e-ISSN: 2757-8836
ORIGINAL ARTICLE
J. Raw Mater. Process. Foods vol.2(2) (2021) 49-57

Recieved: 17/11/2021 Revised: 10/12/2021 Accepted article published: 29/12/2021 Published online: 30/12/2021

Production of *Spirulina platensis* Biomass Under Different Temperature and Nitrogen Regimes

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Abstract

Spirulina **is of the worldwide cultivated and consumed microalgae. It is generally used directly or as an additive in the food industry due to its high protein content. Besides the high protein content Spirulina biomass contain important fatty acids, (e.g., GLA), vitamins, minerals, and some bioactive compounds, that are affected by the parameters of biomass cultivation. In the presented study, the limitation of nitrogen (25%, 50%, 75% and 100% N concentration) and temperature fluctuations (25°C and 30°C) on *Spirulina platensis* growth parameters, biomass, protein, total phenolic, total carotenoids and antioxidant capacity were investigated and the production of *Spirulina platensis* was optimized in terms of biomass and metabolites.While the amount of biomass increased in general with the increase in temperature, dry weight decreased. The highest level of the protein accumulation was determined at 30°C and Spirulina medium with 100% N concentration. Protein, total phenolic substance, and total carotenoid amounts were found at the higher level with** the temperature increase to 30°C in all samples.

Keywords: Spirulina platensis, biomass, nitrogen regime, temperature, growth parameters, bioactive contents

1. INTRODUCTION

Increase in the world population and the scarcity of resources has simulated searching for alternative food sources. Algae, seaweeds and microalgae, have been studied mostly as an alternative food source. Algal researches have been carried since 1950s and microalgae, especially *Spirulina, Chlorella* and *Dunaliella,* have been produced industrially since 1970s and is used in different product groups (Wells et al., 2017).

Spirulina sp. is one of the blue green microalgae with the most interesting among the others, which has been cultivated and consumed for its high protein content, valuable amino acid profile, and rapid growing and easy cultivation behaviors. However, blue-green microalgae species like *Spirulina* sp. are promising resources for polyunsaturated fatty acids (PUFA), primarily gamma linolenic acid (GLA), blue pigment "phycocyanin", and plenty of polyphenols. Many of these compounds, named as bioactive, are having a potential as pharmaceutical for their potential of preventing some chronic diseases such as cancer, improving the triacyl-glycerol level in blood, blood pressure, inhibiting the formation of pro-inflammatory leukotrienes and regulating of immune system function (Gershwin & Belay, 2007; Vonshak et al., 1982). Additionally, *Spirulina* sp. has been approved as GRAS (generally recognized as safe) and suggested as a remedy for malnutrition.

It is known that the cultivation parameters closely affect the biomass production with its chemical composition (Göksan et al., 2007). *Spirulina* sp. growth parameters are directly affected by temperature, pH, light intensity, and nutrient composition. High penetration can occur during the light intensity, nitrogen and phosphorus concentration and temperature changes.

According to these interactions, the aim of the presented study was to determine the effects of medium type, different nitrogen concentrations and temperature on biomass growth parameters, protein, total phenolic, total carotenoid contents, and total antioxidant capacity.

2. MATERIALS AND METHODS

2.1 Strain and Culture Media

Spirulina platensis (UTEX 2356) strains were procured from UTEX, Culture Collection of Algae, Texas, Austin. The whole study was carried out in Yalova University, Armutlu Vocational School, Algae Production Facility.

Cells were maintained with two different media: **Spirulina medium** (13.61 g $NaHCO_3$, 4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 2.50 g $NaNO_3$, 1.00 g K_2SO_4, 1.00 g NaCl, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_2O$. All nutrients were dissolved in distilled water containing (per liter): 6 mL of metal solution (97 mg $FeCl_3 \cdot 6H_2O$, 41 mg $MnCl_2 \cdot 4H_2O$, 5 mg $ZnCl_2$, 2 mg $CoCl_2 \cdot 6H_2O$, 4 mg $Na_2MoO_4 \cdot 2H_2O$), 1 mL of micronutrient solution (50.0 mg Na_2EDTA, 618 mg H_3BO_3, 19.6 mg $CuSO_4 \cdot 5H_2O$, 44.0 mg $ZnSO_4 \cdot 7H_2O$, 20.0 mg $CoCl_2 \cdot 6H_2O$, 12.6 mg $MnCl_2 \cdot 4H_2O$, 12.6 mg $Na_2MoO_4 \cdot 2H_2O$) and 0.15 mg of B12 vitamin), and **Zarrouk medium** (18.0 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1.0 g K_2SO_4, 1.0 g NaCl, 0.04 g $CaCl_2$, 0.08 g Na_2EDTA, 0.2 g $MgSO_4 \cdot 7H2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 1.0 ml micronutrient solution (2.86 mg H_3BO_3; 0.02 mg $(NH_4)_6Mo_7O_{24}$; 1.8 mg $MnCl_2 \cdot 4H_2O$; 0.08 mg Cu_2SO_4; 0.22 mg $ZnSO_4 \cdot 7H_2O$).

For experiments *Spirulina platensis* were pre-cultivated in 250 mL erlenmayer flasks. Precultures were inoculated to 500 mL, 1 L, 2 L and 10 L (Fig. 1) respectively, with the inoculum rate of 2%.

Cultivation pre-trials were carried out with Zarrouk medium, at 25±2°C, 30±2°C and 35±2°C without any changes in medium component concentrations. At 35°C, the growth period was rapid and reached the death phase after 72 hours. For this reason, growth parameters and bioactive contents were analysed in cultures grown at 25°C and 30°C.

Figure 1. Spirulina platensis cultivation

Two different growth media (Spirulina and Zarrouk), temperatures (25±2°C and 30±2°C) and four different "$NaNO_3$" concentrations, which was chosen as the limiting nitrogen source, were chosen for the evaluation of biomass production (Table 1). During the trials, the temperature was maintained by using an air conditioner and a lightening regime with a 14:10 hour light-dark period (32-40 µmol photon $m^{-2}s^{-1}$) was applied. The light intensity of the environment was measured with a light meter (Licor, LI-250).

Table 1. Trial design of the study

Run No	Coded Values	Medium Type	Temperature (°C)	$NaNO_3$ Concentration
4	*1A-100*	Spirulina	25	100
1	*1A-25*	Spirulina	25	25
2	*1A-50*	Spirulina	25	50
3	*1A-75*	Spirulina	25	75
12	*1B-100*	Spirulina	30	100
9	*1B-25*	Spirulina	30	25
10	*1B-50*	Spirulina	30	50
11	*1B-75*	Spirulina	30	75
8	*2A-100*	Zarrouk	25	100
5	*2A-25*	Zarrouk	25	25
6	*2A-50*	Zarrouk	25	50
7	*2A-75*	Zarrouk	25	75
16	*2B-100*	Zarrouk	30	100
13	*2B-25*	Zarrouk	30	25
14	*2B-50*	Zarrouk	30	50
15	*2B-75*	Zarrouk	30	75

Cultures were aerated with 2 L min^{-1} during growth period, harvested by filtration (20 µm mesh) and washed three times with deionized water to eliminate medium salts. Obtained wet biomass was freeze-dried at -60°C overnight to obtain dry biomass, weighed and kept at -80°C for analysis.

2.2 Analytical methods

Daily biomass concentration determined with indirect method based on cell suspension optical density (OD) measured at 670 nm using a UV-Vis spectrophotometer. Final biomass concentration was determined according to Vonshak (1982). Protein was determined by the Kjeldahl method according to AOAC standard methods (AOAC, 2005).

Fatty acid profiles were measured by the method described by Akpınar-Bayizit et al (2014).

Phenolic extraction was assessed as described by Goiris et al (2012). 0.1 g of dry biomass was weighed, and 2 mL ethanol:water (3:1) mixture was added. After shaking the mixture for 30 minutes at room temperature, it was centrifuged at 4 500xg for 10 minutes, this process was repeated 2 times and the supernatants were collected. Total phenols of the extracts were assessed using the Folin-Ciocalteau method (Singleton & Rossi, 1965) and the results were expressed as mg gallic acid equivalent (GAE) g^{-1} dry biomass. Antioxidant capacity of microalgal extracts was determined by free radical scavenging assay DPPH (Wang et al., 2009). A calibration curve was prepared, Trolox and the results were given as µmole trolox equivalent (TE) g^{-1} dry biomass. For total carotenoid content, ethanolic extracts were prepared following the method described above, for phenolic extraction. Total carotenoids were determined spectrophotometrically and calculated by the Lichtenthaler equation (Lichtenthaler & Buschmann, 2001).

$$(1): Ca = 16{,}82 \times A665 - 9{,}18 \times A652$$

$$(2): Cb = 34{,}09 \times A652 - 15{,}28 \times A665$$

$$(3): C(x+c) = 1000 \times A470 - 1{,}63 \times Ca - 14{,}96 \times Cb$$

Ca; Chlorophyll a, Cb; Chlorophyll b, C(x+c); Xanthophyll and Carotenoid, A470; absorbance value at 470 nm, A652; absorbance value at 652 nm and A665; absorbance value at 665 nm.

2.3. Statistical analysis

The descriptive statistics of the data obtained in the study and the correlations between the data were made with JMP (Version 7.0, SAS, Institute Inc. Comp., NC, USA). Results are shown as the mean ± standard deviation of 4 replicate measurements.

3. RESULTS AND DISCUSSION

3.1 Growth parameters and protein

Many studies have been carried out on *Spirulina platensis* in the last two decades. These studies were generally carried out in wastewater (Bezerra et al., 2020; Cardoso et al., 2020; Krishnamoorthy et al., 2019) and in synthetic mediums (Alberto Vieira Costa et al., 2004; Colla, Furlong, et al., 2007; Colla, Oliveira Reinehr, et al., 2007; de Jesus et al., 2018, 2019; Karemore et al., 2020; Mehar et al., 2019; Silva Benavides et al., 2017; J. Wang et al., 2019) for both biofuel, food and pharmaceutical applications. Studies reported some modifications for Zarrouks' medium, using cheaper nitrogen sources like $(NH_4)_2SO_4$ and $NaNO_3$ (Rodrigues et al., 2011) or NH_4Cl (Carvalho et al., 2004). Ragaza et al. (2020) recently revised the culture media modifications, other alternative media and outcomes of these modifications on producing *Spirulina*.

In the presented study two synthetic mediums were used for the cultivation of *Spirulina platensis* with various N concentrations to obtain food grade biomass. The optical density data and the growth parameters and chemical analysis results were given in Figure 2 (25°C), Figure 3 (30°C) and Table 2, respectively. Temperature effects the cultivation time inversely, as can be seen by the comparison of Fig.2 and Fig.3, at 30°C approximately two-fold decrease determined for cultivation time. While an increase in optical density values was observed with the decrease in N ratio at 25°C in Spirulina medium, a decrease was observed in the maximum OD value at 30°C. In Zarrouk medium, however, changes in N ratio did not have a positive effect on OD values. Budiyono et al. (2013) recorded the highest OD value for *Spirulina platensis* in the medium where the C:P:N ratios were 76.3:11.7:1 and reported that the OD values were decreased as the C and N ratios were decreased. However, in the presented study, although the P ratio was constant, the change in the C and N ratios effected the culture growth and the specific growth rate increased from 0.005 to 0.168.

When the post-harvest biomass amounts were examined, although a small decrease was observed in the specific growth rate with the increase in temperature in the Spirulina medium, there was an increase in the amount of biomass. The highest specific growth rate (μ) rate at 25°C was 0.3653 and the amount of dry biomass was 0.4152 g L^{-1} in a culture with a concentration of 25% N. As the temperature was raised to 30°C, the culture with a concentration of 75% N with a μ value of 0.3285 and a dry biomass amount of 0.4462 g L^{-1} gave the best results.

Comparing the culture media on the basis of temperature, in Spirulina medium at 25°C highest μ values were recorded. However, μ values obtained in Spirulina medium were recorded higher than Zarrouk medium in samples with the same nitrogen content in nitrogen limitation. It was determined that the

Table 2. Growth parameters and chemical analysis data

	Biomass concentration	Specific Growth Rate	Protein	Total Phenolic Content	Total Carotenoid Content	Antioxidant Capacity
	g.L^{-1}	μ	%	mg GAE g^{-1}	mg g^{-1}	μg TE g^{-1}
1A-100	1.3904	0.1847	55.7584^{d}	1.3384±0.02^{abc}	12.1899±0.05^{fg}	11.2589±0.18^{cde}
1A-25	1.4298	0.3653	55.0653^{de}	1.1363±0.08^{def}	12.0442±0.63^{fg}	10.1494±0.66^{fg}
1A-50	1.4072	0.2295	53.9848^{ef}	1.3568±0.01^{ab}	14.0595±0.03^{b}	11.9897±0.05^{bc}
1A-75	1.3830	0.1829	53.1311^{f}	1.1225±0.01^{def}	12.3636±3.71^{ef}	12.4875±0.32^{b}
2A-100	1.3950	0.1145	56.1326^{d}	1.2357±0.14^{bcd}	12.7921±0.68^{de}	11.1986±0.02^{cde}
2A-25	1.4358	0.2908	70.1600^{c}	1.3914±0.14^{a}	12.6128±0.22^{ef}	10.9985±0.07^{def}
2A-50	1.3840	0.0631	54.0895^{ef}	1.2032±0.04^{cde}	13.4042±0.08^{c}	11.1045±1.98^{cde}
2A-75	1.3540	0.0545	50.9493^{g}	0.9682±0.14^{g}	11.6418±0.05^{g}	10.7528±0.61^{defg}
1B-100	1.3265	0.1861	80.0419^{a}	1.1764±0.07^{de}	13.2145±0.01^{cd}	10.6338±0.18^{defg}
1B-25	1.3114	0.0824	77.8715^{b}	1.4717±0.30^{a}	10.8343±0.16^{h}	10.3502±0.61^{efg}
1B-50	1.3242	0.1383	49.7915^{g}	1.0051±0.07^{fg}	10.1731±0.12^{i}	11.5430±0.44^{bcd}
1B-75	1.2882	0.3285	78.8284^{ab}	1.4536±0.07^{a}	17.9034±0.02^{a}	10.9913±1.08^{def}
2B-100	1.2826	0.4498	52.9898^{f}	0.6062±0.01^{h}	8.7754±0.08^{j}	14.7877±0.47^{a}
2B-25	1.3307	0.2253	56.6889^{d}	1.0609±0.04^{efg}	13.3240±0.05^{cd}	9.8222±0.47^{g}
2B-50	1.3561	0.3226	49.7159^{g}	1.3754±0.04^{ab}	12.4991±0.21^{ef}	11.1170±0.22^{cde}
2B-75	1.3446	0.1488	53.9633^{ef}	1.0089±0.10^{fg}	14.2897±3.35^{b}	10.8195±0.09^{def}

**A: Spirulina medium, B: Zarrouk Medium, 1: 25°C, 2: 30°C*

***Mean values± standard deviation. Within columns, values with the different superscripts differ significantly from each other ($p<.05$).*

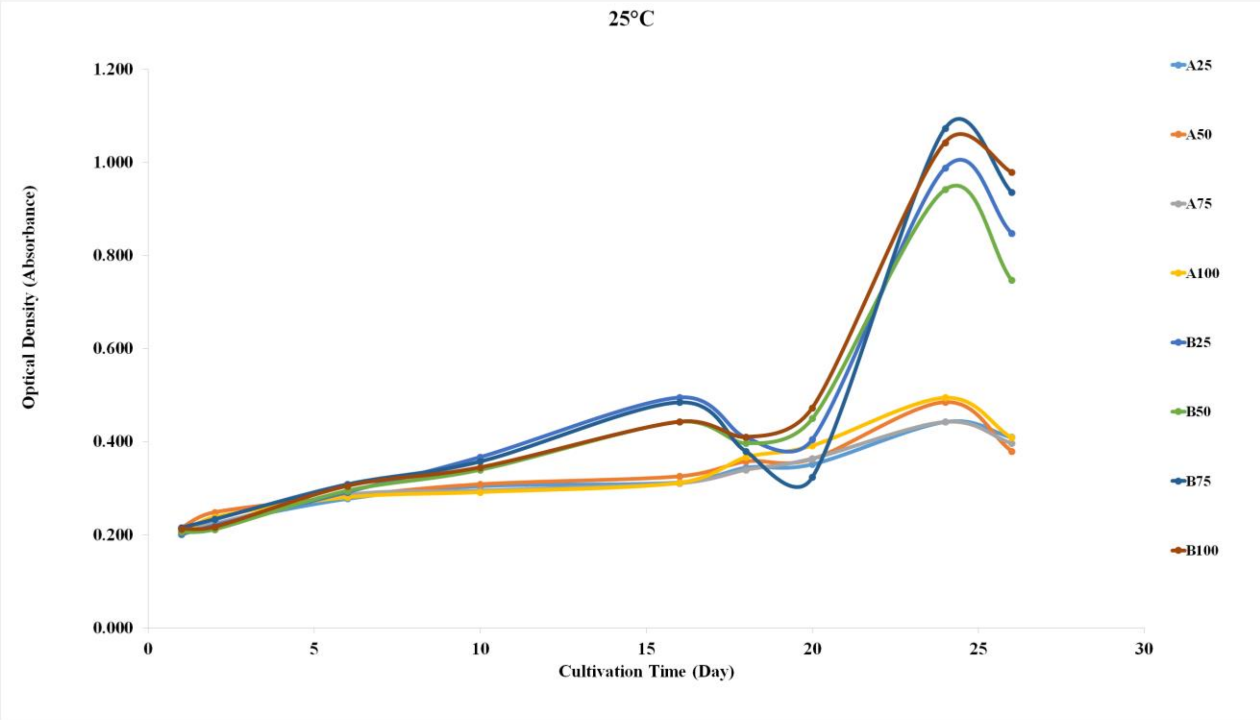

Figure 2. Growth parameters of Spirulina platensis a) at 25℃

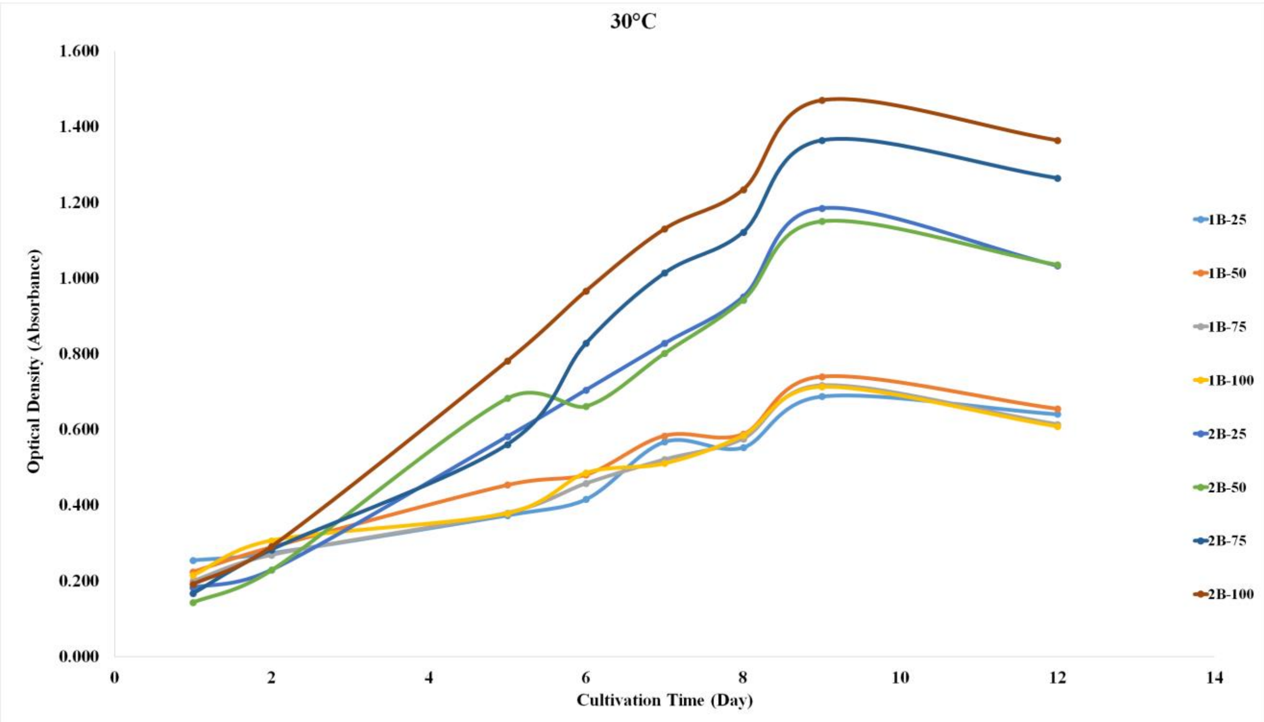

Figure 3. Growth parameters of Spirulina platensis at 30℃

effect the antioxidant activity. Also, high alkaline pH may favour the overproduction of antioxidants and the growth rate as in the presented study findings confirmed.

DPPH free radical scavenging assay gave a maximum IC_{50} value of trolox of 14.78 µg g^{-1}.

Also, total carotenoids changing trend was recorded irregular, the correlation between the amount of phenolic compound and carotenoid were found more than phenolic compound and antioxidant, unless all correlations were found insignificant (data were not shown). At 25°C, decrease N concentration by half (run 3 and run 7) 14.05±0.03 and 13.40±0.08 respectively, increased carotenoid content independent from the growth media type. However, at 30°C high carotenoid content was determined at 75% N concentrations (run 12 and run 16), 17.90±0.02 and 14.28±3.35, respectively. Park et al. (2018), studied carotenoid content of seven commercial spirulina samples and determined values ranging between 0.28 mg/g and 4.48 mg/g. Pigment formation is dependent on the nutrient concentration. Shanab et al. (2012) revealed that nitrogen starvation in the cyanobacteria causes a decrease in especially, phycobiliprotein pigment content. Interestingly, the antioxidant capacity remained unaffected, and the authors explained this with the idea that the production of Although antioxidant substances might decrease steadily during the different processing steps, there can also be a successful enrichment of bioactive substances in the resulting products. TPC, TCC and TAC values were obtained in Spirulina medium at 30°C. In general, these values decrease in nutrient stress. Zarrouk medium is known as the most preferred synthetic medium, it is seen in this study that biomass with high protein content and high phenolic substances may be increased, which compensates for the absence of pigments with antioxidant effects. Therefore, different cultivation conditions may have led to the different results in carotenoid content as compared to the work described by Goiris et al. (2012) and Almendinger et al. (2021).

4. CONCLUSIONS

The presented study tested the temperature regimes of 25°C, 30°C and 35°C for the cultivation *S. platensis* (UTEX-LB 2340). At 35°C, the growth period was rapid and reached the death phase after 72 hours. For this reason, growth parameters and bioactive contents were analysed in cultures grown at 25°C and 30°C. Along with the temperature regime, Spirulina and Zarrouk media were evaluated separately, and the nitrogen source $NaNO_3$ was used by adjusting the rates of 25%, 50%, 75% and 100%.

It can be reported that the effect of the N regime is insignificant at this temperature where 25 degrees is ideal for high biomass content and specific growth rate. The protein ratio was found to be 80% at 100N and 77% at 25N in Spirulina medium at 30°C. In this case, it will be possible to obtain high protein with spirulina medium and 0.625 g $NaNO_3$ amount

ACKNOWLEDGEMENTS

This article is a part of the doctoral thesis of Oya Irmak ŞAHİN-CEBECİ (Supervisor: Arzu AKPINAR-BAYİZİT, 2015, Uludag University, Institute of Science).

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