

# Enhancing Algal Biomass, Lipid and Astaxanthin Production by Mix-Cultivation of *Haematococcus Pluvialis* With *Simplicillium Lanosoniveum* DT06

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## Research Article

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**Enhancing algal biomass, lipid and astaxanthin production by mix-cultivation of  
*Haematococcus pluvialis* with *Simplicillium lanosoniveum* DT06**

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## **Abstract**

To enhance algal lipid and astaxanthin synthesis, the astaxanthin-producing green alga *Haematococcus pluvialis* was mix-cultivated with the antibiotic-synthesizing fungus *Simplicillium lanosoniveum* DT06 under non-aseptic conditions (NM) in this study. Results showed that in contrast to aseptic pure culture (AH) and non-aseptic pure culture (NH) of *H. pluvialis*, the production of cell (biomass), lipids and astaxanthin increased 56% and 119%, 112.4% and 279%, 74% and 175%, reaching 2.45 g/l, 0.837 g/l and 88.84 mg/l respectively; the average growth rate and the average specific growth rate increased 60.8% and 133.1 %, 19% and 31.6%, reaching 194.2 mg l<sup>-1</sup> d<sup>-1</sup> and 0.25 d<sup>-1</sup> respectively; and the average lipid synthesis rate and average specific lipid synthesis rate increased 112.5% and 278.66%, 36.15% and 97%, reaching 69.75 mg l<sup>-1</sup> d<sup>-1</sup> and 28.47 mg g<sup>-1</sup> d<sup>-1</sup> respectively; and also the content of C16-C18 fatty acids that are suitable for biofuels production increased to 83.19%. Therefore, NM provides an efficient and economical way for the production of biofuels.

**Keywords** Mix-cultivation · *Haematococcus pluvialis* · *Simplicillium lanosoniveum* DT06 · Biomass · Lipid · Astaxanthin

## **1 Introduction**

Microalgae are an important source of lipids and other molecules that can be used as feedstocks to produce biofuels and high-value products (1, 2). Nevertheless, algae-based biofuels (biodiesel) are now still economically unviable owing to its low productivity and high production cost (1, 3, 4). To deal with these problems two strategies have been applied: (1) mix-cultivation of microalgae with bacteria or fungi

to promote algal growth and lipid synthesis by taking advantage of their synergistic effects (complementary nutritional metabolisms) (5-9) and (2) co-production of high-value products along with biofuels so as to increase income and in turn reduce production costs (10-12).

*Haematococcus pluvialis* is a unicellular green alga, capable of synthesizing high-value astaxanthin along with lipids (13), and thus a suitable candidate for biofuel production (14-16). Whereas, *H. pluvialis*'s low cell growth rate constrains its lipid productivity, which is attributed, at least partially, to its susceptibility to harmful bacteria and fungi that inhibit its growth severely (9, 17). As a matter of fact, contamination of harmful microorganisms is a major impediment to any algae for substantial production of biofuels, because aseptic cultivation of algae outdoor on an industrial scale is unachievable. Therefore, suppressing or even eliminating harmful bacteria and fungi is essential to promote cell growth and lipid synthesis of *H. pluvialis* and other algae.

In the previous studies, we isolated a symbiotic fungus *simplicillium lanosoniveum* DT06 from the culture broth of cyanobacterium *Chroococcus sp.* (18). DT06 can synthesize a new antibiotic inhibiting gram-negative bacteria and some fungi (19) and promote *Chlamydomonas reinhardtii*'s growth and lipid production in aseptic cultures (20). Therefore, in the present study, we mix-cultivated *H. pluvialis* with DT06 in non-aseptic cultures to mimic the actual outdoor culture regime of *H. pluvialis* and find out if enhancements of cell, lipid and astaxanthin production can be achieved.

## 2 Materials and methods

### 2.1 Microorganisms and media

*H. pluvialis* was obtained from the Institute of Hydrobiology of Chinese Academy of Science, and maintained at 4 °C in a liquid BBM medium (13). Seed culture was prepared by inoculating 5 ml activated *H. pluvialis* culture into 250 ml flasks containing 100 ml of BBM medium, and incubated in an orbital shaker with top cool white fluorescent lamps at 25 °C, 110 rpm, 60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

The fungus *simplicillium lanosoniveum* DT06 was isolated from the culture broth of cyanobacterium *Chroococcus sp.* (18), which was streaked on PDA (21) agar plate and incubated at 28 °C for 7 days to facilitate spore production. The fungal spores were collected from the agar plate with 20 ml sterile distilled water and counted using a hemacytometer under the light microscope.

### 2.2 Pure and mixed-cultures

Aseptic pure culture of *H. Pluvialis* (AH): 250 ml flasks containing 100 ml culture sterilized BBM medium were inoculated with 10% (v/v) seed culture of *H. pluvialis* with a cell concentration of  $2.5 \times 10^5$  /ml.

Non-aseptic pure culture of *H. Pluvialis* (NH) were conducted in the same way as AH, except that the BBM medium was not sterilized.

Non-aseptic mixed-culture *H. Pluvialis* and DT06 (NM): 250 ml flasks containing 100 ml non-sterilized BBM medium were inoculated seed culture of *H. pluvialis* ( $2.5 \times 10^5$  /ml) and DT06 spore suspension ( $1.25 \times 10^7$  /ml) to reach a cell ratio 50: 1 (*H. Pluvialis*: DT06).

All cultures were incubated in an orbital shaker at 25 °C, 110 rpm, 60  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ .

## 2.3 Analytical methods

### 2.3.1 Biomass and growth kinetic

#### 2.3.1.1 Biomass

*H. pluvialis* biomass (dry cell weight, DCW  $\text{g l}^{-1}$ ) was calculated using Eq. (1) (22).

$$\text{Dry cell weight} = [-4.2 \times \{(\text{OD}_{680} - \text{OD}_{750}) / \text{OD}_{680}\} + 1.4] \times \text{OD}_{680}$$

where,  $\text{OD}_{680}$  and  $\text{OD}_{750}$  were the absorbances of culture broths at 680 nm and 750 nm respectively, which were measured with a Visible spectrometer (723N, Shanghai, China).

#### 2.3.1.2 Growth kinetics

The cell growth rate ( $\text{g l}^{-1} \text{ d}^{-1}$ ) of *H. pluvialis* was calculated through Eq. (2).

$$r_g = \frac{X_n - X_0}{t_n - t_0}$$

The specific cell growth rate ( $\text{d}^{-1}$ ) of *H. pluvialis* was calculated through Eq. (3).

$$\mu = \frac{\ln X_n - \ln X_0}{t_n - t_0}$$

where  $X_0$  and  $X_n$  is the biomass ( $\text{g l}^{-1}$ ) at day  $t_0$  and  $t_n$  respectively.

### 2.3.2 Lipid production and synthesis kinetic

#### 2.3.2.1 Lipid production

0.05 g of dried algal cells was blended with 5 ml of chloroform/methanol (2: 1) and the mixture was stirred at 2000 rpm on a magnetic stirrer for 20 min. The procedure was carried out 2 times. All the chloroform layers were collected, combined and evaporated to dryness at 60 °C. The total lipid ( $G_L$ ) was weighed on an analytical

balance.

### 2.3.2.2 Lipid synthesis kinetics

The total lipid content ( $\text{mg g}^{-1}$ ) of *H. pluvialis* was calculated through Eq. (4).

$$G(\text{mg g}^{-1}) = \frac{G_L}{VX}$$

where  $G_L$  is the total lipid (mg),  $V$  is the culture volume (l),  $X$  is biomass ( $\text{g l}^{-1}$ ).

The lipid synthesis rate:  $r_s(\text{mg l}^{-1} \text{d}^{-1})$  of *H. pluvialis* was calculated through Eq. (5).

$$r_s(\text{mg l}^{-1} \text{d}^{-1}) = \frac{X_n G_n - X_0 G_0}{t_n - t_0}$$

The specific lipid synthesis rate of *H. pluvialis* was calculated through Eq. (6).

$$q(\text{d}^{-1}) = \frac{r_s}{X_n}$$

where  $X_0$  and  $X_n$  is the biomass ( $\text{g l}^{-1}$ ) at day  $t_0$  and  $t_n$ , while  $G_0$  and  $G_n$  is the corresponding lipid content ( $\text{mg g}^{-1}$ ) at day  $t_0$  and  $t_n$ , respectively.

### 2.3.3 Fatty acid composition

Fatty acid methyl ester (FAME) was prepared by extraction-transesterification (23). The extracted lipid from 0.05 g of dried cells was blended with 6 ml mixture of methanol, concentrated sulfuric acid and chloroform (2.55: 0.45: 3) and kept in a water bath at 90 °C for 90 min. Upon completion of the reaction, the chloroform layer containing FAME was carefully collected. Algal fatty acid composition was analyzed using a gas chromatograph (GC-Agilent 7890B) equipped with a flame-ionized detector (FID) and an INNOWAX capillary column (Agilent, USA; 30 m  $\times$  0.32 mm  $\times$  0.5  $\mu\text{m}$ ). The initial column temperature was set at 80 °C for 2 min, raised to 140 °C ( $12 \text{ }^\circ\text{C min}^{-1}$ ), then 240 °C ( $20 \text{ }^\circ\text{C min}^{-1}$ ) and held for 20 min. The temperatures of the

injector and detector were 250 °C and 280 °C, respectively. FAME in the sample was identified and quantified by comparing the peak area with the standards (FAME Mix C8-C22 standard, 18920-1AMP; Sigma-Aldrich, St. Louis, MO, USA) using C17:0 as the internal standard.

#### **2.3.4 Astaxanthin production**

Astaxanthin was analyzed according to the method (13).

#### **2.3.5 Nitrate nitrogen**

Nitrate nitrogen was analyzed according to the method (24).

#### **2.3.6 pH**

pH was measured using a pH meter.

### **3 Results**

#### **3.1 Cell growth**

##### **3.1.1 Biomass**

The growth of *H. pluvialis* was promoted in NM but inhibited in NH in comparison with AH. As shown in Fig. 1, the biomass of AH increased slowly on the first 2 days (lag phase) and rapidly from then on till the 8th day and hereafter remained relative stable at 1.57 g/l (stationary phase). The biomass of NH changed in a manner similar to that of AH but was lower than those of AH and reached 1.12 g/l at the end of experiment, which was 29% lower than that of AH. By contrast, the biomass of NM elevated drastically from day2 till day10 and reached the highest value of 2.45 g/l on day12, which increased 56% and 119% compared to those of AH and NH, respectively.

### **3.1.2 Growth kinetic**

As shown in Table 1, the average growth rate ( $194.2 \text{ mg l}^{-1} \text{ d}^{-1}$ ) and average specific growth rate ( $0.25 \text{ d}^{-1}$ ) of *H. pluvialis* in NM were the highest in three cultures, which were 60.8% and 19%, 133.1 % and 31.6% higher than those of AH ( $120.8 \text{ mg l}^{-1} \text{ d}^{-1}$ ,  $0.21 \text{ d}^{-1}$ ) and NH ( $83.3 \text{ mg l}^{-1}$ ,  $0.19 \text{ d}^{-1}$ ), respectively.

### **3.2 Lipid synthesis**

#### **3.2.1 Lipid concentration and lipid content**

As shown in Fig. 2, the highest lipid concentration ( $0.837 \text{ g/l}$ ) and lipid content ( $341.8 \text{ mg/g}$ ) were achieved in NM, which increased 112.4% and 36%, 279% and 73% in comparison with those of AH ( $0.394 \text{ g/l}$ ,  $251.4 \text{ mg/g}$ ) and NH ( $0.221 \text{ g/l}$ ,  $197.5 \text{ mg/g}$ ), respectively.

#### **3.2.2 Lipid synthesis kinetics**

The results of kinetic analyses were listed in Table 2. The average lipid synthesis rate ( $69.75 \text{ mg l}^{-1} \text{ d}^{-1}$ ) and average specific lipid synthesis rate ( $28.47 \text{ mg g}^{-1} \text{ d}^{-1}$ ) of NM were the highest among the three cultures, which were 112.5% and 36.15% higher than those of AH ( $32.83 \text{ mg l}^{-1} \text{ d}^{-1}$ ,  $20.91 \text{ mg g}^{-1} \text{ d}^{-1}$ ) and 278.66% and 97% higher than those of NH ( $18.42 \text{ mg l}^{-1} \text{ d}^{-1}$ ,  $14.45 \text{ mg g}^{-1} \text{ d}^{-1}$ ), respectively.

### **3.3 Astaxanthin production / Astaxanthin content**

The astaxanthin production (concentration) and astaxanthin content in three cultures (Fig. 3) were consistent with the lipid production and content (Table 2), which were highest in NM ( $88.84 \text{ mg/l}$ ,  $36.26 \text{ mg/g}$ ) followed AH ( $51.04 \text{ mg/l}$ ,  $32.51 \text{ mg/g}$ ) and NH ( $32.31 \text{ mg/l}$ ,  $28.85 \text{ mg/g}$ ), and thus the astaxanthin production and astaxanthin

content of NM were 74%, 11.53% and 175%, 25.68% higher than those of AH and NH, respectively.

### **3.4 Fatty-acid composition**

As results shown in Table 3, the fatty acids profiles of the total lipids of *H. pluvialis* in all cultures were in the range of C14-C22 and mainly C16-C18 that are suitable for biodiesel production (25). The content of C16-C18 fatty acids of NM (83.19%) was higher than those of AH (82.6%) and NH (80.71%), and more importantly oleic acid (C18:1), a key indicator of biodiesel quality (26), accounts for 21.69 % of the total fatty acids of NM, which was 8.72% and 23.3% higher than those of AH (19.95%) and NH (17.59%), respectively. In addition, the percentage of saturated fatty acid (SFA) of NM (36.12%) increased 4.3% and 7.5% in comparison with those of AH (34.62%) and NH (33.59%), respectively.

### **3.5 Nitrate nitrogen**

Variation of nitrate nitrogen concentration of three cultures (Fig. 4) followed a similar pattern. The nitrate nitrogen concentration of AH and NH declined drastically within the first 2 days, from 250 mg/l to 73.43 mg/l and 106.43 mg/l, and then slowly to 11.34 mg/l and 18.53 mg/l respectively at the end of the experiment. By contrast, the nitrate nitrogen concentration of NM decreased more drastically than those of AH and NH to 40.56 mg/l on the 2th day, and became undetectable on the 8th day.

### **3.6 pH**

As shown in Fig. 5, the pH of AH and NH elevated quickly and continuously with the onset of experiment and reached 9.67 and 9.98 respectively at the end of the

experiment. By contrast, the pH of NM increased slowly to 8.52 after 8 days, and then remained relatively constant in the range of 8.48 to 8.57.

#### **4 Discussion**

The results of this study demonstrated that the production of cell (biomass) (Fig. 1), lipid (Fig. 2) and astaxanthin (Fig. 3) of *H. pluvialis* elevated in NM in comparison with those of AH and NH.

(1) The elevation of *H. pluvialis* biomass in NM was caused definitely by the enhancement of cell growth as evidenced by the elevation of the average cell growth rate and specific growth rate (Table 1), which, in turn, might be attributed to the following factors:

(a) DT06 suppressed or even eliminated the harmful microorganisms (bacteria and fungi) by synthesizing antibiotic. Since NM and NH were both non-aseptic cultures, the decrease of biomass in NH compared to that of AH (aseptic culture) indicted the existence and inhibition of harmful microorganisms against *H. pluvialis*; while, on the other hand, the fact that the biomass of NM was higher than that of AH implied the suppression or even elimination of the harmful microorganisms in NM.

(b) The synergistic effects between DT06 and *H. pluvialis* in metabolism (complementary nutritional metabolisms). The growth of *H. pluvialis* in photoautotrophic condition depended solely on photosynthesis taking up CO<sub>2</sub> but releasing O<sub>2</sub>, and thus were limited by both the insufficiency of former and accumulation of latter; while, on the contrary, DT06 metabolically absorbed O<sub>2</sub> but liberated CO<sub>2</sub>, and thus promoted the growth *H. pluvialis* in NM, a case similar to

those in other alga-microorganism co-cultures (13, 27, 28, 29, 30).

(c) The metabolism of DT06 stabilized the pH of NM. The quickly elevated pHs in AH and NH (Fig. 5) inhibited cell growth, which were resulted primarily from the uptake of physiologically alkaline salts e.g.,  $\text{NaNO}_3$  (Fig. 4) and secretion of  $\text{NH}_4^+$  (31) by *H. pluvialis*; while in NM, the release of  $\text{CO}_2$  and uptake of  $\text{NH}_4^+$  by DT06 lowered and relatively stabilized the culture pH (Fig. 5), which was in favor of *H. pluvialis* growth. Additionally, it is noteworthy that after the uptake of  $\text{NH}_4^+$ , DT06 probably secreted organic nitrogen for the growth of *H. pluvialis*, which could explain the reason for the growth of *H. pluvialis* after day8 (Fig.1) when nitrate nitrogen was depleted (Fig. 4).

(2) The elevation of lipid production in NM was ascribed to (a) the elevated biomass of *H. pluvialis* (Fig. 1) that set the cell base for enhanced synthesis of lipid, and (b) the enhancement of lipid synthesis as evidenced by the elevation of lipid content (Fig. 2), average lipid synthesis rate and specific lipid synthesis rate of *H. pluvialis* (Table 2). The reasons for this seemed to come from two aspects: firstly, the quickly depletion of nitrate nitrogen (Fig. 4), which resulted in nitrogen starvation that promoted lipid synthesis by directing the carbon flux toward the specific pathway (32), and secondly  $\text{CO}_2$  generated by DT06 increased  $\text{CO}_2$  concentration, which not only facilitated lipid synthesis (33-35) but also influenced the composition of fatty acid (36-38), and thus might be the cause for the enrichment of C16-C18 fatty acids, particularly oleic acid in NM (Table 3) as well.

(3) The reasons for the elevated astaxanthin production in NM (Fig. 3) were similar to

or even the same as those for the enhanced lipid yield (Fig. 2). Since astaxanthin is lipid-soluble dispersing in lipid droplets in *H. pluvialis* cells (15, 16, 39), it is not surprising that lipid and astaxanthin syntheses are closely associated, and conditions favor lipid synthesis also enhance astaxanthin production (40-42).

## **5 Conclusion**

Mix-cultivation of the antibiotic-synthesizing fungus *simplicillium lanosoniveum* DT06 with the astaxanthin-producing green alga *Haematococcus pluvialis* in non-aseptic condition promotes algal growth as well as lipid and astaxanthin syntheses, and thus provides an efficient way for co-production of biofuels and high-value products.

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## **Compliance with Ethical Standards**

## **Conflict of Interest**

The authors declare that they have no conflict of interest.

## **Declarations**

## **Funding**

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## **Conflict of Interest**

The authors declare that they have no conflict of interest.

**Availability of data and material**

Not applicable

**Code availability**

Not applicable

**Ethics approval**

Not applicable

**Consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Authors' contributions**

Xiang-Ying Xing and Qing-Lin Dong conceived and designed this study. Ran Yan, performed the experiments and analyzed the data along with Xian-Yong Yu and Kang-Li Shi. Ran Yan and Qing-Lin Dong wrote the manuscript.

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## **Figure Captions**

**Fig. 1** The growth curve of *H. pluvialis*

**Fig. 2** Lipid concentrations and lipid contents of different cultures

**Fig. 3** The astaxanthin production and astaxanthin content in different cultures

**Fig. 4** Variation of nitrate nitrogen concentration in different cultures

**Fig. 5** Fluctuation of pH in different cultures

**Table 1** The growth kinetic parameters of *H. pluvialis* of different cultures

Different cultures	Average growth rates / ( $\text{mg l}^{-1} \text{d}^{-1}$ )	Average specific growth rate / ( $\text{d}^{-1}$ )
AH	120.8 $\pm$ 3.13	0.21 $\pm$ 0.02
NH	83.3 $\pm$ 2.28	0.19 $\pm$ 0.03
NM	194.2 $\pm$ 4.12	0.25 $\pm$ 0.01

**Table 2** The lipid synthesis kinetic parameters of different cultures

Different cultures	Average lipid synthesis rate / (mg l <sup>-1</sup> d <sup>-1</sup> )	Average specific lipid synthesis rate / (mg g <sup>-1</sup> d <sup>-1</sup> )
AH	32.83±0.12	20.91±0.14
NH	18.42±0.63	14.45±0.21
NM	69.75±0.42	28.47±0.22

**Table 3** The fatty acid profiles of *H. pluvialis* in different cultures

Fatty acids	AH	NH	NM
C14:0	1.32±0.15	2.87±0.12	2.04±0.05
C16:0	18.62±1.35	15.74±1.15	19.14±1.21
C16:1	4.44±0.22	5.63±0.13	4.61±0.22
C16:2	3.02±0.11	3.76±0.56	2.93±0.08
C16:3	3.94±0.18	4.11±0.84	2.12±0.09
C18:0	4.67±0.21	4.62±0.35	4.96±0.15
C18:1	19.95±1.31	17.59±1.36	21.69±1.32
C18:2	18.64±1.16	16.47±1.33	19.23±1.26
C18:3	9.32±0.33	12.79±0.98	8.51±0.35
C20:0	7.43±0.17	7.93±0.47	7.63±0.21
C20:1	3.17±0.06	3.7±0.31	2.95±0.04
C22:0	2.58±0.13	2.43±0.24	2.35±0.07
C22:1	2.9±0.11	2.36±0.27	1.84±0.08
SFA	34.62±1.47	33.59±1.62	36.12±1.51
C16-C18	82.6±2.13	80.71±1.99	83.19±1.78

# Figures

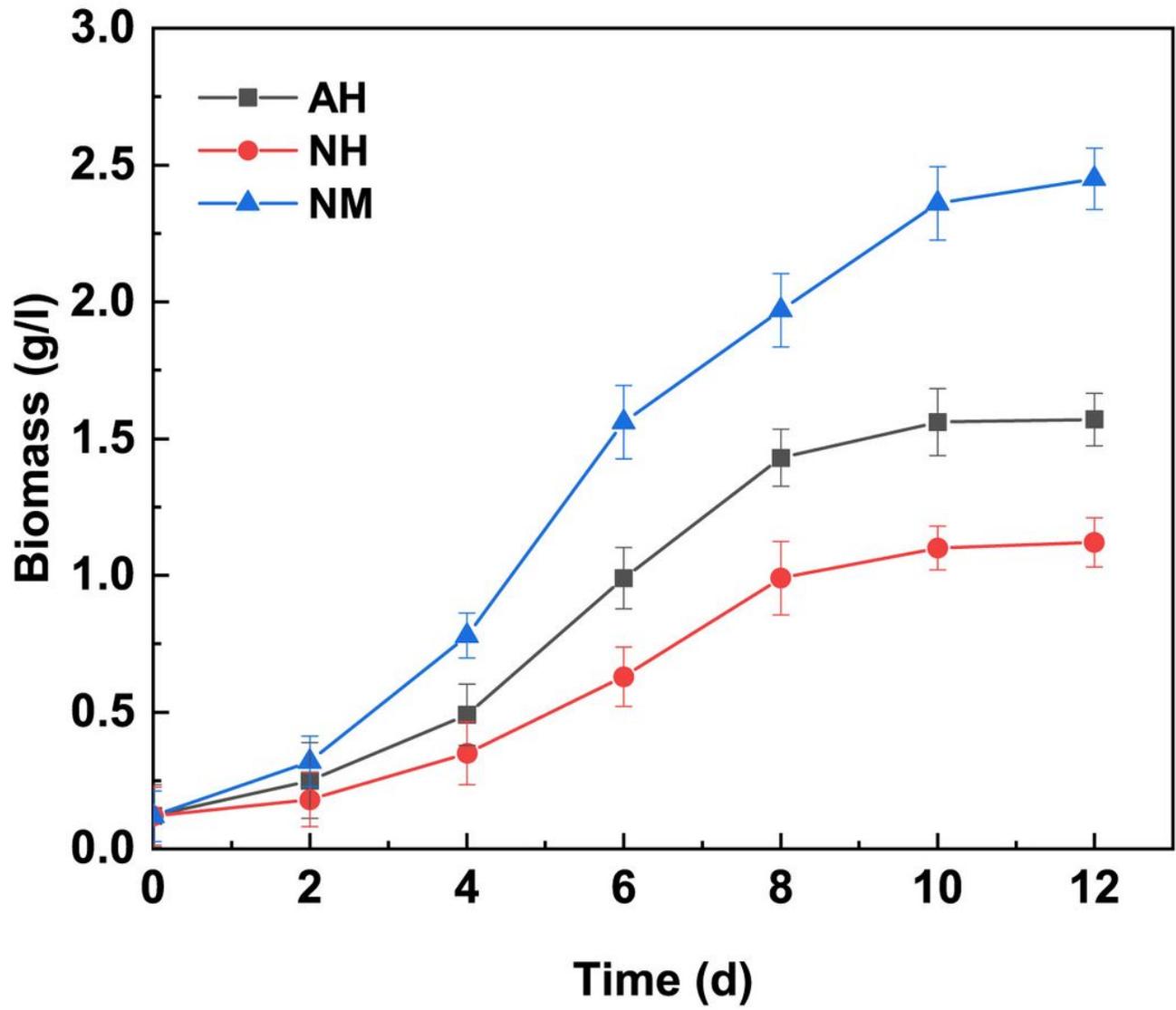


Figure 1

The growth curve of *H. pluvialis*

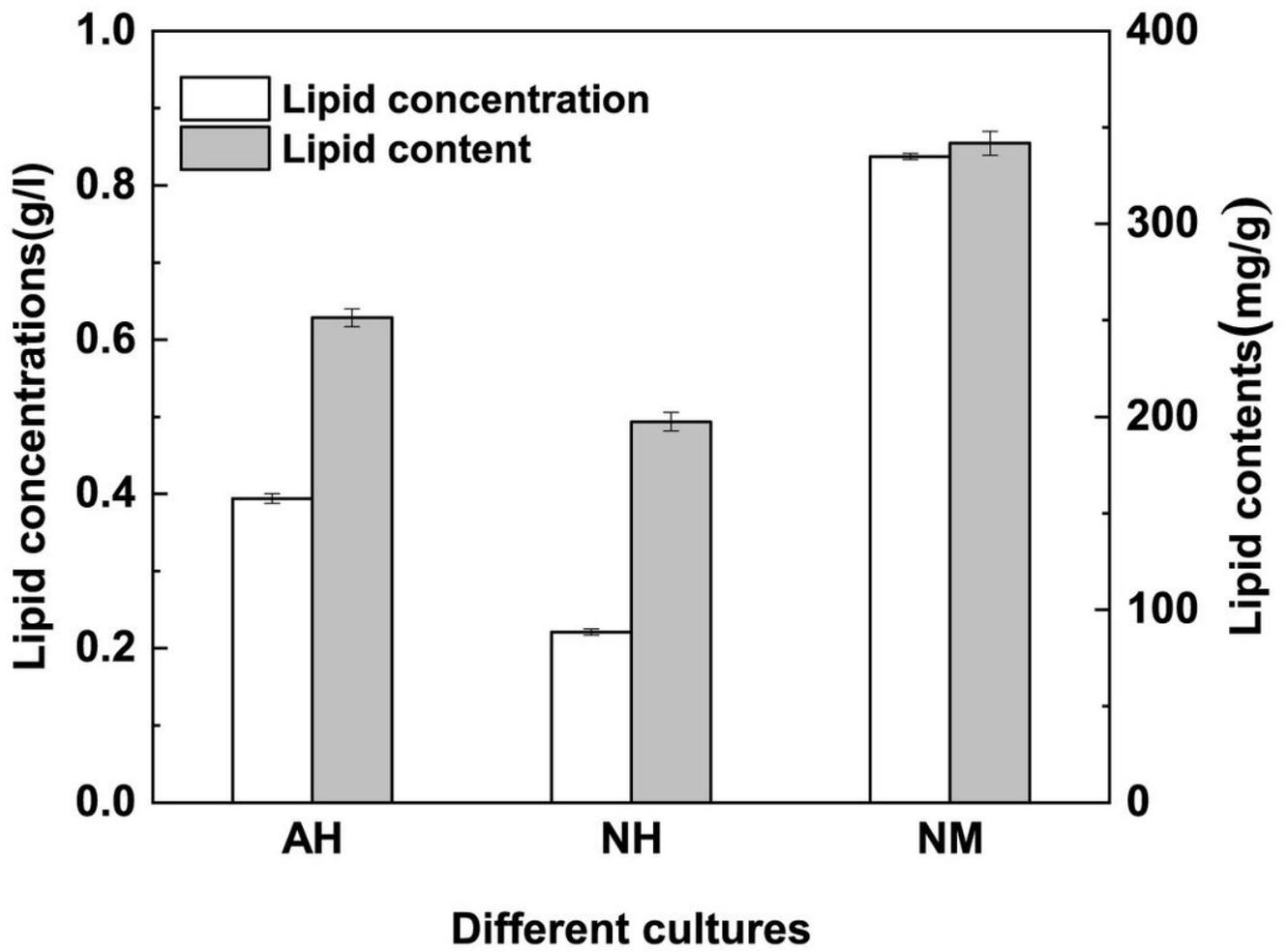


Figure 2

Lipid concentrations and lipid contents of different cultures

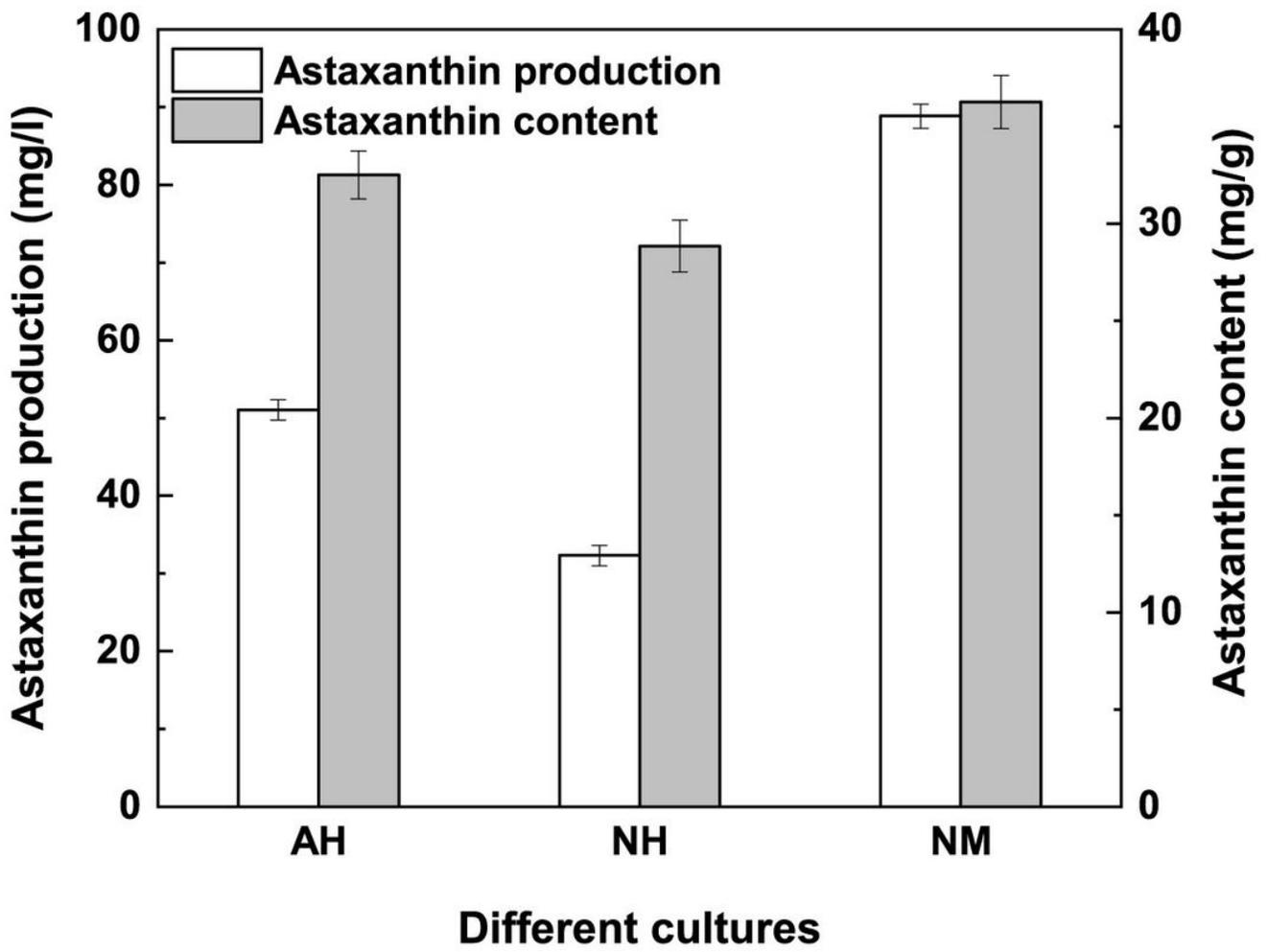


Figure 3

The astaxanthin production and astaxanthin content in different cultures

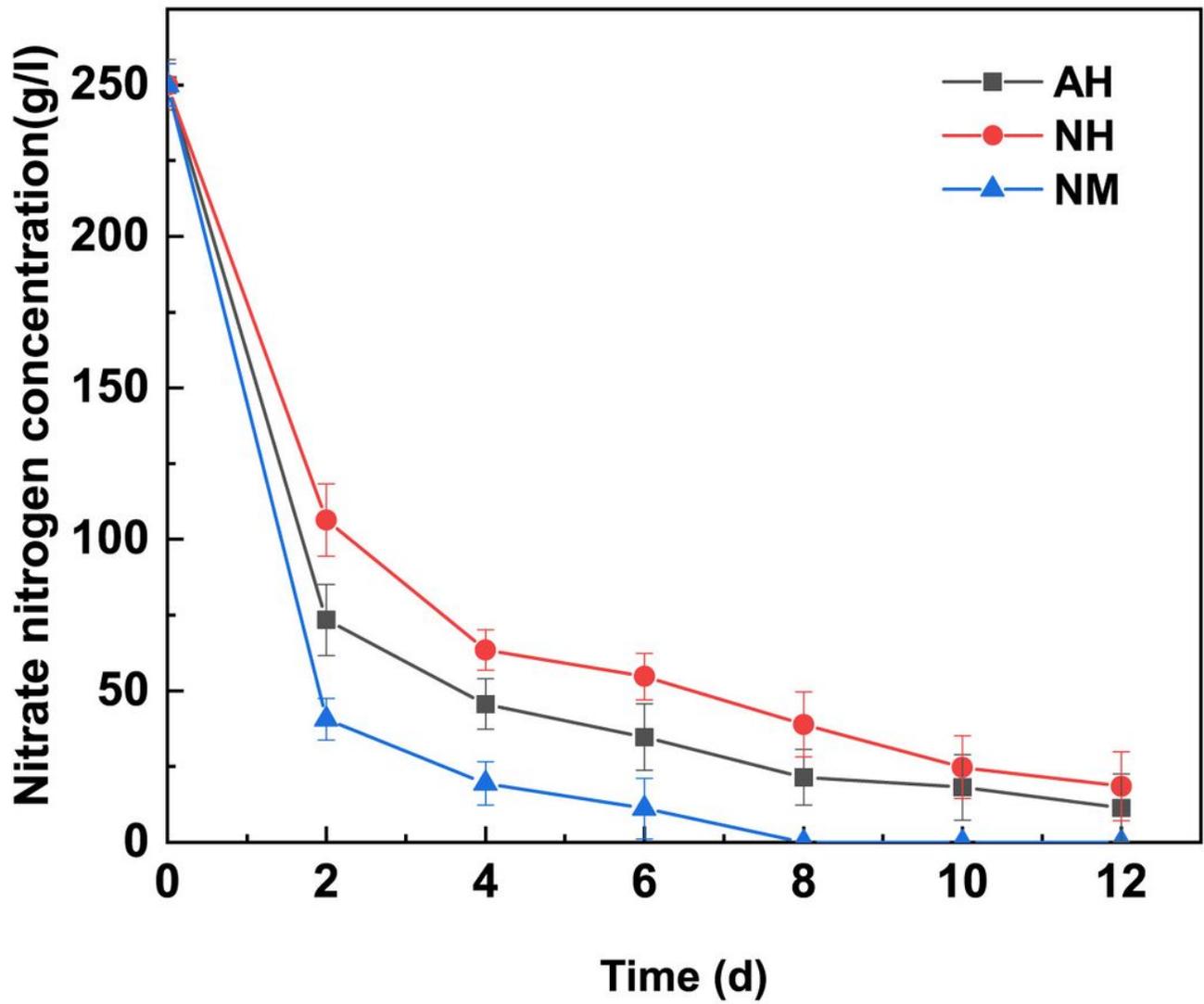


Figure 4

Variation of nitrate nitrogen concentration in different cultures

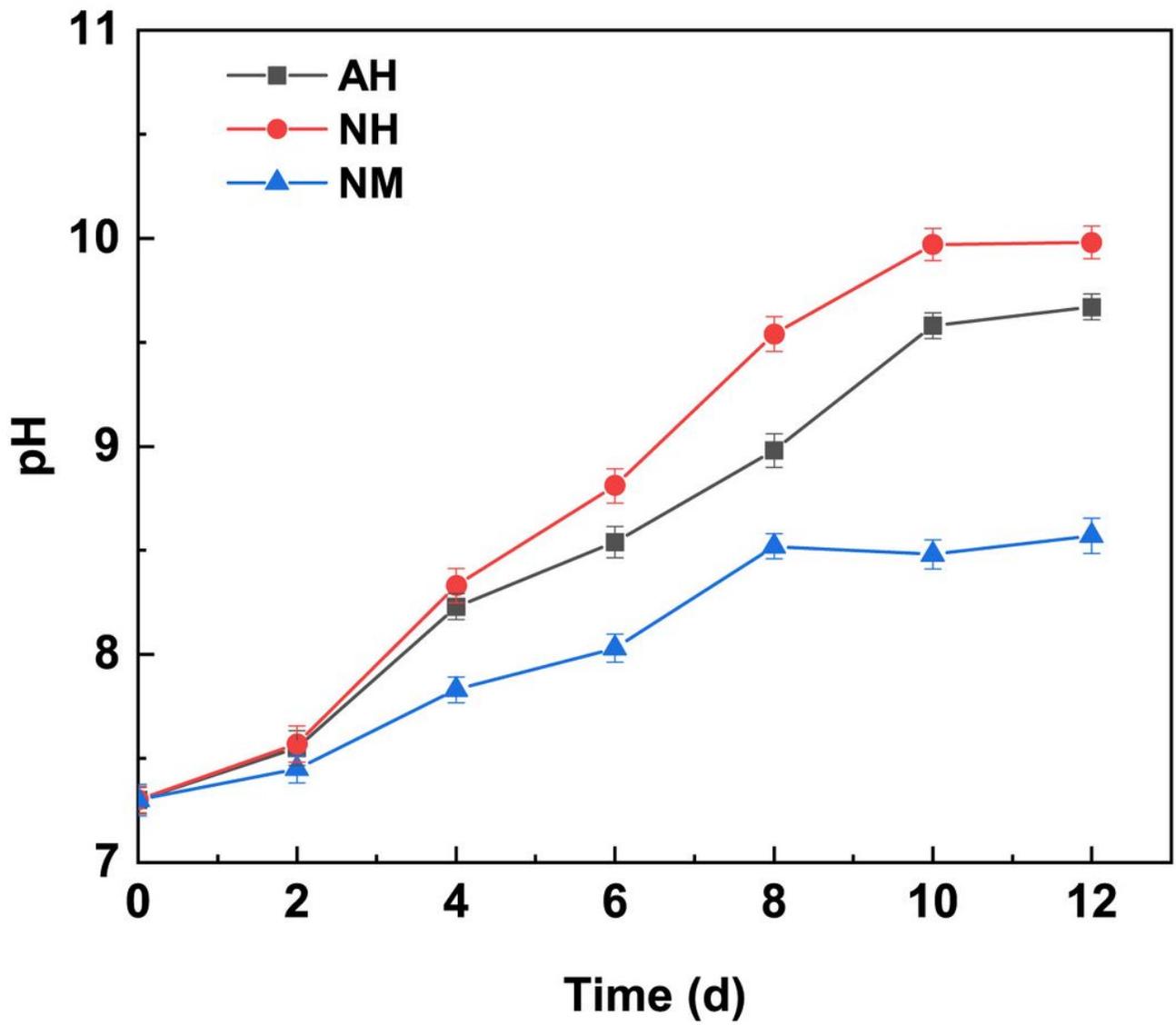


Figure 5

Fluctuation of pH in different cultures