 mM NH_4HCO_3 every day and 6 mM NH_4HCO_3 every two days, respectively (Fig 5A-5D). While the growth rate in fed-batch with 3 mM NH_4HCO_3 every day (1.187 d^{-1}) was slightly higher than 6 mM NH_4HCO_3 every two days feeding (1.143 d^{-1}). However, the growth rate decreased in case of fed-batch with 6 mM NH_4HCO_3 every day indicating that this level is over toxicity limit.

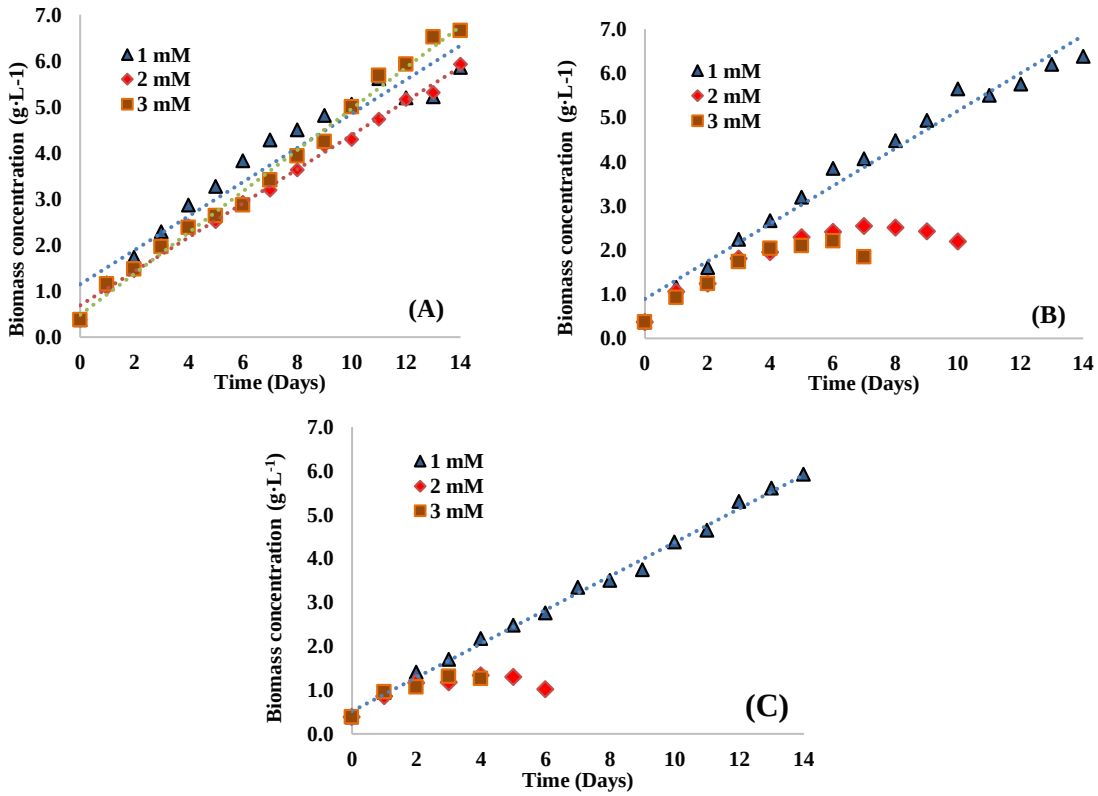

Fig.4 Biomass concentration of fed-batch cultivation of *Spirulina platensis* by pulse-feeding every day (A) Ammonium bicarbonate (NH_4HCO_3) (B) Ammonium Chloride (NH_4Cl) and (C) Ammonium sulfate $(NH_4)_2SO_4$

Table 2. The effect of ammonium concentration on kinetic parameters of fed-batch cultivation of *Spirulina platensis*.

Ammonia concentration (mM)		W ($g \cdot L^{-1}$)	P_x ($g \cdot L^{-1} \cdot d^{-1}$)	μ (d^{-1})	P_{CPC} ($mg \cdot L^{-1} \cdot d^{-1}$)	% CPC
NH_4HCO_3	1.0	5.857	0.391	0.196	176.7	17.67
	2.0	5.927	0.396	0.197	192.1	19.21
	3.0	6.649	0.448	0.205	217.7	21.77
NH_4Cl	1.0	6.385	0.429	0.202	150.6	15.06
	2.0	2.548	0.210	0.176	123.9	12.39
	3.0	2.207	0.182	0.168	n/a	n/a
$(NH_4)_2SO_4$	1.0	5.915	0.396	0.197	113.2	11.32
	2.0	1.327	0.219	0.164	n/a	n/a
	3.0	1.304	0.106	0.101	n/a	n/a

n/a is cannot found because of ammonium inhibition during culture.

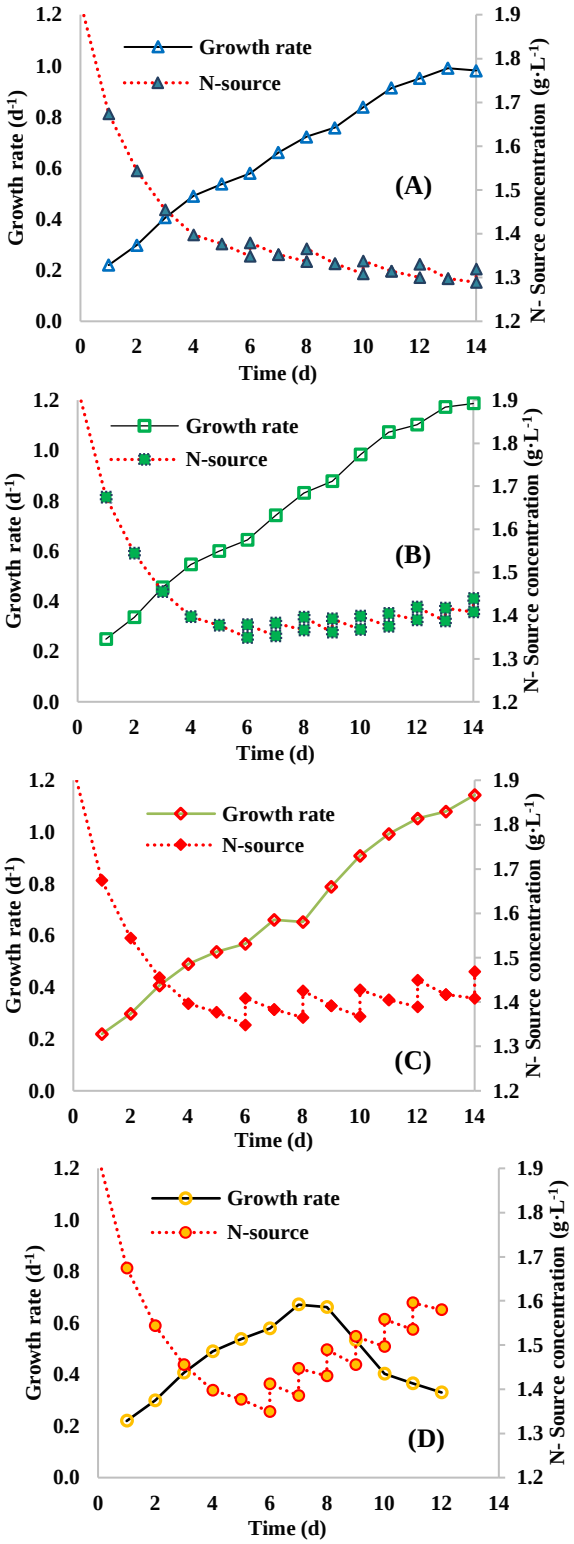

Fig.5 Time-course profiles of growth rate(empty symbol), N-source concentration (full symbol) during the culture fed-batch with (A) 3 mM NH_4HCO_3 every two days (B) 3 mM NH_4HCO_3 everyday (C) 6 mM NH_4HCO_3 every two days (D) 6 mM NH_4HCO_3 everyday.

Fig. 6 show that the CPC concentration of *Spirulina platensis* was increased with the prolonged time of fed-batch operation (3 mM NH_4HCO_3 every day). This CPC concentration was higher than that obtained in the batch culture with sodium nitrate concentration 3.5 $g \cdot L^{-1}$ around 53% (Table 1). This result was indicated that adjusting the feeding ammonium concentration could enhance the accumulation of CPC, and fed-batch cultivation with the NH_4HCO_3 of 3.0 mM seemed to be a feasible strategy for enhancing CPC accumulation.

Table 3 was present the comparison of the performance of biomass production, biomass productivity, CPC production, CPC productivity between this result and the literature. The biomass production and biomass productivity from 3 mM NH_4HCO_3 feeding was higher than other study and indicated that biomass production not only requires nitrogen sufficient condition but also need other nutrients. However, The CPC production obtained from 3 mM NH_4HCO_3 feeding was enhanced around 75% from reported in previous literature.

3.3 Kinetic Model for CPC Production

The changes in biomass concentration and CPC production with cultivation time in batch and fed-batch cultivations were plotted in Fig. 3 and Fig. 6, then the experimental data were fitted by the regression tool as a function of non-linear regression in statistic program, the following kinetic models were established in this work given as the following equation:

Batch cultivation

$$\mu = 0.600\left(\frac{C_{S1}}{0.414+C_{S1}}\right)\left(\frac{C_{S2}}{0.056+C_{S2}}\right) \quad r^2 = 0.938 \quad (14)$$

$$P_{CPC} = 0.335\left(\frac{C_{S1}}{0.413+C_{S1}}\right)\left(\frac{C_{S2}}{0.055+C_{S2}}\right) \quad r^2 = 0.937 \quad (15)$$

Fed-Batch cultivation

$$\mu = 0.400\left(\frac{C_{S1}}{0.414+C_{S1}+\frac{C_{S1}^2}{13{,}885.6}}\right)\left(\frac{C_{S2}}{0.056+C_{S2}+\frac{C_{S2}^2}{0.190}}\right) \quad r^2 = 0.992 \quad (16)$$

$$P_{CPC} = 0.223\left(\frac{C_{S1}}{0.413+C_{S1}+\frac{C_{S1}^2}{17{,}234.4}}\right)\left(\frac{C_{S2}}{0.055+C_{S2}+\frac{C_{S2}^2}{0.188}}\right) \quad r^2 = 0.993 \quad (17)$$

The experimental data fitted the model quite well (r^2 =0.937-0.992). The maximum growth rate constant (μ_m) and maximum CPC growth rate constant ($P_{CPC,m}$) in fed-batch was higher than batch cultivation. The concentration of nitrate (C_{N1}) and concentration of ammonium (C_{N2}) are 0.41 and 0.05 $g \cdot L^{-1}$, respectively.

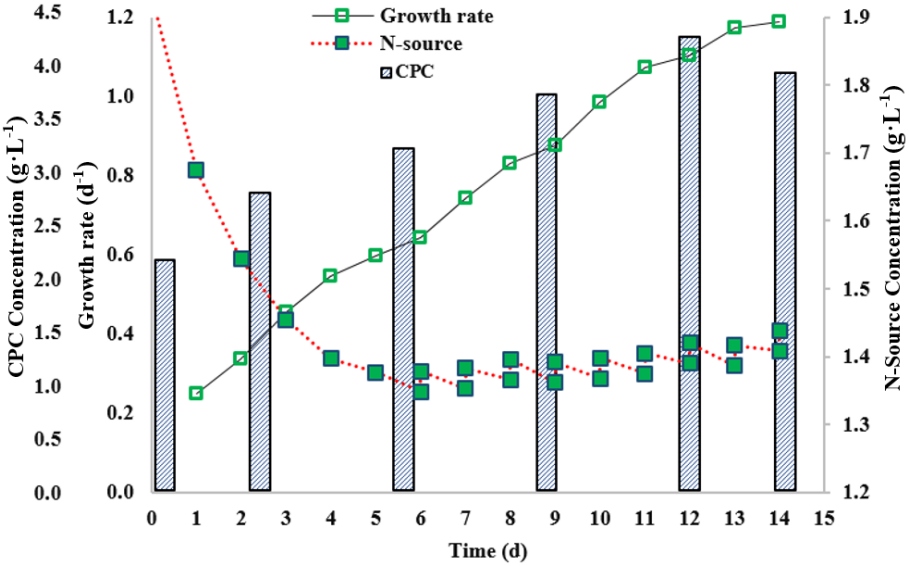

Fig.6 Time-course profiles of growth rate (empty symbol), N-source concentration (full symbol) and CPC concentration (column) during the fed-batch with 3 mM NH_4HCO_3.

Table 3. Comparison of kinetic parameters under fed-batch operation from this work with those reported in the literature.

Operation strategies	Biomass production ($g \cdot L^{-1}$)	Biomass productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	CPC productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	Reference	
Batch	0.770	92.4	67	Sassano *et al*., 2007	[11]
Batch	2.250	740.0	125	Chen *et al*., 2013	[10]
Batch	3.114	n/a	14	Leema *et al*, 2010	[12]
Fed-batch	1.759	113.9	n/a	Nascimento *et al.*, 2014	[1]
Fed-batch	6.780	588.2	94	Xie et al., 2015	[13]
Fed-batch	6.649	448.0	98	This study	

This result was an illustration of *Spirulina platensis* cultivation that refers about the nitrogen source was not only show an adverse impact on growth rate, the productivity of Spirulina platensis but also significantly in term of increasing the CPC formation. For nitrate inhibition on growth, the value of nitrate inhibit (C_{I1}) is 17,234.4 $g \cdot L^{-1}$, which means that when the nitrate concentration was lower than the result, it would not occur the inhibition on the growth rate and CPC growth rate. Moreover, the value of ammonium inhibition (C_{I2}) was 0.188 $g \cdot L^{-1}$, indicating that the amount of ammonium concentration should be lower than this value and it could use as the optimal inhibition ammonium concentration for fed-batch cultivation.

In commercial scale cultivation, the controlling is well relative for establishing "Smart Farm" culture modeling, production controlling and feed monitoring that is invariable underpinning by sensors and sensor networks. In a control system, a controller unit would record continuously such as the culture temperature, pH, and biomass growth by the sensor. This information of cultivating was continuously transferred to the computer data logger where the data would be analyzed, displayed and recorded.

$$\frac{d(C_{NN})}{dt} = (0.615\text{-}2.030\ P_{CPC})\, C_{S1} C_{S2} \quad r^2 = 0.902 \qquad (18)$$

The kinetic model in fed-batch cultivation Eq. (16) was used for estimated nitrogen consumption (C_{NN}) according to the relationship between the CPC growth rate and N-source concentration as shown in Eq. (18). When the nitrogen consumption exceeded the pre-set value, the nutrients feeding system was activated for a predefined period, so that the nitrogen source feeding solution could be fed into the culture. This system was verified by comparison with the constant feeding method. When the constant feeding was used, owing to the amount of ammonium fed being larger than of the cell growth demanded, the ammonium in the medium accumulated to a certain concentration to inhibit cell growth.

4. CONCLUSIONS

The cultivation of microalgae *Spirulina platensis* was feasible using ammonium salt such as ammonium bicarbonate (NH_4HCO_3) ammonium chloride (NH_4Cl) and ammonium sulphate ($(NH_4)_2SO_4$). The application of ammonium salt as nitrogen source presented several advantages. Besides being a cheaper source compared to the traditional ones (nitrates), it was readily assimilated by the microorganism, without any expenditure of energy. Ammonia toxicity could be circumvented by the application of the fed-batch process with exponentially-increasing feeding rates. The fed-batch cultivation with ammonium bicarbonate feeding was proved to be an effective method to further enhance the CPC production, giving the maximum biomass production (6.649 $g \cdot L^{-1}$) and productivity (0.448 $g \cdot L^{-1} \cdot d^{-1}$). Further studies on the extraction and purification method by using high technology such as ultrasonic microbubble temperature differential etc.

5. ACKNOWLEDGMENTS

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