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Production of Biomass and γ-Linolenic Acid Production by *Spirulina platensis* Under Different Temperature and Nitrogen Regimes

Oya Irmak Şahin[1*], Arzu Akpınar-Bayizit[2]

[*1] *Yalova University, Engineering Faculty, Chemical Engineering Department, Yalova, Turkey.*
[2] *Bursa Uludag University, Agriculture Faculty, Food Engineering Department, Bursa, Turkey.*

*Corresponding author : isahin@yalova.edu.tr
Orcid No: https://orcid.org/0000-0003-2225-7993

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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 contains important fatty acids, (e.g. GLA), vitamins, minerals and other bioactive compounds. These important compounds 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* biomass yield, lipids and fatty acid profile were investigated with the comparison of Spirulina medium and Zarrouk medium. In the present investigation, the production of *Spirulina platensis* was optimized in terms of biomass and metabolites. With the increase in temperature, while the amount of biomass increased in general, dry weight decreased. The highest level of lipid accumulation was determined as 12.31 ± 1.72 % for the sample 25°C, Spirulina medium and 50% N concentration. Protein, lipid, total phenolic substance, and total carotenoid amounts were found at the highest level with the temperature increase to 30°C in all samples except the sample with the highest oil content. Consequently, the highest PUFA values were found in 30°C, Zarrouk medium and 75% N concentration as 42.610%, whereas GLA was 25°C, Zarrouk medium and 100% N concentration as 24.735%. On the other hand, GLA values were determined significantly high both during growth at 25°C and 30°C in Zarrouk medium.

Keywords: *Spirulina platensis*; biomass; fatty acid; GLA; nitrogen regime; temperature regime

1 Introduction

The use of "microorganisms" as well as plants as a source of "lipid" and "fatty acid" has been investigated for many years. Microbial oils have been researched at the industrial level in many developed countries since the 19th century, especially against famine, the decrease in traditional oil raw materials and the increase in the world population during the 1st and 2nd World Wars, and these researches have been carried out in secrecy for long periods (Ratledge 2004, 2006; Gunstone et al. 2007).

Microbial oils obtained from microorganisms and called single cell oil (THY, SCO: single cell oil) attract great attention all over the world today due to the therapeutic and nutraceutical PUFAs they contain (Ratledge 2005; Ratledge and Cohen 2008). Many groups of microorganisms (yeast, mold, bacteria and microalgae) have the ability to accumulate neutral oil under certain specific conditions. These microorganisms can accumulate 25% or more lipid in their cells and are described as “oleaginous” (Ratledge 2006; Vance and Vance 2008).

The reason for the concentration of studies on *Spirulina* is that it is a rich source of food components such as protein, vitamins (especially vitamin B12 and provitamin-A), essential amino acids, mineral substances (especially Fe) and essential fatty acids (especially GLA). 60-70% of the dry weight of Spirulina is protein and at least 20% of the total fat is composed of GLA (Ötleş and Pire 2001, Belay 2002, Colla et al. 2007). Although microalgae contain less fat than yeast and molds, the unsaturated fat profile is interesting.

The biomass yield and the change of the obtained metabolites are inevitable as a result of development in changing media compositions and temperatures, so the diagnosis and optimization of the growth conditions for the target metabolite gain importance. This study aims to determine the biomass and fatty acid composition, especially GLA, which has nutraceutical and therapeutic value, of *Spirulina platensis*, which was developed using different temperatures and nitrogen concentrations in two different growth environments.

2 Materials and Method

2.1 Material

Spirulina platensis (UTEX LB2356) strains were procured from UTEX, Culture Collection of Algae, Texas, Austin.

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 $CaCl_2 \cdot 2H_2O$, and 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 $CoCl_2 \cdot 6 H_2O$, 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 $ZnSO_4 \cdot 7H_2O$).

2.2 Growth Conditions

Two different growth media (Spirulina and Zarrouk), temperatures (25±2°C and 30±2°C) and four different "$NaNO_3$" concentrations (defined as; 100%, 25%, 50% and 75%) were chosen for the evaluation of biomass production, the trial design is described in Table 1.

Table 1 Trial groups

	100 % N	*25 % N*	*50 % N*	*75 % N*
25°C - Spirulina Medium	A	A25	A50	A75
25°C - Zarrouk Medium	B	B25	B50	B75
30°C - Spirulina Medium	C	C25	C50	C75
30°C - Zarrouk Medium	D	D25	D50	D75

The temperature was maintained by using an air conditioner and a lightening regime of 32-40 µmol photon $m^{-2}s^{-1}$ with a 14:10 hour light-dark period was applied. Cultures were aerated with 2 L min^{-1} during the growth period and harvested by filtration (20 µm mesh). Obtained wet biomass was freeze-dried at -60°C overnight to obtain dry biomass, weighed and kept at -80°C for analysis.

2.3 Biomass and Lipid Analysis

Biomass concentration was measured daily by optical density (OD) at 680 nm (Optizen-Pop UV-Vis spectrophotometer, Korean). Lipid extraction was determined according to Folch et al. (1957) and fatty acid profiles were measured by the method described by Akpınar-Bayizit et al. (2014).

2.4 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 Discussions

In this study, the changes in the GLA content of *Spirulina platensis* microalgae belonging to the *Oscillatoriaceae* family against different temperature and N source limitations were investigated. Optical density analyses were performed on *S. platensis*, which was cultured in 4 different groups until it reached the stationary phase. After the cultures that reached the stationary phase were harvested, they were dried, and their biomass amounts were calculated (Table 2).

Table 2 Growth parameters of *Spirulina* cultures

	OD_{final}	OD_{max}	*Biomass concentration*	*Lipid*
			(g L^{-1})	%
A	0.409	0.495	0.252^{ef}	0.945^{g}
A25	0.410	0.443	0.415^{bc}	2.178^{ef}
A50	0.380	0.485	0.284^{e}	12.310^{a}
A75	0.397	0.443	0.205^{f}	2.964^{cde}
B	0.979	1.043	0.314^{d}	1.591^{fg}
B25	0.848	0.988	0.418^{bc}	3.559^{bc}
B50	0.747	0.942	0.369^{cd}	2.885^{cde}
B75	0.936	1.073	0.218^{f}	3.762^{bc}
C	0.609	0.714	0.388^{c}	3.282^{cd}
C25	0.641	0.688	0.283^{e}	3.571^{bc}
C50	0.655	0.740	0.397^{c}	2.597^{de}
C75	0.614	0.717	0.446^{b}	4.339^{b}
D	1.365	1.471	0.511^{a}	1.560^{fg}
D25	1.033	1.185	0.428^{b}	3.119^{cd}
D50	1.036	1.151	0.332^{d}	0.952^{g}
D75	1.265	1.365	0.303^{de}	1.441^{fg}

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

The lipid synthesizing capacity of microorganisms is limited by some parameters. First of all, lipid production is directly related to the presence of primary nutrients and is induced by the reduction of nitrogen sources in the environment. Similarly, the increase in the amount of carbon source increases the amount by accelerating the lipid synthesis. While the decrease in nitrogen source causes an increase in lipid synthesis, it causes a slowdown in protein and nucleic acid synthesis (Ratledge and Wynn 2002; Ratledge and Cohen 2008; Ratledge 2008).

The importance of algae in human nutrition is due to the high amounts of protein, vitamins, amino acids and mineral substances in its structure. Biochemical properties in algal cultures vary depending on parameters such as species, growing region, season, water temperature, light intensity and exposure time. Compared to other seafood, the lipid content of algae is low and generally varies between 1–5% (Aguilera-Morales et al. 2005; Dawczynski et al. 2007). While the amount of lipid in green algae varies between 0.6% and 4.3%, this rate is stated as 2-12.3% for other microalgae (Chernova et al. 2001; Bigogno et al. 2002).

Table 1 Fatty acid composition of *Spirulina platensis* biomass samples

%	C16:0	C18:0	C16:1	C18:1	C18:2	C18:3 (GLA)	GLA in PUFA
A	27.558±0.057^{de}	3.068±0.007^{c}	4.470±0.0065^{cd}	13.966±0.0264^{def}	9.085±0.0274^{ef}	17.587±0.0412^{de}	60.43
A25	11.127±0.094^{f}	7.337±0.068^{b}	0.299±0.0044^{e}	35.008±0.2768^{a}	25.228±0.1134^{a}	2.970±5.1456^{ı}	1.71
A50	22.401±10.323^{e}	9.156±3.896^{a}	1.049±1.5065^{e}	19.763±4.7544^{b}	4.766±7.0257^{g}	0.510±0.0025^{ı}	22.19
A75	22.786±0.078^{e}	2.358±0.012^{cd}	3.861±0.0013^{d}	16.405±0.0622^{c}	8.637±0.0932^{fg}	14.803±0.0497^{fg}	54.76
B	28.578±0.480^{cde}	2.450±0.048^{cd}	6.228±0.1258^{a}	10.994±0.1535^{ghı}	9.074±0.1592^{ef}	24.735±0.4245^{a}	73.16
B25	38.587±0.049^{a}	2.238±0.019^{cde}	5.514±0.0817^{abc}	11.570±0.0245^{fgh}	16.919±0.0197^{bc}	11.878±0.0131^{gh}	38.97
B50	29.521±0.06^{bcde}	1.859±0.009^{cde}	5.674±0.0148^{ab}	12.611±0.1089^{efg}	10.666±0.0295^{ef}	22.978±0.0513^{ab}	65.27
B75	33.378±0.005^{abcd}	1.876±0.01^{cde}	6.113±0.0054^{a}	8.502±0.0094^{jk}	10.704±0.0073^{ef}	23.785±0.0072^{a}	68.96
C	33.486±0.234^{abcd}	2.272±0.125^{cde}	4.730±0.0354^{bcd}	15.000±0.1042^{cde}	16.139±0.1158^{bcd}	17.520±0.1213^{de}	50.68
C25	39.882±0.054^{a}	1.457±0.017^{de}	5.779±0.3540^{ab}	8.456±0.0141^{jk}	17.018±0.0190^{bc}	12.239±0.0133^{gh}	40.48
C50	35.844±0.081^{abc}	0.984±0.004^{e}	5.153±0.0125^{abc}	7.969±0.0222^{jkl}	17.591±0.0503^{b}	15.720±0.0472^{ef}	45.99
C75	36.942±0.101^{ab}	1.879±0.006^{cde}	4.743±0.0043^{bcd}	15.969±0.0534^{cd}	12.271±0.0418^{def}	9.378±0.0290^{gh}	41.07
D	35.561±0.103^{abc}	1.873±0.006^{cde}	4.748±0.1200^{bcd}	7.309±0.2361^{kl}	16.130±0.2223^{bcd}	22.142±0.0566^{abc}	53.53
D25	37.177±0.082^{a}	2.862±0.013^{cd}	5.171±0.0262^{abc}	9.062±0.0306^{ijk}	15.522±0.0244^{bcd}	20.189±0.0294^{bcd}	56.53
D50	35.301±0.068^{abc}	2.526±0.0172^{cd}	5.361±0.0076^{abc}	10.000±0.1279^{hıj}	17.249±0.0864^{bc}	19.104±0.0269^{cd}	52.55
D75	35.536±0.099^{abc}	1.986±0.007^{cde}	4.824±0.0186^{bcd}	6.817±0.0252^{kl}	17.666±0.0393^{b}	23.205±0.0623^{ab}	54.46

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

The lipid content of *S. platensis* was determined between 0.945 and 12.310 % values, and the highest value was observed in the samples of 25°C + 50% N Spirulina medium. It is reported that 6.4% to 14.3% of the dry biomass of *S. platensis* consists of lipids (Badzhanov et al. 2004; Kachroo et al. 2006). Colla et al. (2007), cultivated *S. platensis* at 4 different N concentrations at 30°C and 35°C, and determined lipid contents varied between 6.69±0.27% and 10.37±0.63. Piorreck et al. (1984) stated that the total amount of lipid in cyanobacteria does not depend on the nitrogen concentration in the medium and that nitrogen is the growth parameter limiting lipid synthesis in eukaryotic algae.

In research that optimizing the lipid ratio, values ranging from 5.21±0.1% to 18.02±0.4% were determined (Xue et al. 2010, Uslu et al. 2011, Madkour et al. 2012, Azgın et al. 2014). Griffiths et al. (2011) in their study with 10 microalgae, reported that the lipid ratio in the biomass increased with the decrease in N ratios. The data obtained in the presented study are in harmony with this study, the relationship of lipid ratio with varying N concentration, temperature and biomass amounts could not be determined clearly. The change in temperature and nutrient medium concentration applied to *S. platensis* was determined statistically, and it was determined that there was a difference in the lipid ratio ($p < 0.05$). In microbial growth, it is expected that the amount of lipid will increase as the temperature decreases and N concentration decreases in the presence of high C in the nutrient medium (Rodolphi et al. 2009, Dean et al. 2010). In this case, the best lipid synthesis occurred in 25°C + 50% N Spirulina medium (12.310). In the Zarrouk medium at the same temperature, the medium provided the best lipid value with 75% N (3.762%). This is thought to be due to the fact that the C concentration in the Spirulina medium is higher than that in the Zarrouk medium. Similarly, when the two mediums at 30°C are compared, it is seen that lipid synthesis is higher in the Spirulina medium (Table 3).

Although it varies according to the species, it is noted that the ratios of EPA, ARA, ALA and GLA in total lipid were significantly higher, especially in *S. platensis* (Habib et al. 2008). In recent years, the importance of algae has been associated with the composition of essential fatty acids, which are similar or alternative to other fatty acids of vegetable and animal origin (Vazhappily and Chen 1998; de Swaaf et al. 1999, Rosa et al. 2005; Mendes et al. 2007). 17% of the fatty acids of *S. platensis* consist of essential pigments, paraffin, sterol and terpene alcohols, which are unsaponifiable (Hoseini et al. 2013). ω-6 fatty acids in sterol structure, which make up half of the total fatty acids, are found in the galactolipid structure of algal cell walls (Certik and Shimizu 1999). However, although this content is low, it is also stated to be a good source of ω-3 and ω-6, especially GLA.

S. platensis contains approximately 49% GLA, making Spirulina the best source of GLA after breast milk and evening primrose and borage oils (Petkov and Furnadzieva 1988). SFA values of S. platensis grown in Spirulina broth only at 25°C showed a significant change with varying N concentrations. In other groups, changes in temperature and nitrogen concentration were not found to have a significant effect on SFA values. The SFA values obtained in the study were 70.3% for the culture developed by Durmaz and Gökpınar (2006) at 26°C, and Ambrozova et al. (2014) were found to be lower than the values of 63.18% of the culture

they developed in the photobioreactor under sunlight. Ötleş and Pire (2001) reported the SFA ratios as 55.72%, 51.64% and 51.96% in the analysis results of Spirulina powders offered for sale in the market.

It was determined that GLA accumulation was very low at high temperature and low nitrogen concentration, where MUFA synthesis accelerated in Spirulina medium (Table 3). Similar results (Piorreck et al. 1984, Griffiths et al. 2012) has also been reported. Production of PUFA and GLA is closely related to the presence of dissolved oxygen in the nutrient medium, and the absence of carbon sources. De Morais et al (2019) observed that the increase in unsaturated fatty acids, especially oleic acid, and reduction of saturated fatty acids, mainly palmitic, were proportional to the increase in the glycerol concentration in the medium.

It was reported by Knothe (2005) that the PUFA yield would increase with a decrease in temperature. However, in the presented study, it was observed that PUFA yield increased when the temperature increased from 25°C to 30°C in other algal growths, except for the samples grown at 25°C + 75% N Spirulina medium. This increase in PUFA yield was found more prominently in the 50% N sample, especially in the Spirulina medium. While Durmaz and Gökpınar (2006) reported that GLA synthesis was not affected by culture temperature, Colla et al. (2004) stated that temperature directly affects the fatty acid synthesis and that the best amount of GLA can be obtained with growth at 30°C. GLA values were detected higher in samples at 30°C than growth at 25°C. The highest GLA value was observed in the Zarrouk medium 25°C + 100% N sample group with 24.73% of total fatty acids and 73.16% of PUFA.

4 Conclusion

It is known that oleaginous microorganisms tend to accumulate lipids with high PUFA content inside the cell in the presence of N limitation and a high amount of C source in the nutrient medium, under optimum growth temperatures. However, unlike these microorganisms, it was not observed that the PUFA values in the biomass increased with the increase in temperature, regardless of the N limitation. Additionally, at low temperatures, algal cultivation time took longer in both media (t25°C: 25 days, t30°C: 12 days).

It can be concluded that cultivation of *Spirulina platensis* at 25°C in Zarrouk medium and at higher than 50% concentration of $NaNO_3$ approximately 20 % of GLA could be observed. Also at these conditions, GLA concentrations were above 50% of PUFA.

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