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An Optical-Density-Based Feedback Feeding Method for Ammonium Concentration Control in *Spirulina platensis* Cultivation

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Cultivation of *Spirulina platensis* using ammonium salts or wastewater containing ammonium as alternative nitrogen sources is considered as a commercial way to reduce the production cost. In this research, by analyzing the relationship between biomass production and ammonium-N consumption in the fed-batch culture of *Spirulina platensis* using ammonium bicarbonate as a nitrogen nutrient source, an online adaptive control strategy based on optical density (OD) measurements for controlling ammonium feeding was presented. The ammonium concentration was successfully controlled between the cell growth inhibitory and limiting concentrations using this OD-based feedback feeding method. As a result, the maximum biomass concentration (2.98 g/l), productivity (0.237 g/l·d), nitrogen-to-cell conversion factor (7.32 gX/gN), and contents of protein (64.1%) and chlorophyll (13.4 mg/g) obtained by using the OD-based feedback feeding method were higher than those using the constant and variable feeding methods. The OD-based feedback feeding method could be recognized as an applicable way to control ammonium feeding and a benefit for *Spirulina platensis* cultivations.

Keywords: Optical-density-based feedback, ammonium bicarbonate, *Spirulina platensis*, feeding methods, fed-batch cultivation

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Microalgae are capable of utilizing light and carbon dioxide to synthesize organic substances, such as proteins, carbohydrates, pigments, essential fatty acids, vitamins, and antioxidants [9]. *Spirulina platensis* is one of the widely cultured commercial microalgae that can provide raw materials for food, pharmaceuticals, animal feed, and bioenergy [1, 30] owing to its peculiar properties such as high nutritional value [12] and ease of harvest [29].

Nitrogen is an essential microalgae nutrient that affects the biomass synthesis rate and biomass composition [11, 15, 24]. In most commercial *Spirulina platensis* cultivations, nitrate salts were used as the nitrogen source because it could ensure high growth rate with easy operation [14]. As up to 25% of the microalgae production cost was related to the culture medium [2], and nitrogen was the second abundant element in *Spirulina platensis* biomass, using ammonium salts or wastewater containing ammonium as alternative nitrogen sources for *Spirulina platensis* production was attractive from the economic viewpoint [8, 34]. Furthermore, the negative effect of using nitrate salts, generating high salinity effluents, could be prevented [26].

The main reason to restrict using ammonium salts in *Spirulina paltensis* cultivations was that *Spirulina platensis* only grows satisfactorily in medium with an optimum ammonium concentration. Growth inhibition or even cell death would take place when the concentration of ammonium was at a relatively high level [3]. A fed-batch process is a good option to solve this problem, which could avoid nutrient sources inhibition and ensure high biomass productivity [6, 7]. In previous reports, traditional feeding methods such as constant [5] and variable feeding methods [7, 10, 29] were employed in *Spirulina platensis* cultivations for carbon dioxide, glucose, and ammonium salts addition. However, in most of the studies, the nutrients were fed in an open-loop, feed-forward control scheme in which nutrients were added according to historical date or predicted growth [16, 29], and the nutrient concentrations could not be controlled accurately because such strategies are not robust and may easily be affected by unplanned process perturbation [29]. As all the direct online ammonium analyzer technology required sample taking, chemical reagents addition, and calibration and cleaning cycles [22], it is complex to detect and control the ammonium concentration online by an online ammonium analyzer. An ammonium

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control system based on indirect feeding parameters such as pH, dissolved oxygen, and cell concentration is preferred to be used to regulate the feed rate of the nutrients automatically.

Optical density (OD) is one of the most important parameters in *Spirulina platensis* cultivation. Measuring the OD of cell growth is useful for quantifying various culture parameters such as biomass concentration and production [21, 27]. In this study, an indirect online feeding method based on online OD measurement was introduced to control ammonium concentration at an expected level in *Spirulina platensis* cultivations. The advantage of this method was investigated to avoid the inhibition effect of ammonium and to improve biomass production.

Materials and Methods

Microorganism and Cultivation Conditions

Spirulina platensis was obtained from Yantai University (Yantai, China). The inoculum was grown in Zarrouk medium [35] at 30°C, shaking at 100 rpm for 72 h. Continuous light intensity was kept at 100 μmol/m^2·s by means of twelve 30 W fluorescent lamps and an illuminance meter (FGH-1; Photoelectric Instrument Factory of Beijing Normal University, China). The biomass that was prepared for subsequent cultivation was collected by filtration and washed by nitrogen-free Zarrouk medium to remove sodium nitrate.

Fed-batch cultivations were carried out in a 2.5 L closed airlift column photobioreactor that contained 2 L of culture medium. The initial cell concentration was set at 0.15 g/l. The culture temperature was set at 30 ± 0.5°C and the pH value was kept at 9.6 ± 0.1 by pH feedback-controlled CO_2 feeding at a rate of 0.02 L/min. Sterile air at a flux of 0.4 L/min was used to mix the culture medium. Continuous light intensity was kept at the value of 100 μmol/m^2·s. The cultivation volume was maintained through the addition of distilled water and nitrogen source feeding solution.

Online OD-Based Fed-Batch Cultivation Control System

The schematic diagram of the OD-based fed-batch cultivation control system is shown in Fig. 1. A controller unit continuously recorded the culture temperature and pH values measured by sensors submersed in the photobioreactor. In order to minimize the interferences from air flow, biomass growth was monitored online by an OD probe (GX-2; Nanning ADS Co. Ltd., China) with an optical path length of 1 cm at 650 nm in a measurement chamber where the sample could be degassed. The data of culture temperature, pH, and OD were continuously transferred through the local area network (LAN) to a computer where the data were analyzed, displayed, and recorded using LabView 8.6 software (National Instruments, USA). The OD value was converted to the biomass concentration according to the relationship [Eq. (3)] between the OD value and the biomass concentration by the software program in LabView. The accuracy of the OD probe showed a maximum error of approximately 5% of the total biomass concentration.

A feeding system was proposed to realize real-time control of the ammonium concentration in the culture based on the online measured OD values. At the initial stage of the cultivation, the OD value of the cell suspension was recorded as OD_0. Every 5 min, the controller program calculated the increment of biomass concentration (ΔX) according to the difference between the OD_0 and real-time OD value measured online (OD_t). When the ΔX exceeded the preset value, the nutrients feeding pump (BQ 50-1J; Longer Pump, China) was activated for a predefined period, so that the nitrogen source feeding solution could be fed into the photobioreactor. At the same time, the value of OD_0 was substituted by the OD value at the feeding time (OD_{t1}). Next time, the controller program calculated the ΔX between the online OD (OD_t) and OD_{t1}. When the ΔX

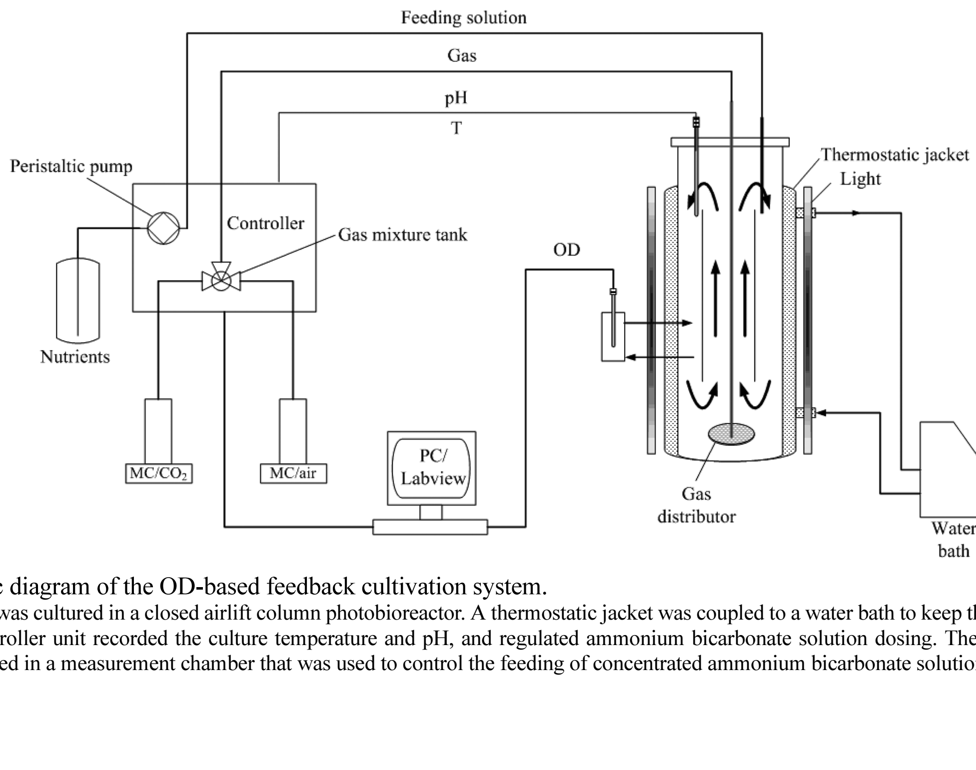

Fig. 1. Schematic diagram of the OD-based feedback cultivation system.

Spirulina platensis was cultured in a closed airlift column photobioreactor. A thermostatic jacket was coupled to a water bath to keep the culture temperature at 30 ± 1°C. A controller unit recorded the culture temperature and pH, and regulated ammonium bicarbonate solution dosing. The optical density (OD) values were measured in a measurement chamber that was used to control the feeding of concentrated ammonium bicarbonate solution.

Table 1. The cultivation results of different feeding methods.

Feeding method	Feeding protocol	X_m (g/l)	P_X (g/l·d)	$Y_{X/N}$ (gX/gN)	Protein (%)	Chlorophyll (mg/g)
Manual feeding	$F_N = 2\text{-}C_N$ [a]	3.06 ± 0.08	0.242 ± 0.007	7.16	63.1 ± 2.3	13.1 ± 0.9
Constant feeding	$F_N = 1.26$ [b]	1.17 ± 0.08	0.172 ± 0.013 [d]	5.54	- [e]	- [e]
Variable feeding	$F_N = -0.0208t^2 + 0.2211t + 0.8786$ [b]	2.60 ± 0.10	0.205 ± 0.008	6.35	60.3 ± 1.2	11.5 ± 0.6
OD-based feedback feeding	$F_N = 1.00$ [c]	2.98 ± 0.11	0.237 ± 0.009	7.32	64.1 ± 1.8	13.4 ± 1.1

[a] Runs were performed by adding ammonium bicarbonate according to the residual ammonium concentration analyzed every 0.5 day, where F_N (mmol/l) is the ammonium bicarbonate feeding concentration per time, C_N (mmol/l) is the residual ammonium concentration in the culture medium.
[b] Runs were performed by adding ammonium bicarbonate according to the feeding protocol every 0.5 day; t(d) is the cultivation time.
[c] Runs were performed by adding ammonium bicarbonate based on ΔX, which was calculated according to the equation $\Delta X = 0.0688\Delta OD^2 + 0.4977\Delta OD$, where ΔX (g/l) is the increment of biomass concentration and ΔOD is the increment of OD value of cell suspension. The feeding was activated per ΔX = 0.1 g/l.
[d] P_X (g/l·d) was calculated using the data of 0–6 days.
[e] Considering the death and disintegration of *Spirulina platensis* occurred because of ammonium inhibition, the biomass composition was not determined.

exceeded the preset value, the nutrients feeding controller was activated again. The nutrients feeding procedure was repeated until the end of the feeding.

Ammonium Feeding

In all the experiments, 0.25 mol/l ammonium bicarbonate was used as nitrogen source feeding solution.

According to the proper initial ammonium concentration (0.56–1.1 mmol/l) [29] and the inhibitory ammonium level (2 mmol/l) [7] of *Spirulina platensis* reported previously, a manual control fed-batch cultivation (Table 1) was carried out to establish different feeding protocols. The initial ammonium bicarbonate concentration was set at 1 mmol/l and the nitrogen feeding solution was fed manually every 0.5 day based on the residual ammonium concentration measured offline in the culture medium. The residual ammonium concentration of the medium was maintained in the range of 0.5–2 mmol/l.

From the curve of ammonium-N consumption versus time, it was possible to calculate the amount of ammonium that should be added daily to sustain the expected cell growth. The constant protocol (Table 1) was carried out by derivation of the equation, which was obtained by describing the curve using linear fit. The variable protocol (Table 1) was carried out by derivation of the equation, which was obtained by describing the curve using polynomial fit. During the experiments carried out with the constant and variable feeding protocols, the nitrogen source feeding solution was pulse-fed every 0.5 day.

From the curve of ammonium-N consumption versus biomass accumulation, the ratio of ammonium added to biomass could be obtained. Because the OD value could be converted to the biomass concentration according to a calibration curve, an OD-based feedback feeding protocol was carried out (Table 1) on the basis of the relationship between the ammonium-N consumption and biomass production. The nitrogen source (0.25 mol/l ammonium bicarbonate solution) feeding was coupled to the nutrient feeding system based on the OD that would be activated for a constant time when the increment of biomass concentration (ΔX) exceeded 0.1 g/l (Table 1).

Analytical Methods

Offline biomass concentrations were determined by measuring the OD of the microalgae suspension at 560 nm using a spectrophotometer (723N; Shanghai Precision&Scientific Instrument Co. Ltd., China) [19]. The OD values were converted to biomass concentration according to a pre-established calibration curve [33]. The concentration of ammonium was measured by an ammonium electrode (A-501; Jiangfen, China). The biomass was collected and washed three times with distilled water. Then the protein and chlorophyll contents were determined according to Lowry *et al*. [20] and Bennett and Bogorad [4], respectively. Every experiment was repeated three times, and the experimental errors were less than 5%.

Parameters Calculation

The cell productivity (P_X, g/l·d) was calculated as the ratio of the total amount of cells produced per volume to the cultivation time, as shown in Eq. (1):

$$P_X = \frac{X_m - X_i}{t} \quad (1)$$

where the X_m and X_i were the maximum and initial biomass concentration (g/l), respectively. The t (d) was the cultivation time.

The biomass yield based on nitrogen ($Y_{X/N}$, gX/gN) was calculated as the ratio of the total amount of biomass produced to the total nitrogen added (N_t, g) as shown in Eq. (2):

$$Y_{X/N} = \frac{X_m - X_i}{N_t/V} \quad (2)$$

where the V (L) was the cultivation volume.

RESULTS AND DISCUSSION

Interrelationships Between the Biomass Accumulation and Ammonium Consumption

In the manual control fed-batch cultivation of *Spirulina platensis*, the cells grew to give the maximum biomass concentration of 3.06 g/l at 12 days (Fig. 5); then the cell growth ceased because of the shadowing effect of high cell density [25].

Both the biomass concentration and the amount of ammonium-N consumed increased with the culture time in the cell growth phase (before 12 days). It was found that

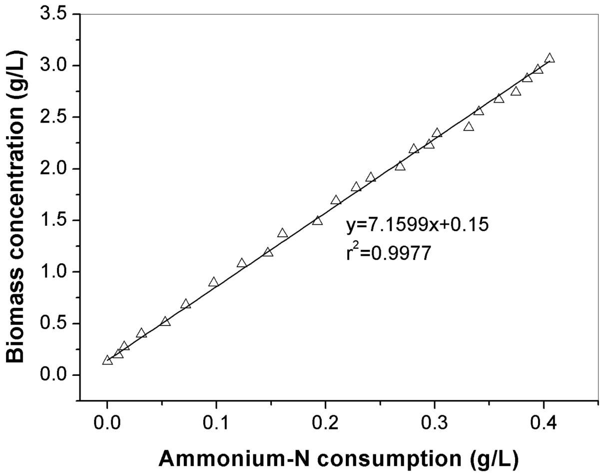

Fig. 2. Relationship between the ammonium-N consumption and biomass production during the fed-batch cultivation of *Spirulina platensis*.

The plots were obtained during the cell growth phase.

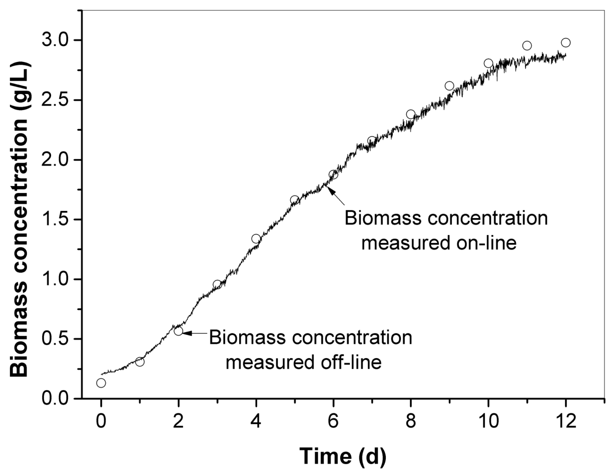

Fig. 3. Application of optical density sensors in monitoring the growth of *Spirulina platensis* in the fed-batch cultivation with 1 mmol/l initial ammonium concentration.

the consumption of ammonium-N was proportional to the biomass production (Fig. 2). The value of $Y_{X/N}$ was estimated to be 7.16 gX/gN ($r^2 = 0.9977$), which was constant and independent of time. The linear relationship between the ammonium-N consumption and biomass production meant that the ammonium feeding could be controlled according to the increment of biomass production.

Relationship Between the Online OD and Biomass Concentration

The online OD at 650 nm could be converted to the biomass concentration within the biomass concentration range of this culture experiment. A polynomial relationship was observed to describe the relationship between the online OD and the biomass concentration, as shown in Eq. (3):

$$X = 0.0688 \times OD_{650}^2 + 0.4977 \times OD_{650} \quad (3)$$

where OD_{650} was measured by the online OD probe. A correlation coefficient of 0.9985 was achieved, which suggested that the polynomial function was fit for the conversion between the online OD and biomass concentration.

An example of online continuous measurement of biomass concentration using the optical density sensor is shown in Fig. 3. A good agreement between the biomass concentrations measured offline and the ones measured online suggested that the online OD probe was applicable to measure the biomass concentration online.

OD-Based Feedback Fed-Batch Culture

In the fed-batch culture, the ammonium bicarbonate was added to the medium in an OD-based feedback-controlled manner in order to eliminate the substrate inhibition (2 mmol/l) [7] and prolong the growth phase. Three initial ammonium bicarbonate concentrations (1, 2, and 3 mmol/l) were tested and the time courses of biomass production and ammonium concentration in the culture medium are shown in Fig. 4.

It was reported that the ammonium concentration was a key factor for the growth of *Spirulina platensis* [7]. Therefore, an appropriate ammonium concentration should be maintained during the cultivation. As shown in Fig. 4, when the OD-based feedback feeding method was used, the ammonium concentration could be maintained in the range of 0.50−1.33, 1.34−2.89, and 2.06−3.30 mmol/l with the initial ammonium concentration of 1, 2, and 3 mmol/l,

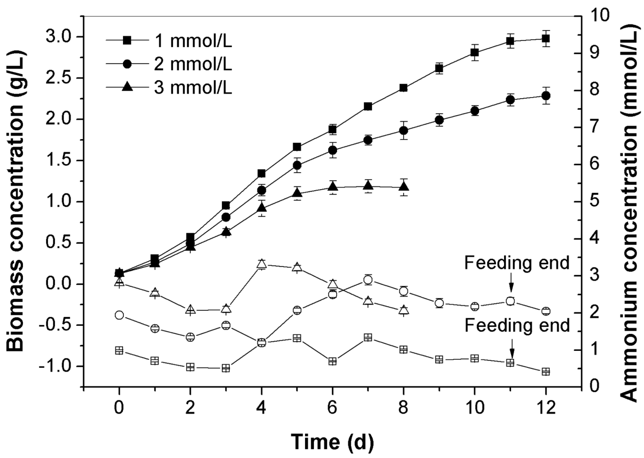

Fig. 4. Time course of biomass concentration (full symbols) and ammonium concentration (empty symbols) during OD-based feedback fed-batch cultivations with various initial ammonium concentrations.

The ammonium bicarbonate feeding started at the beginning and ended at day 11.

respectively. This result implied that the feeding rates of ammonium coincided with the growth rates of *Spirulina platensis*. Thus, the residual ammonium concentration in the medium could be kept at a certain range by this feeding method.

Fig. 4 also shows the changes of biomass concentrations at different initial ammonium concentrations (from 1 mmol/l to 3 mmol/l). The culture with 1 mmol/l ammonium obtained the maximum biomass concentration of 2.98 g/l, which was comparable with that of the manual control experiment (3.06 g/l). The cell growth of the culture with 2 mmol/l ammonium was relatively slow and the maximum biomass concentration of 2.29 g/l was attained, 23.2% lower than that of the culture with 1 mmol/l ammonium. It implied that the growth of *Spirulina platensis* was slightly inhibited when the ammonium concentration was maintained around 2 mmol/l. When the ammonium concentration increased to 3 mmol/l, the inhibition of cell growth caused by ammonium became worse. The cell growth ceased at day 6 and only a maximum biomass concentration of 1.18 g/l was attained, 60.4% lower than that of the culture with 1 mmol/l ammonium. These results were consistent with the previous studies, which showed that the proper initial ammonium concentration was in the range of 0.56–1.1 mmol/l [29], and 2–6 mmol/l ammonium would inhibit *Spirulina platensis* growth [3, 7].

The phenomenon that high concentration ammonium inhibited the growth of microalgae was not only observed in *Spirulina platensis*. In the cultivation of *Scenedesmus*, Park *et al.* [23] reported that the final biomass concentration gradually decreased when the ammonium concentration increased from 7.14 mmol/l to 71.4 mmol/l. Similar result was also found in the cultivation of *Chlorella vulgaris*. When the concentration of ammonium exceeded 17.9 mmol/l, the maximum cell number observed was significant lower than those when the ammonium concentration was below 17.9 mmol/l [31]. It was suggested that maintaining a relatively low ammonium concentration in the culture medium was a benefit for microalgae growth. In the current study, the ammonium concentration of the medium was kept at a low range of 0.50–1.33 mmol/l with the initial ammonium concentration of 1 mmol/l using the OD-based feeding method, and *Spirulina platensis* growth would neither be inhibited for ammonium accumulation nor be limited for nitrogen source scarcity.

Comparison of Different Feeding Methods

Runs with 1 mmol/l initial ammonium bicarbonate concentration performed using other feeding methods (manual, constant, and variable feeding methods) were compared with the OD-based feedback feeding method. The results are shown in Table 1 and Fig. 5.

As shown in Fig. 4 and 5, the advantage of the OD-based feedback feeding method in automatically controlling

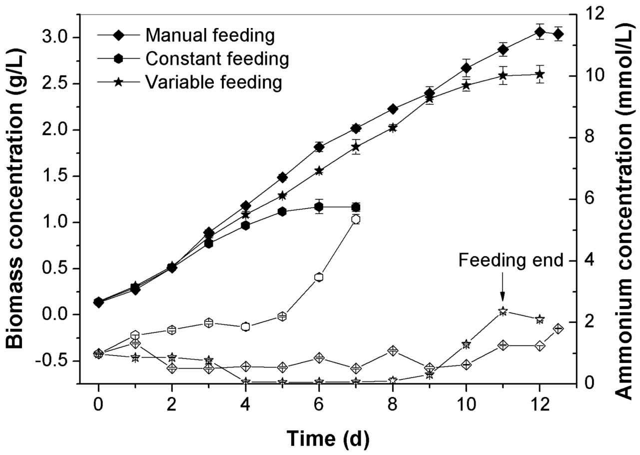

Fig. 5. Time course of biomass concentration (full symbols) and ammonium concentration (empty symbols) during fed-batch culture of *Spirulina platensis* with the manual, constant, and variable feeding methods.

residual ammonium concentration was obvious compared with the constant and variable feeding methods. When the constant feeding method was used, owing to the amount of ammonium fed being larger than that of the cell growth demanded, the ammonium in the medium accumulated to a certain concentration to inhibit cell growth. The cell growth nearly stopped after day 5, mainly because the ammonium concentration (>2.19 mmol/l) exceeded the inhibitory level (2 mmol/l) of *Spirulina platensis* [7]. Furthermore, the biomass turned its color from green to yellow and disintegrated. The same phenomenon was observed by Ferreira *et al.* [9] with a higher ammonium concentration at about 15–16 mmol/l. As a result, both the final biomass concentration and productivity were lower than those of the fed-batch cultivation using the OD-based feedback feeding method. Similar to our results, Soletto *et al.* [29] fed ammonium sulfate as nitrogen source at five different constant feeding rates (0.19, 0.36, 0.56, 1.1, and 1.7 mmol/l·d), but low final biomass concentrations were obtained with all feeding rates owing to the occurrence of excess ammonium inhibition or scarce ammonium limitation of the growth. It was demonstrated that for the constant feeding method, it was hard to control the ammonium concentration at a stable level.

When the variable feeding method was used, the cell growth was limited for ammonium scarcity in the medium (nearly zero) between day 4 and day 8, and a final biomass concentration of 2.60 g/l was obtained. Compared with the final biomass concentration (2.98 g/l) obtained using the OD-based feedback feeding method, no obvious decrease in the final biomass concentration of the variable feeding method was observed because *Spirulina platensis* could degrade phycocyanins to be used as a nitrogen reserve for continued biomass accumulation under nitrogen-limited condition [13]. Our result was in agreement with the previous result that the biomass concentration was not significantly affected at different nitrogen concentrations [32]. Thus, it is suggested that if the feeding methods could reduce the accumulation of ammonium, it will lead to higher biomass concentration. However, considering that nitrogen is a fundamental element of protein and chlorophyll, the protein and chlorophyll syntheses could be affected in nitrogen shortage condition [17, 32]. As expected, both the protein and chlorophyll contents using the variable feeding method (with nitrogen limited) were lower than those using the OD-based feedback feeding method (without nitrogen limited). However, the difference of protein and chlorophyll contents between the variable feeding and OD-based feedback feeding was not so much, perhaps because a rapid restoration of total protein occurred after nitrogen source replenishment (after day 8) in the variable feeding cultivation [13].

Similar to the variable feeding method in our study, other feeding methods (exponentially increasing feeding method and linearly increasing feeding method) were also studied previously using urea as the nitrogen source in *Spirulina platensis* cultivations [29]. However, ammonia significantly accumulated with different feeding methods during the cultivations. Under such conditions, the cell growth and the biomass chemical compositions would be affected [28, 29]. All the results suggested that a suited ammonium-feeding method by which the ammonium concentration was maintained at proper levels was important to obtain satisfactory cell growth and chemical compositions.

As mentioned previously, by using the OD-based feedback feeding method, the range of residual ammonium concentration in the culture medium was always kept at the appropriate levels (0.50−1.33 mmol/l), which was similar to the manual feeding run (0.50−2.00 mmol/l). Owing to the ammonium concentration control, a relatively high final biomass concentration (2.98 g/l) with the protein content of 64.1% and chlorophyll content of 13.4 mg/l was obtained, which was also comparable to that of manual feeding run. The satisfactory results enable the use of the low-cost ammonium bicarbonate to take the place of traditional nitrogen sources (nitrate salts) in commercial *Spirulina platensis* production.

Furthermore, the biomass yield based on nitrogen ($Y_{X/N}$) was improved by using the OD-based feedback feeding method. As shown in Table 1, the maximum $Y_{X/N}$ (7.32 gX/gN) was obtained using the OD-based feedback feeding method, whereas the minimum $Y_{X/N}$ (5.54 gX/gN) was obtained with constant feeding method owing to the excessive nutrients feeding. This was confirmed by the significant increasing of ammonium concentration in the medium during the whole cultivation, which demonstrated that a large part of the available nitrogen source was not uptaken. This result was similar to the previous study [11] showing that low $Y_{X/N}$ was obtained when the ammonium concentration in the medium exceeded the biomass requirements. A relatively higher $Y_{X/N}$ (6.35 gX/gN) was obtained when the variable feeding method was employed, but still lower than that of the OD-based feedback feeding method (7.32 gX/gN). This should be mainly ascribed to the overfeeding of ammonium in the later phase of cultivation. Similar results were found in the fed-batch cultivations of *Alteromonas putrefaciens* [18], which showed that the cell yield from the nutrients supplied to the culture system could be raised by automated feeding nutrient utilizing the online OD values. It was demonstrated that the OD-based feedback feeding method could improve the nitrogen source utilization efficiency by feeding nutrient approximately to the stoichiometric ratio of nutrient consumption.

In the commercial production of *Spirulina platensis*, factors such as biomass productivity, chemical compositions, and the cost of production should be taken into account. Application of ammonium bicarbonate instead of nitrate salts as nitrogen source could decrease the production cost, depending on the culture medium. Its toxicity when present at high levels can be circumvented and its utilization efficiency can be improved by application of the fed-batch process with the OD-based feedback feeding method. Although the constant and variable feeding methods were commonly used in fed-batch cultivations, they were open-loop feed-forward methods in which the nutrient feed was increased at a predetermined time of cultivation. Thus, the changes of microalgae growth may lead to a significant deviation of nutrient concentration from the expected situation. In contrast, the OD-based feedback feeding method was based on the relationship between nutrients consumption and biomass production, which would not be affected by the cell growth changes of microalgae. The ammonium feeding rate using this feeding method was changed in proportion to its consumption rates, so a proper ammonium level was maintained during the *Spirulina platensis* cultivation. Using this feeding method could obtain the maximum biomass productivity while maintaining high levels of protein and chlorella contents. It was revealed to be the optimum feeding method for ammonium salts feeding in *Spirulina platensis* cultivation.

All the preceding results reported had clearly demonstrated that the proposed OD-based feedback feeding method was quite effective in controlling ammonium concentration in *Spirulina platensis* cultivation. It provided a simple way to use ammonium salts as an alternative nitrogen source in microalgae cultivation. Compared with the other traditional feeding methods, the main advantages of this OD-based feedback feeding method are ensuring high biomass productivity with good quality, and improving the substrate utilization efficiency. All of these advantages could promote its large-scale application. Furthermore, it has the potential to be employed for other microalgae cultivations.

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