Seeking for optimum culture medium for chlorella pyrenoidosa and analysis of the amino acid component

Wei Li¹, Xiaojiang Chen²*, Mengmeng Zheng³, Zhicheng Li⁴, Haiyang Li⁵
¹,²,⁴,⁵ Jiangsu Agri-animal Husbandry Vocational College
³Jiangyan County Agriculture Commission of Taizhou Prefecture

*Corresponding author and e-mail: Xiaojiang Chen, cq_cxj@126.com

Abstract. Chlorella Pyrenoidosa is one of the widely used algae in aquaculture. In order to find out a proper culture medium for high-density culturing to meet the demand of fish reproduction and water quality control, five normal algae culture media were test via multiple comparisons. The densities of the five groups, with six parallel samples each, which were cultured in corresponding medium, were determined by blood counting chamber on the beginning (0d), the 3rd day (3d), the 6th day (6d) and 9th day (9d). Then the growth curves were described and the growth radio were calculated and compared by One-Way ANOVA (Turkey method) using the SPSS 21.0 software. The multiple comparisons results revealed that HGZ group could reproduced a rapid and stable speed at high density, while SE group and Aquatic 4# reproduced faster than the HGZ group. Amino acid component results revealed that C. Pyrenoidosa contains 18 kinds of amino acid (42.19 ~ 43.14% of the body mass). There are significant differences on total amino acid, fish essential amino acid and non-essential amino acid between groups based on the multiple comparisons. However, the amino acid could not meet the demand of fish or shellfish. Thus, extra amino acid, such as tryptophan and methionine should be added and the HGZ medium was the best choice for producing C. Pyrenoidosa to satisfy the aquaculture.

1. Introduction
Chlorella Pyrenoidosa is unicellular green algae, who belongs to Chlorella and be of with different cell sizes, goblet-shaped chromatophore. It has been suggested that the consumption of natural “whole foods” rich in macronutrients has many healthful benefits. Besides of rich in protein, vitamins and minerals, C. Pyrenoidosaare of high reproductive ability via their special autospor e method [1]. Besides of their nutrition, C. Pyrenoidosaare also the ideal diet or feed supplement for aquatic larvae for their ability in controlling of water quality or for their small size in initial feed application. Because of rich nutrition, small shape and no pollution to water quality, it is difficult to replace them with artificial diet [1]. They are widely used in aquatic animal breeding and water quality control [2, 3]. Especially in the process of rotifer cultivation and reproduction of shellfish.

However, there are some barriers in aquiculture application. Such as, it is difficult to control the quality of different batches and to obtain algae in large numbers in a short time to match up the requirements of production. In this article, five different commonalgaeculture media were investigated to find the ideal medium for culturing the C. Pyrenoidosain a high concentration and speed to meet the requirements of larva.
2. Materials and methods

2.1. Materials

Species: *Chlorella Pyrenoidosa* (Provide by Qingdao Microalgae Biotechnology Co., Ltd., China)

Main Instruments: Artificial climate Chamber (Shanghai Duteo Scientific Instruments Co., Ltd., GZP-150)

Medium: Basal, Aquatic Medium 4# [4], SE (Brostol’s solution) [5], HGZ-145[6] and WAV medium [7]

2.2. Cultivation Methods and Procedure

Prepare thirty clean conical flask (50ml); Add 15 mL algae medium of *C. Pyrenoidosa* and 35 mL corresponding medium in five groups (Basal, Aquatic 4#, SE, HGZ and WAV), with 6 repeated trials.

The algae medium cultured in artificial climate Chamber (28°C, 10 000 Lux, photoperiod of 16L∶8D). Shake the flask twice a day to guarantee the homogeneity. The concentration of *C. Pyrenoidosa* were counted via blood counting chamber.

2.3. Growth ratio formula

Growth ratio represents the average relative growth rate (or reproduction rate) of *C. Pyrenoidosa* of different periods.

\[ \mu = \frac{\ln C_2 - \ln C_1}{t_2 - t_1} \]

Where

- \( \mu \) = growth ratio of *C. Pyrenoidosa*
- \( C_1 \) = concentration of *C. Pyrenoidosa* at \( t_1 \) (10^5 ind/L)
- \( C_2 \) = concentration of *C. Pyrenoidosa* at \( t_2 \) (10^5 ind/L);
- \( t_1 \) = start point of the test period;
- \( t_2 \) = termination point of the test period.

2.4. Analysis of the Amino Acid Component

*C. Pyrenoidosa* of Basal group, Aquatic 4# and HGZ were harvested at the 9th day, with 3 repeat trials. Then, *C. Pyrenoidosa* were vacuum frozen drying at low temperature for amino acid component analysis.

3. Results

3.1. Growth ratio and curve of 5 different culture media

The concentrations of *C. Pyrenoidosa* of different cultural medium were presented in table 1 and figure 1 at 0th d, 3rd d, 6th d, and 9th d. At 0th d, there is no difference between the groups. At 3rd d, group SE is primary high concentration, while group Aquatic 4# secondary. At 6th d, group Aquatic 4# came up to be the primary. At 9th d, HGZ showed highest concentration and growth ratio which were significant higher than the other groups (Fig 1 tab1) (Turkey method).
Figure 1. Line chart of *C. Pyrenoidosa*’s concentrations of groups at stages.

Table 1. Growth ratio of *C. Pyrenoidosa* of groups at different periods

| Medium  | 0-3 d     | 3-6 d     | 6-9 d     |
|---------|-----------|-----------|-----------|
| Aquatic 4# | 0.15±0.00c,m | 0.10±0.00d,l | 0.09±0.00b,l |
| Basal   | 0.07±0.00a,n | 0.03±0.01a,m | 0.01±0.00a,l |
| SE      | 0.17±0.01d,n | 0.06±0.00b,l | 0.09±0.00b,m |
| HGZ     | 0.13±0.01c,m | 0.11±0.00d,l | 0.14±0.00c,m |
| MAV     | 0.11±0.02b,m | 0.08±0.01c,m | 0.13±0.01c,l |

Tips: a, b, c, d and e showed the significant difference between groups in a column; l, m and n showed the *significant* difference between groups in a line; different letter stands for significant difference between groups or periods, while same letter means no significant difference in a column or line.

3.2. Analysis of the Amino Acid Component

According to the results (Tab. 2), *C. Pyrenoidosa* consisted of 18 amino acid (42.19 ~ 43.14%), which contained 8 human essential amino acids (hEAA) with a high concentration (16.12 ~ 16.44%). While the concentration of fish essential amino acid (fEAA) was 18.36 ~ 19.67%. Essential amino acid (EAA) is an important nutrition index, the EAA of *C. Pyrenoidosa* were higher than the other plant feedstuff. Such as defatted soybean cake (12.64%), defatted peanut cake (9.81%) and alfalfa meal (5.81%). Thus, *C. Pyrenoidosa* is a good resource for feedstuff.

Post Hoc Multiple Comparisons of different groups results revealed that the medium could affect the amino acid component of *C. Pyrenoidosa*. 
Table 2. Amino Acid Component of different groups

| Amino Acid                     | HGZ       | Aquatic 4# | Basal      |
|--------------------------------|-----------|------------|------------|
| Total Amino Acid               | 43.14±0.11A | 43.10 ±0.35B | 42.19 ±0.15B |
| human essential amino acid     | 16.44±0.09a | 15.71±0.18c | 16.12±0.13b |
| fish essential amino acid      | 19.67±0.06M | 18.36±0.28O | 18.86±0.04N |
| fish non-essential             | 23.47±0.11m | 24.74±0.08m | 23.32±0.17n |
| fEAA/TAA                       | 0.46  | 0.43       | 0.45       |
| fEAA/NEAA                      | 0.84  | 0.74       | 0.81       |

tips: A and B showed the difference of total amino acid content in each group.
a, b and c showed the difference of human essential amino acids in each group.
M, N and O showed the difference of essential amino acids in fish in each group.
M and n showed the difference of nonessential amino acids in fish in each group.

4. Conclusion

*C. Pyrenoidosa* is rich in amino acids and high ratio of EAA/TAA. For their high protein quality, *C. Pyrenoidosa* could satisfy the basic growth needs of animals and reach the FAO "protein model" standard. All fish essential amino acids could be detected, and the concentration of several fEAA, such as leucine, lysine, valine, phenylalanine, arginine and threonine, were higher than 5%. According to Yuping Deng’s and Kai Lu’s researches, leucine can significantly improve the fish quality of grass carp and the intestinal antioxidant condition, who would improve immunity of fish and promote production [8-9]. Most fish’s lysine requirement was 3.3-8.5% of diet. Lacking of lysine, fish would hesitate to intake fodder and be easy to get sick even to lost their lives.

Besides high nutrition, *C. Pyrenoidosa* is a kind of high-quality initial feeding for fish and shellfish. Unlike other fodder used in aquiculture, *C. Pyrenoidosa* could also improve the water quality and avoid fodder corruption in water. However, methionine (0.35%) and tryptophan (0.38%) could be the primary and secondary limiting amino acid for most fish for their low concentration. It is said by JIA Peng and Luo that methionine content of fodder was positively correlated with fish quality (protein and fat) [10-11]. Therefore, it is necessary to pay attention to the supplement of methionine, cysteine and other amino acids. In addition, *C. Pyrenoidosa* also contains a lot of nutrient material, such as vitamin, carotene, which could play a positive role in improving immunity.

The growth rate of *C. Pyrenoidosa* varied significantly among different culture media and conditions. The variation of amino acids components was relative modest. Neither HGZ nor Aquatic No. 4 can meet all the amino acids needs of fish. Extra ones would be added into the fodder. Therefore, the application of *C. Pyrenoidosa* as feeding or additive should take more consideration of its growth rate and reproducing stability (at high density) rather than the components of amino acid. Thus, HGZ would be the best choice for aquaculture application.

Furthermore, *C. Pyrenoidosa* is also widely used in water quality and algal bloom control [12-13]. However, there are many problems in the application of aquaculture, such as unstable algae quality. Therefore, the research on how to standardize the chlorella culture system, concentration, storage and transportation should be carried out to make greater and wider usage in aquiculture.
Acknowledgment
This research was financially supported by “Qing Lan Project of Jiangsu Province”. This work was supported by “311 Talents Project in Taizhou”, Research projects of the Jiangsu agri-animal husbandry vocational college (No. NSF201711; NSFHP201910) and Investigation and evaluation on culture system of Chlorella Pyrenoidosa (11710119010)

References
[1] Yang, et al., Growth of Chlorella pyrenoidosa in wastewater from cassava ethanol fermentation. World Journal of Microbiology & Biotechnology, 2008. 24(12): p. 2919-2925.
[2] Shen, N.N. and LI, Application of Chlorella pyrenoidosa and Bacillus lichenifirmis for water quality control in shrimp culture. Marine Fisheries Research, 2008.
[3] Jiang, D.S., et al., Acute toxicity of three typical pollutants to aquatic organisms and their water quality criteria. Environmental Science, 2014. 35(1): p. 279-285.
[4] Wang, J., Effects of transgenic Bt rice on several typical aquatic organisms. 2014, Yangzhou University.
[5] LiFei, GUOJian-lin, and ZHANGAi-ju, Effects of three culture media on the growth of Chlorella and its application in culture of freshwater rotifers. Journal of Biology, 2016. 33(1): p. 109-111.
[6] Ma, X., W. Xue, and Y. Wei, Effects of Different Mediums and Shake on the Growth of Mieoreystis Aeruginosa and Production of Mieorcystins. Journal of Shanxi Agricultural University(Natural Science Edition), 2009. 29(2): p. 157-160.
[7] Gao, X., et al., A comparative study on total lipid and fatty acid composition of five newly isolated marine diatoms. Journal of Biology, 2014. 31(1): p. 60-63.
[8] Deng, Y., Effects of graded levels of dietary leucine on growth, flesh quality, intestinal immunity and antioxidant status in young grass carp (Ctenopharyngodon idella). 2014, Sichuan Agricultural University.
[9] Zhou, F., Study on effects of dietary lysine and arginine on growth performance and the arginine/lysine antagonism mechanism in juvenile black sea bream, Acanthopagrus schlegelii. 2011, Zhejiang University.
[10] JIAPeng, et al., Effects of dietary methionine levels on the growth performance of juvenile gibel carp (Carassius Auratus gibelio). Acta Hydrobiologica Sinica, 2013. 37(2): p. 217-226.
[11] Zhi, L., et al., Dietary l-methionine requirement of juvenile grouper Epinephelus coioides at a constant dietary cystine level. Aquaculture, 2005. 249(1): p. 409-418.
[12] Xinjian, Z., et al., Study on Chlorella pyrenoidosa and photosynthetic bacteria as water quality modulator in turbot larvae culture. MARINE FISHERIES RESEARCH, 2008. 29(6): p. 116-121.
[13] Olguin, et al., Comparative sensitivity of Scenedesmus Acutus and Chlorella Pyrenoidosa as Sentinel Organisms for Aquatic Ecotoxicity Assessment: studies on a highly polluted urban river. Environmental Toxicology, 2015. 15(1): p. 14-22.