**Research Article**

**Effect of Vitamin-B_{12} and Vitamin-H on the Growth and Astaxanthin Content of *Haematococcus pluvialis* CH-1**

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**Abstract:** An economic microalgae *Haematococcus pluvialis* CH-1 was used as experimental material. An experiment of adding six grades of concentrations of Vitamin-B_{12} and Vitamin-H respectively was conducted. Cell density, biomass and astaxanthin content were measured. The results showed that the growth of *H. pluvialis* was accelerated significantly by adding of Vitamin-B_{12} and Vitamin-H, respectively. The optimal adding concentration of Vitamin-B_{12} and Vitamin-H respectively for *H. pluvialis* was 50 and 500 µg/L. Under the optimal concentration for *H. pluvialis*, cells density, biomass and astaxanthin content were enhanced with Vitamin-B_{12}: 29.5, 30.0 and 28.3%; Vitamin-H: 17.1, 29.2 and 22.6% higher than the blank, respectively. On the mass culture of motile cells of *H. pluvialis*, properly adding Vitamin-B_{12} and Vitamin-H, respectively was effective for increase cells density, biomass and astaxanthin content.

**Keywords:** *Haematococcus pluvialis*, vitamin-b_{12}, vitamin-h, astaxanthin

**INTRODUCTION**

The unicellular fresh water microalgae, *Haematococcus pluvialis* Flotow (Volvocales, Chlorophyceae) is green-colored, biflagellate and motile in its vegetative stage. In its growth stages, it has both motile and non-motile forms (Esra et al., 2007). In the life cycle of *H. pluvialis*, green vegetative cells with two flagella can grow autotrophic ally in the light or heterotrophic ally in extreme environment, such as high temperature, nutrient deficiencies, the dark (Ranjbar et al., 2008). Most of the reports showed astaxanthin accumulation in encysted cells when grown in heterotrophic conditions under high light or salinity stress. The red secondary carotenoid astaxanthin, which is widely used as a color additive in aquaculture, food and feed industries and has recently, attracted attention as nutraceutical food, cosmetics, pharmaceuticals, due to its high antioxidant activity (Miki, 1991; Palozza and Krinsky, 1992; Fukuzawa et al., 1998; Martin and Mark, 2003; Bjerkeng and Johnsen, 1995). The Chlorophyte alga *Haematococcus pluvialis* is believed to accumulate the highest levels of astaxanthin in nature.

It is reported, adding VB_{12}, VB_{12} and VH into culture medium can effectively promote the growth of microalgae. Ford (1958) proved there is a close relationship between adding concentration of VB_{12} and cell division rate of *Isochrysis galbana*; The results showed proliferation effect order of adding VB_{1}, VB_{12} and VH for *Prorocentrum micans* was VB_{1}>VH>VB_{12} (Wang et al., 1996). Liu et al. (2002) pointed out that growth promotion effect is the best for the transgenic *Anabaena* while adding VB_{1}, VB_{12} and VH together However; it is unknown how to influence on cell density, biomass and astaxanthin content of *H. pluvialis* when respectively adding two kinds of vitamins, VB_{12} and VH. In this study, the effect of different concentrations of VB_{12} and VH on cell density, biomass and astaxanthin content of *H. pluvialis* was studied and the results may provide the support of theory and technology for the high density cultivation of *H. pluvialis*.

**MATERIALS AND METHODS**

**Algal strain, medium and cultivation conditions:** *Haematococcus pluvialis* CH-1 used in the present work was obtained from the Research Center of Hydrobiology of Jinan University (Guangzhou, China), Stock culture of *H. pluvialis* CH-1 was grown photoautotrophic ally in BBM medium (Lorenz and Cysewski, 2000) at 24±1°C under 12 h: 12 h photoperiod (60 µmol/m²/s) in 25 mL flask.. The cells of logarithmic growth phase were inoculated into 150 mL culture medium; inoculation density is 5-10×10^{3} cells/mL.

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Table 1: Treatments on three kinds of Vitamin with six concentration grades (µg/L)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration grades</th>
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<tbody>
<tr>
<td>VB₁₂</td>
<td>0        0.05  0.5  5  50  500</td>
</tr>
<tr>
<td>VH</td>
<td>0        0.5   5   50  500 5000</td>
</tr>
</tbody>
</table>

Experimental design: The experiment was conducted in the two groups, added-vitamin B₁₂ groups and added-vitamin H groups; each group was divided into 6 concentrations grades treatment, including 3 replicates per treatment (Table 1). The concentration grade of 0 treatments was without any vitamin, which was the blank.

Measure indexes and methods:
Cell density: The sample was measured every 48 h, using hemocytometer counting.

Biomass: 10 mL algae liquid was filtrated by 0.45 µm cellulose acetate membrane every 6 d, then drying 36 h in 80°C, kept in a desiccators to room temperature, finally weighing by electronic balance (Sartorius BS210S).

Astataxanthin content: According to Boussiba’s (Boussiba and Vonshak, 1991) method for the extraction and measure of astataxanthin: astataxanthin concentration (mg/L) = OD₄₉₀×4.5

Data analysis: Analyses were done using the Excel program (Microsoft) and the SPSS software package version 11.5 of SPSS Inc. (Chicago, IL, USA.)

RESULTS AND DISCUSSION

Cell density: The results showed that, adding VB₁₂ and VH respectively could obviously increase the cell density of *H. pluvialis* (Fig. 1). In VB₁₂ group, cell density increased with the increase of the adding concentration of VB₁₂. When adding the concentration was 50 µg/L, the cell density was the maximum (Fig. 1a) and was significantly higher than other treatments. It reached 4.96×10⁵ cells/mL in the 18th day, enhanced 29.5% than the blank (p<0.01). But when the highest concentration (500 µg/L) was adding, the cell density was lower but still higher than the blank. The optimal concentration of adding VB₁₂ was 50 µg/L.

In the adding VH test group, the cell density increased with the increase of the adding concentration of VH in 0.5-500 µg/L (Fig. 1b). When adding concentration was 500 µg/L, the cell density was higher than other treatments in the late culture period (12-22 d) and reached 4.36×10⁵ cells/mL in the 22th day, enhanced 17.1% than the blank (p<0.05). When adding the concentration was 5000 µg/L, the cell density was lower than the blank.

The biomass: The effect of adding VB₁₂ and VH for the biomass of *H. pluvialis* was showed in Fig. 2. In VB₁₂ group, when the adding concentration is less than 50 µg/L, the biomass increased with the increase of the adding concentration and reached the maximum value 0.57 g/L at the concentration 50 µg/L, enhanced 30% than the blank. But when the adding concentration is 500 µg/L, the biomass significantly decreased (Fig. 2a). In the VH group, the biomass was significantly higher than other treatments (p<0.01) besides the blank, at the concentration level 500 µg/L, reached 0.54 g/L, enhanced 29.2% the blank (Fig. 2b). At the adding concentration 0.5 µg/L, the biomass was slightly lower than the blank.

It was concluded the trend of the effect (change) of adding VB₁₂ and VH for the biomass of *H. pluvialis* are consistent with the trend of the effect (change) for the cell density, it indicated they were high correlation among the cell density and the biomass which can represent the growth rate of *H. pluvialis*.

Based on our research, higher vitamin concentration not always promoting the growth of *H. pluvialis*, when the adding concentration exceeds a certain range, the effect of promotion declined obviously, even control the growth. It showed under the sufficient nutrient condition, growth rate of *H. pluvialis*
Fig. 2: Effects of Vitamin-B$_{12}$ and Vitamin-H on the biomass of *H. pluvialis*
don’t have obvious difference between low and high nutrient conditions. On the view of economy, adding VB$_{12}$ to culture *H. pluvialis* is effective and economic.

**The astaxanthin content:** Respectively adding VB$_{12}$ and VH made the changes of the astaxanthin content of *H. pluvialis* (Fig. 3). In VB$_{12}$ group, the astaxanthin content increased with the increase of the adding concentration, reached maximum 28.35 mg/L at the adding concentration 50 µg/L, enhanced 28.3% than the blank (Fig. 3a). But when the highest concentration (500 µg/L) of VB$_{12}$ was added, the astaxanthin content was lower than the blank. In VH group, the astaxanthin content increased with the increase of VH When adding concentration was 500 µg/L, the astaxanthin content was also higher than other treatments, enhanced 22.6% than the blank (p<0.05). When adding the concentration was 5000 µg/L, the astaxanthin content was lower than the blank (Fig. 3b).

*H. pluvialis* Flo tow (Chlorophyceae) has a complex life-cycle involving several stages from motile flagellated zooids through to palmella and encysted stages. The conditions for the astaxanthin production are known to be considerably different from those for the growth of *H. pluvialis*. Therefore, to obtain high productivity of astaxanthin-hyperaccumulated *H. pluvialis* biomass, a two-stage culture system is likely to be more effective (Choi *et al*., 2002). The first stage is for high-rate growth of green motile cells under optimum conditions, astaxanthin is completely absent from the cells.

The second stage is for the hyperaccumulation of astaxanthin in red cells upon exposure of the cells to growth-limiting conditions, where a morphological and biochemical transformation occurs from green motile cells into inert red cysts. Various factors and/or methods promoting astaxanthin formation have been suggested: high irradiation, nitrogen deficiency, phosphate deficiency, magnesium deficiency, acetate addition, ferrous ion addition and salt addition or high temperature (Harker *et al*., 1996). Astaxanthin is synthesised and accumulated within oil droplets (Mendes-Pinto *et al*., 2001).

In the two test groups of adding vitamin, through comprehensive analysis, the rule of promoting astaxanthin content of *Haematococcus pluvialis* CH-1 and the rule of promoting the growth rate of *H. pluvialis* CH-1 is basically the consistent. It is
concluded that adding vitamin mainly promoting the growth rate of \textit{H. pluvialis} CH-1 and indirectly increase the astaxanthin content., because adding the active micronutrient can promote to increase the content of cellular material in \textit{H. pluvialis} CH-1,which can transfer into the astaxanthin under growth-limiting conditions.

**CONCLUSION**

In BBM medium added VB\textsubscript{12} and VH can obviously increase the growth of the \textit{H. pluvialis} motile cell, respectively. Adding VB\textsubscript{12} can significantly improve the cell density, biomass and astaxanthin content, while promotion effect of adding VH is lower. The suitable concentration of VB\textsubscript{12} and VH is different, the optimal adding concentration of Vitamin-B\textsubscript{12} and Vitamin-H respectively for \textit{H. pluvialis} was 50 µg/L and 500 µg/L. Adding suitable concentration of VB\textsubscript{12} can obviously improve astaxanthin content in the commercial production process of natural astaxanthin of \textit{H. pluvialis}.

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