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The Effects of Nutritional Media and Initial Cell Density on the Growth and Development of *Spirulina platensis*

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Abstract: The main objective of the study was to determine the effects of nutritional media and initial cell density on the growth and development of *Spirulina platensis*. This study was carried out at the Center for Incubation and Technology Transfer, Hue University from May 2015 to August 2016. In the first experiment, *S. platensis* was cultured in four different nutritional media, including Zarrouk, Spi-RIA3, Nisole and Thuoc medium (Vietnamese). Resulted showed that *S. platensis* grew significantly in the Zarrouk medium rather than in other nutritional medium at the same time, and gained the maximum density of 2,736.5 mg/L after 13 d inoculums, with specific growth rate of 4.987% per day and the time to doubling population of 0.139 d. In the second experiment, *S. platensis* was cultured in six different initial cell densities: 100, 400, 700, 1,000, 1,300 and 1,600 mg/L, and the optimum nutritional medium was Zarrouk medium selected from experiment one. Results showed at the initial culture density of 400 mg/L, the highest biomass was gained with the maximum density of 3,071.2 mg/L after 21 d inoculums, with specific growth rate of 9.15% per day and time to double population of 0.076 d. *S. platensis* biomass from culture in Zarrouk medium at initial cell density 400 mg/L obtained total protein of 51.98%, total lipid of 1.75%, total carbohydrate of 0.75%, carotene of 0.36% and total minerals of 5.2%, on a basis of dry matter.

Key words: *Spirulina platensis*, nutrient medium, initial density, growth rates.

1. Introduction

Spirulina platensis is a microalgae species with high economic and nutritional value. Most studies have shown that *Spirulina* is rich in protein, up to 60%-70%, while this number is just 21% in beef, 20.3% in chicken, 19% in pork and 20% in fish. It also contains other ingredients, such as lipids 4%-7%, carbohydrates 13.6%, unsaturated fatty acids 18%, xanthophyll 0.22%, chlorophyll 1.0% and phycocyanin 14% [1]. *Spirulina* contains more than 18 kinds of amino acids, with all eight essential oils, like isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, and fatty acids non-substitute, such as linoleic acid (C18:2) and γ-linoleic acid (C18:3) [1, 2]. Especially, the highest levels are leucine (10.9% total amino acid), valine (7.5%) and isoleucine (6.8%) [3]. Due to the absence of cellulose cell walls, *Spirulina* is easy to digest [4]. In addition, *Spirulina* is not only an excellent source of vitamins, such as vitamin A, E and B (B1, B2, B6 and B12), but is also rich in essential minerals, such as potassium, calcium, magnesium, iron, zinc and fiber. Furthermore, *Spirulina* also contains many important anti-aging substances, such as phycocyanin, chlorophyll and carotene.

Due to the enormous positive impact on human health, *Spirulina* has been considered as an ideal nourishment food for twenty-first centuries [5]. Therefore, *Spirulina* is a very popular food source in some developed countries, such as Japan, USA and

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Germany, etc., whereas Vietnam is not paid much attention because of the complex and expensive breeding method. Therefore, the utilization of cheap and available materials in household to product *Spirulina* biomass not only meets the demand about culturing *Spirulina* at home, but can also take the advantages of human health improvement. Although it is a small culture system, it also needs to be standardized for optimal production and economic effectively. *Spirulina* is a type of multicellular and filamentous cyanobacterium which needs to synthesize nutrition for itself by photosynthesis. Sunlight is the main source of energy for the growth and development of algae. In addition, the nutritional components, like carbon, nitrogen, phosphorus and micronutrients directly affect the algal growth, especially in the high-density conditions [6-9]. The pH level is also a prominent factor for the development of algae [10].

This paper aimed to evaluate the effect of the nutrient medium and initial cell density on the production of *S. platensis* in greenhouse condition with light control, natural temperature and using domestic water for aquaculture environment.

2. Materials and Methods

2.1 S. platensis for Multiplication

Pure *S. platensis* was supplied by the Institute of Microbiology and Biotechnology, Hanoi National University, Vietnam. Before multiplication, the algae seeds were tested for contamination and then cultured in Zarrouk medium which was contained in glass bottles or clear plastic bottles to produce primary seedling pots for research. The *Spirulina* biomass was harvested with a 20 µm diameter filter. Next, it was weighted by analytical balance to measure the quantity before adding to the different treatments.

2.2 Experimental Layout

2.2.1 Experiment 1: Effect of Various Nutrient Media on the Growth and Development of *S. platensis*

The algae were respectively cultured in four nutrient media: Zarrouk medium (E1) [11], Spi-RIA3 medium (E2) [12], Nisole medium (E3) [12] and Thuoc medium (E4) [13]. The initial cell density culture was 500 mg/L.

2.2.2 Experiment 2: Effect of Initial Cell Density on Growth and Development of *S. platensis*

The experiment included six treatments with different initial cell densities: 100 mg/L (D1), 400 mg/L (D2), 700 mg/L (D3), 1,000 mg/L (D4), 1,300 mg/L (D5) and 1,600 mg/L (D6). The optimum nutritional media to culture *Spirulina* algae was selected from experiment one.

The treatments for these two experiments are randomly placed to 5 L transparent plastic containers with three replications. Greenhouse condition is affected by natural condition, such as light and temperature, and aeration is maintained continuously for all treatments. The water used is chlorine-treated household water. Measurable indicators are algae growth rate, pH, temperature, time to duplicate the generation and algae density.

2.3 Monitoring Methods and Indicators Measurement

2.3.1 Environmental Factors

The pH was measured with a pH meter (Hanna HI98127) with pH from 2.0 to 16.0 and pH accuracy ± 0.1, and temperature was measured with Hanna HI98127 in a range from 5.0 ℃ to 60.0 ℃ and temperature accuracy ± 0.5 ℃. The frequency of measurement was two times per day at 8:00 am and 14:00 pm, respectively.

2.3.2 Measurement of Algae Growth Indicators

The optical density at 420 nm (OD_{420nm}) was measured on the spectrophotometer machine [12].

The specific growth rate (*SGR*; %/d) is calculated according to Eq. (1) [14]:

$$SGR = \frac{\ln(OD_t) - \ln(OD_o)}{t} \times 100\% \qquad (1)$$

where, OD_o: initial optical density; OD_t: maximum optical density; t: time of algae reaching the maximum density.

The time to double generation (t_d) is defined by Eq. (2) [14]:

$$t_d = \frac{\ln 2}{SGR} \quad (2)$$

S. platensis biomass carrying out from optimum conditions was analyzed by the following biochemical composition:

(1) Fresh biomass after harvesting was dried in a Panasonic oven MOV-212-PK at 0 ℃ and then analysed;

(2) Total protein content was determined by the Bradford method [7];

(3) Total lipid content was determined by the Soxhlet method [15];

(4) Total mineral content is determined by the principle: organic substances are completely burned by heat (550-600 ℃), and then the rest one is weighted and calculated the percentage of total minerals in the samples [16];

(5) Total carbohydrate content was determined by phenol-sufuric acid method [17];

(6) The carotene content was determined by Tzirel's method with 0.072% potassium bicromat scale [16].

2.4 Statistical Analysis

Data were statistically analysed using Minitab software version 16.2.0 and Microsoft Excel 2007 to calculate the mean and standard deviation. ANOVA was used to identify significantly different means compared between the nutrient media treatments and between the initial density treatments at a probability level of $P \leq 0.05$.

3. Results and Discussion

3.1 Effects of Four Nutrient Media on Growth and Development of S. platensis

The growth and development of *S. platensis* in Zarrouk (E1), Spi-RIA3 (E2), Nisole (E3) and Thuoc medium (E4) is shown in Table 1 and Fig. 1. After 17 d multiplication, there was no significant difference in the temperature and pH factors between these four nutrient media. The temperature was from 30.6 ℃ to 33.1 ℃ and pH from 9.04 to 10.62.

3.1.1 The Maximum Density of Algae

S. platensis with the same initial cell density of 500 mg/L in the different nutrient media had an adaptive phase during the first 5 d of the culture period. At this time, the cell density of *S. platensis* in these nutrient media is no substantially different. However, from day 6 onwards, there was a significant difference in *S. platensis* cell density between the treatments. The algal density increased rapidly and reached the highest level at day 15 in E2 medium, day 13 in E1 and E3 medium. In particular, in E1 medium, there was the highest maximum density of 2,736.5 mg/L, followed by E3 and E2 with the highest density of 2,573.7 mg/L and 2,559.5 mg/L, respectively. In these nutrient media, *S. platensis* had a balanced phase from day 12 to day 15 of breeding process, and time of the dead phase was after the day 15.

However, in the E4 medium, the algae cell density increased slowly and reached a peak after 9 d (880.7 mg/L), the day 10 was the time of death phase and the death phase in this nutrient medium happened quicker

Table 1 Growth and development indicators of *S. platensis* in different nutrient media.

Treatments	Maximum optical density (mg/L)	Specific growth rate (%/d)	Time to reach maximum optical density (d)	Time to duplicate generation (d)
E1	2,736.5 ± 39.4^{a}	4.987 ± 0.316^{a}	13	0.139 ± 0.009^{b}
E2	2,559.5 ± 85.1^{b}	3.600 ± 0.253^{b}	15	0.193 ± 0.014^{a}
E3	2,573.7 ± 15.4^{b}	4.454 ± 0.459^{ab}	13	0.157 ± 0.015^{b}
E4	880.7 ± 14.5^{c}	-	9	-

-: undefined.

$^{a\text{-}c}$ Values with different letters on the same column showed significant difference ($P < 0.05$).

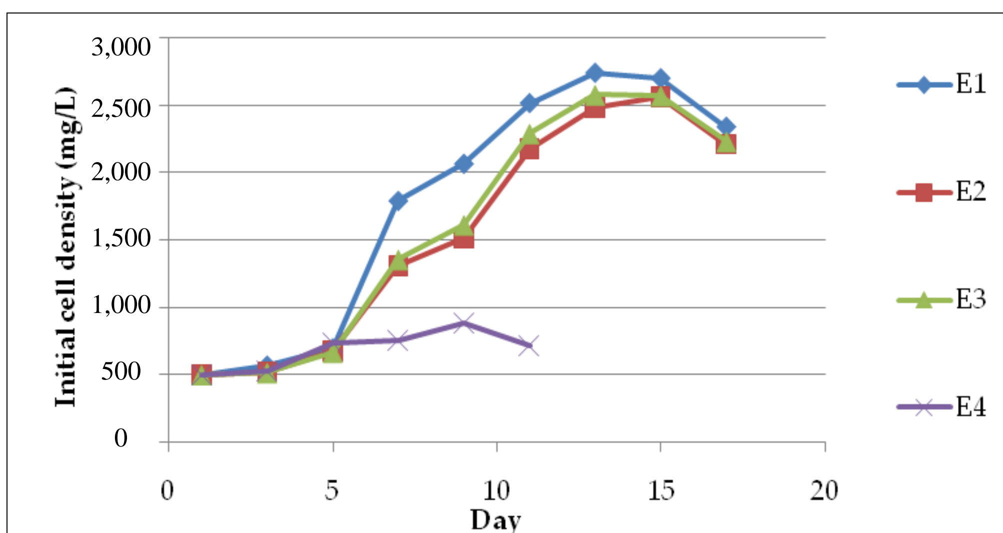

Fig. 1 Growth curve of *S. platensis* in different nutrient media.

than that in three others. Thus, the fluctuation of algal cell density in E4 medium revealed that E4 medium was not suitable for the growth of *S. platensis*.

3.1.2 Double Generation Time

Double generation time of *S. platensis* was not the same in different nutrient media. The longest time was in E2 medium (0.193 d) and the statistical analysis indicated a significant difference to other treatments ($P < 0.05$). After that, the time to duplicate generation in E3 and E1 were 0.157 d and 0.139 d, respectively. There was no significant difference between E3 and E1 nutrient media ($P > 0.05$).

3.1.3 Specific Growth Rate (%/d)

The specific growth rate of *S. platensis* was the highest in E1 (4.987%/d), followed by E3 (4.454%/d) and the lowest in E2 (3.600%/d). The statistical analysis showed that the growth rate of *S. platensis* in E1 was not significantly different from E3 ($P < 0.05$), but the difference was statistically significant compared to E2 ($P < 0.05$).

The substance compositions in different nutrient media affect the maximum cell density and specific growth rate of algae. Although all four nutrient media contain N, P and C, there is a difference of the N and P sources. While N and P sources in E4 are from $(NH_4)_2SO_4$ and $Ca(H_2PO_4)_2$, correspondingly, others are from $NaNO_3$ and K_2HPO_4, respectively. However, all four nutrient media have the same C sources ($NaHCO_3$). In addition, the comparison between the nutrient media indicates that $NaHCO_3$ content in E2 and E3 media have lower than that in E1 and E4 media. On the other hand, E4 medium has the lowest level of N content, which may lead to a lower growth of *S. platensis* in this medium. Although E2 and E3 mediums are similar in N, P and C content, the E2 medium composition does not have micronutrients. Therefore, the specific growth rate of *S. platensis* in E2 was lower than that in E3 medium.

In the investigated nutrient media, Zarrouk (E1) medium has the highest of $NaNO_3$ and $NaHCO_3$ and micronutrients content, so *S. platensis* grew better than other media. The results in this study are similar to the findings of Dineshkumar et al. [18] on *S. platensis* in Zarrouk, F/2, Conway, BG11 and seawater media. Based on the above results, the medium of zarouk was selected as the culture medium for *S. platensis* to carry out subsequent experiments.

3.2 Effects of Initial Cell Density on Growth and Development of S. platensis

In addition to the nutrient media that directly affects to the growth of algae, initial cell density is also a factor to consider because it directly impacts the competition in nutritional demand as well as light

demand of algae. The results of initial cell density effect on the growth and development of *S. platensis* are presented in Table 2 and Fig. 2. In this experiment, the temperature varied from 27 ℃ to 34.5 ℃ and pH ranged from 8.5 to 10.8.

3.2.1 The Maximum Density of Algae

The results showed that D1 treatment with the lowest initial density (100 mg/L) reached to the maximum density after 23 d culture. In contract, in the initial cell densities of 400 mg/L (D2), 700 mg/L (D3) and 1,600 mg/L (D6), *S. platensis* reached the maximum density after day 21, 19 and 13, respectively. Particularly, in two treatments of 1,000 mg/L (D4) and 1,300 mg/L (D5), algae reached the maximum density after day 15. The highest maximum density at the balanced stage was in D2 treatment with 3,071.2 ± 120.0 mg/L, and it was significantly different from the treatments including D1, D4, D5 and D6 ($P < 0.05$), but not different from D3 ($P > 0.05$). The maximum cell density level in others treatments was 2,664.7 mg/L in D1, 2,763.7 mg/L in D3, 2,640.6 mg/L in D4, 2,683.7 mg/L in D5 and 2,586.1 mg/L in D6, and these treatments were not statistically significant difference ($P > 0.05$).

3.2.2 Specific Growth Rate (%/d)

The specific growth rate of *S. platensis* differentiated in different initial densities. The *S. platensis* cultured in D1 treatment showed the highest growth rate (11.33%/d), followed by 9.15%/d in D2 and 5.61%/d in D3, and this figure gradually decreased in others treatments, like D4 (4.32%/d), D5 (4.38%/d) and D6 (4.26%/d). The statistical analysis showed that the typical growth rate of *S. platensis* was significantly different between D1, D2

Table 2 Growth and development of *S. platensis* indicators in different initial cell densities.

Treatments	Maximum density (mg/L)	Specific growth rate (%/d)	Time to reach the maximum density (d)	Time to duplicate generation (d)
D1	2,664.7 ± 138.3^{b}	11.33 ± 0.21^{a}	23	0.061 ± 0.001^{c}
D2	3,071.2 ± 120.0^{a}	9.15 ± 0.18^{b}	21	0.076 ± 0.001^{c}
D3	2,763.7 ± 144.5^{ab}	5.61 ± 0.26^{c}	19	0.124 ± 0.006^{b}
D4	2,640.6 ± 43.0^{b}	4.32 ± 0.10^{d}	15	0.161 ± 0.004^{a}
D5	2,683.7 ± 101.0^{b}	4.38 ± 0.23^{d}	15	0.159 ± 0.008^{a}
D6	2,586.1 ± 132.7^{b}	4.26 ± 0.37^{d}	13	0.163 ± 0.013^{a}

$^{a\text{-}d}$ Values with different letters on the same column showed significant difference ($P < 0.05$).

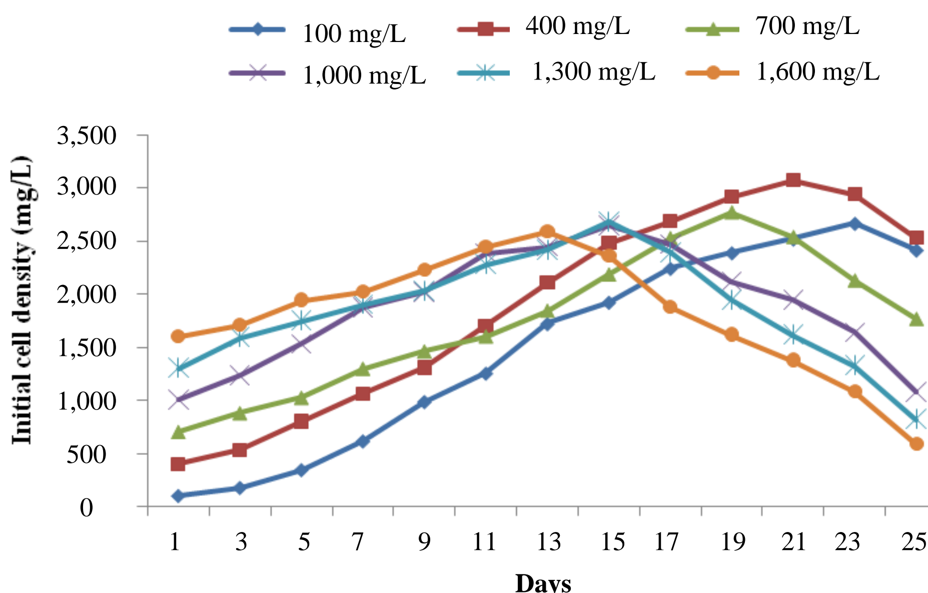

Fig. 2 Growth curve of *S. platensis* in different initial cell densities.

and D3 treatment, and this number differentiated from the other treatments as well ($P < 0.05$). Specific growth rates of algae in D4, D5 and D6 were not significantly different from each other ($P > 0.05$), but significant differences as compared to other treatments ($P < 0.05$).

3.2.3 Time to Double Generation

Time to double generation of *S. platensis* was the shortest in D1 (0.061 d) and D2 (0.076 d), and the statistic was significantly different in comparison with others ($P < 0.05$). It took 0.124 d to duplicate generation of *Spirulina* in the D3 treatment, and there was significant difference compared with the other treatments ($P < 0.05$). The longest duplication time was 0.161 d in D4, 0.159 d in D5 and 0.163 d in D6, respectively. There was no significant difference between these treatments ($P > 0.05$), but significantly different from the other treatments ($P < 0.05$).

In the initial high-density plots, the number of algae cells developed over lag phase which was larger than the low-density plots. After experiencing proliferation, dividing by exponent phase, the number of cells grew faster than that in low-density plots so that the maximum cell density reached a peak more quickly. But the higher of the algal density is, the greater of the light covert between the algal cells will be, so it leads to the limited photosynthesis, resulting in the algae typical growth rate in the high-density plots lower than that in the low-density plots [12].

According to a study done by Vonshak et al. [19], the photosynthetic capacity of *S. platensis*is will decrease when the density is from 0.4 g/L to 1.0 g/L. Furthermore, about 80% *S. platensis* is sometimes obscured and must be in the dark at a density of 0.5 g/L. Gitelson et al. [20] report that specific growth rates of *S. platensis* will tend to decrease, if it is bed in high-density due to the effect of the cover. These authors conclude that when culture conditions are ideal, the specific growth rate of algae can reach at high level in the initial low-density system. Radmann et al. [21] indicate that the specific growth rate of algae will be high level, while the cell density is low at the beginning. To illustrate, the typical algal growth rate in the plots with an initial density of 0.4 g/L was higher than those of 0.6 g/L and 0.8 g/L. However, the study results of Vonshak et al. [22] confirm that if the cell density of algae is lower than 0.1 g/L, it will cause photosynthetic resistance because of light intensity to individual algal cells.

Thus, the culture of *S. platensis* at the initial density of 400 mg/L (D2) is the best in this research by comparing the maximum density and doubling times generation. On the other hand, if the treatments are compared to the specific growth rate and the time of duplicated generation, the initial density of 100 mg/L (D1) provides the best performance. However, for the purpose of obtaining the highest biomass of *S. platensis* (maximum density), the most appropriate initial cell density should be 400 mg/L.

3.3 Content of Nutrients in S. platensis Biomass

To evaluate the quality of algae biomass obtained at the initial density of 400 mg/L in Zarouk medium after harvesting, the fresh algae was dried in a dry oven at 50 ℃ and then used to analyze biochemical components.

The biochemical components in *S. platensis* biomass were 51.98% total protein content, 1.75% total lipid content, 0.75% total carbohydrate, 0.36% carotene and 5.2% total minerals, all on a basis of dry matter (DM) (Table 3).

According to Thuoc et al. [23], when *S. platensis* is bred in Vinh Hao mineral water with supplemental fertilizer, biomass is obtained with protein content of 65.94% DM and lipid content of 1.12% DM,

Table 3 Biochemical components of *S. platensis*.

Biochemical indicator	Content (%DW)
Total protein	51.98
Total lipid	1.75
Total carbohydrate	0.75
Total mineral	5.20
Carotene	0.36

respectively. Nam and Hong [24] used Thanh Tan mineral water to feed *S. platensis* C1 in Zarrouk medium, and the biomass of *S. platensis* C1 had protein content from 51% to 52% DW. In particular, with Z8 medium [25], *S. platensis* biomass has lipid content of 11.86% DW. Lien and Vi [26] reported that *S. platensis* cultured in Thanh Tan mineral water with supplemental fertilizers produced biomass with 55.88% DW protein, 6.56% DW lipid and 18.7% DW carotene, while algae biomass containing 52.78% DW protein, 7.20% DW lipid and 21.0% DW carotene when it is cultured in Z8 medium.

In this study, the biomass of *S. platensis* cultivated in domestic water has a lower carotene content than that in mineral water. However, protein and lipid content are quite high. Therefore, it is possible to mix Zarrouk medium in domestic water to feed *S. platensis* to reduce production costs while obtaining good quality products.

4. Conclusions

The study showed that the different nutrient media and initial cell densities significantly effected the production of *S. platensis*. *S. platensis* grew and developed well with the highest biomass efficiency in the Zarrouk medium. In addition, when changing the initial cell density, the growth and development of *S. platensis* were different depending on the density. The initial density of 400 mg/L gave the highest biomass of *S. platensis* with the highest density of 3,071.2 mg/L after 21 d culturing. Furthermore, biochemical compositions of *S. platensis* cultured in Zarrouk medium with 400 mg/L initial density were obtained as the following: total protein content of 51.98% DW, total lipid of 1.75% DW, total carbohydrate of 0.75% DW, carotene of 0.36% DW and mineral of 5.2% DW, respectively. Therefore, the use of Zarrouk media to culture with 400 mg/L initial density *S. platensis* is significantly efficient for biomass production in the household system.

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