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Statistical Optimization of Total Fatty Acids and Protein Content in *Spirulina platensis* Biomass

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Abstract

Protein and fatty acid deficiency in human nutrition is a major concern for developing countries. The filamentous blue-green microalga *Spirulina platensis* is one of the best algal species that can be used as a food supplement. This study aims to optimise biomass, the total protein, and the fatty acid content of *Spirulina platensis*. Statistics optimization was carried out using the response surface methodology via dry weight and the Kjeldahl method for protein determination, while the fatty acid composition was determined by the conversion of oil to fatty acid methyl esters (FAMEs), which was measured on a gas chromatography (GC) device. The results revealed that the optimum conditions for maximum biomass, total protein, and fatty acid content were in the presence of $NaHCO_3$ at (16.8 g/l) and $NaNO_3$ (2.5 g/l) at temperature (20°C), pH (9), and LI (6000 lux). A prospective study to estimate both essential amino acids and essential fatty acids in *S. platensis* biomass is recommended.

Keywords: Biomass, Fatty acids, Proteins, Response Surface Methodology, *Spirulina platensis.*

1. Introduction

Microalgae have received a lot of attention recently since they are one of the most promising sources of biomolecules that could be employed as functional ingredients. Microalgae contain metabolites with high nutritional value such as proteins, lipids, carbohydrates, pigments, and minerals, making them an appealing dietary supplement for humans and animals [1]. Protein deficiency in human nutrition is a major concern for developing countries. Therefore, there is a need to diversify protein sources and develop new unconventional ones. Various microalgal species have high protein content, making them an alternate source of this nutrient [2]. *S. platensis* is a filamentous blue-green microalgae or cyanobacteria that belongs to the family Oscillatoriaceae [3]. It is known for its high nutritional value, which includes high protein content (60–70%, dry weight), an amino acid rich source of vitamins, mainly vitamin B12 and pro-vitamin A, minerals, especially iron, and polyunsaturated fatty acids [4,5]. Polyunsaturated fatty acids like linoleic acid and -linolenic acid (GLA) [6] are important in human metabolic pathways, particularly as precursors of one type of prostaglandin E1 [7]. GLA has been utilised in a variety of medical applications, including the treatment of diabetes, dermatitis, and premenstrual syndrome [8,9]. Moreover, *S. platensis* contains phycocyanin, -carotene, xanthophyll pigments, -tocopherol, and phenolic compounds, which are responsible for the antioxidant activities of these microalgae [10]. *S. platensis* has been widely used in several countries as a portion of new food and feed products and it is approved by the FDA (U.S.A.) [11]. It is considered safe for consumption by humans and animals as new food and feed products [12, 13]. *S. platensis* cell walls are made up of soft mucopolysaccharides rather than cellulose. This makes it simple to digest and assimilate. At 85 to 95 percent, it is easily edible. This ease of digestion is especially significant for people suffering from digestive problems [14, 15]. The environmental factors, especially light intensity, pH, temperature, and C and N sources, have a significant impact on the growth and propagation of microalgae and cyanobacteria for biomass production, as well as their protein and fatty acid composition [16,17]. The current study aimed to optimize *S. platensis* biomass

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production with a high content of total protein and fatty acids using response surface statistical models
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2. Materials and Methods

2.1. Culturing of S. platensis

In the present study, *S. platensis* was obtained from the Algal Culture Collection at Al-Azhar University (ACCAZ). It was grown on Zarrouck's medium [**18**]. It consists of (part A) $NaNO_3$ 2.5g, K_2HPO_4 0.5g, $CaCl_2.2H_2O$ 0.04g, $MgSO_4$ $7H_2O$ 0.2g, NaCl 1.0 g, K_2SO_4 1.0g, $FeSO_4.7H_2O$ 0.01g, EDTA 0.08g, and $NaHCO_3$ 16.8g/l and (part B) trace elements mixture 1ml/l H_3BO_3 2.86g, $MnCl_2.7H_2O$ 1.81g, $ZnSO_4.7H_2O$ 0.22g, $CuSO_4.5H_2O$ 0.08g, Co $(NO_3)2.6H_2O$ 0.04g, Na_2MoO_4 0.0177 g/l. with exposure to fluorescent light 12/12 dark and light and aeration. Experiments were performed in 5 L flasks containing 2.5 L of sterile Zarrouck's medium inoculated with 500 ml of the *S. platensis* culture.

2.2. Growth measurement (dry weight)

After three weeks of *S. platensis* cultivation, the biomass was harvested by centrifugation. Biomass was collected in pre-weighed Petri dishes, and oven dried at 50°C for three hours until constant weight was obtained, to obtain the dry weight of microalgae.

2.3. Total protein estimation

Protein content was determined by the modified Kjeldahl method [19]. This method mainly involved digestion, distillation, and titration (Kel-Plus, Elite Ex 8L, Pelican equipment, India). For microalgae and cyanobacteria undergoing rapid growth, the recommended Kjeldahl nitrogen-to-protein conversion factor used was 5.95 instead of 6.25 [20].

2.4. Fatty acid extraction

Fatty acids were extracted from *S. platensis* biomass according to the modified method of (Hamdy A. Zahran and Hesham Z. Tawfeuk, 2019) [21]. The fatty acid composition of the extracted *S. platensis* microalgae oil was determined using gas liquid chromatography (GLC) located at the central lab., Egyptian Atomic Energy Authority, its conditions: Fatty acid methyl ester was performed using Hewlett Packard (hp) Model 6890 gas. The Chromatograph instrument is equipped with: BPX capillary column, 60 m * 320 m * 0.25 m. Oven: 120 °C for 1 minute, then 210 °C at a rate of 8 °C/min, then 225 °C at a rate of 2 °C/min and stayed for 5 minutes. Detector (FID) temperature of 300 °C, air flow rate of 400 ml/min, H2 flow rate of 35 ml/min, injector temperature of 250 °C, split ratio of 20:1, and carrier gas N2 flow rate of 3.5 ml/min.

2.5. Statistical Optimization Model

The half-fraction factorial design (25-1) was adopted to study the effects of five independent variables, including temperature, pH, light intensity (LI), NaHCO3, as a carbon source (CS), and NaNO3, as a nitrogen source (NS), concentration to improve the biomass productivity of *S. platensis* and achieve the highest total protein and total fatty acid contents. The half-fraction factorial design describes the correlation between the factors and response (here were dry weight, total protein, and fatty acid content) using cube points to fit a linear (first order) model to evaluate the main and interaction effects between factors and also evaluate the pure experimental error. The mathematical and graphical outputs of the half-fraction factorial design allow the model to create the optimization curves that predict the final optimal settings of the interacted factors that maximize algal biomass (dry weight), total protein, and fatty acid content. The data obtained from both the half-factorial and the optimization were analysed by the analysis of variance (ANOVA) test. The regression of tested variables and their interactions, model significance, and the coefficient of determination (r2) of the generated models were estimated. Minitab® version 18 (2017) was used to generate and analyse all experimental data, which was supplemented with statistical and graphical software packages.

2.6. Design and setup of experiments

Seventeen 5L Erlenmeyer flasks were individually filled with 2.5 L of *S. platensis* cultures. According to the CCD matrix, three temperatures (20, 29, and 38 °C), three pH values (7, 9, and 11), three light intensities (2400, 4300, and 6000 lux), three concentrations of $NaHCO_3$ (0, 8.4, and 16.8 g), and three concentrations of $NaNO_3$ (0, 1.25, and 2.5 g) were used. The flasks were incubated under the appropriate growth conditions with a 12/12 h dark/light cycle.

2.7. The optimization curves

The response optimizer, a tool included in the Minitab® DoE (Design of Experiments) statistical package, was used to generate the optimization curves to determine the final combination settings of the interacted factors that maximize the dry weight, total protein, and fatty acids content (Fig. 7). Individual (d) and composite (D) desirability were used to assess The predicted settings maximise the response from 0 to 1 scale. This stage was ended by additional confirmation experiments (n = 5 replicated runs) to validate the predicted settings resulting from the optimization curves (Fig. 8).

Table (1): Design matrix of 2^5 half factorial CCD and results of *S. platensis* total fatty acids (mg/1g) DW (g/L), and total protein content (%) in response to all levels of interacted factors.

Run Order	Temp. (°C)	pH	LI (LUX)	NS	CS	Dry Weight (g/l)	Total Protein (%)	Total Fatty Acids (mg/g)
1	20	7	2600	0	16.8	0.3385	3.85	0.705
2	38	7	2600	0	0	0.1030	3.48	0.375
3	20	11	2600	0	0	0.3397	4.83	0.741
4	38	11	2600	0	16.8	0.3365	7.17	0.425
5	20	7	6000	0	0	0.3297	5.77	0.962
6	38	7	6000	0	16.8	0.3533	7.7	0.805
7	20	11	6000	0	16.8	0.5439	7	0.923
8	38	11	6000	0	0	0.3084	5.05	0.540
9	20	7	2600	2.5	0	0.2876	6.12	0.863
10	38	7	2600	2.5	16.8	0.2651	10.08	1.020
11	20	11	2600	2.5	16.8	0.5018	11.44	1.184
12	38	11	2600	2.5	0	0.2633	9.95	0.805
13	20	7	6000	2.5	16.8	0.4918	11.44	1.475
14	38	7	6000	2.5	0	0.2564	9.1	1.145
15	20	11	6000	2.5	0	0.5465	11.31	1.114
16	38	11	6000	2.5	16.8	0.4705	11.66	1.145
17	29	9	4300	1.25	8.4	0.3513	7.85	0.871

3. Results and discussion

3.1. Dry Weight

The main effect plots (Fig. 1) were created to represent the regression analysis results by representing deviations of the average between the high and low levels within each factor. The results revealed that temperature, pH, light intensity, carbon, and nitrogen concentrations had a significant difference $p \leq 0.05$ between low and high levels of the *S. platensis* dry weight. The low level of temperature resulted in a higher mean of the response, whereas a higher mean of the response was achieved by the high level of the other factors. The relative importance and the significance of the main effects were established by the Pareto chart (Fig. 2 A).

The normal probability plot (Fig. 2B) determines the actual (real) effect of each term; therefore, it indicates if the results occurred by chance (random) or not. Moreover, normal probability plots determine whether the term causes a negative or positive effect on the response, where a positive effect means an increase in the factor causes an increase in the response and vice versa for a negative effect. Each effect is given a single point on the plot, and the points that are close to a fitted line (which refers to the position where the effects were zero) represent the estimated factors that have no significant effect on the response, while the actual (real) term effect is represented by points far away from the fitted line.

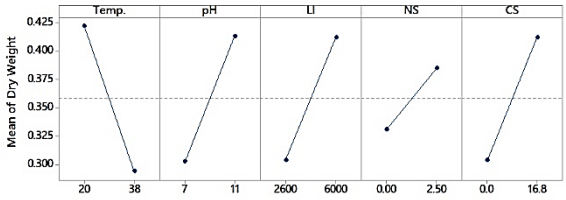


Fig. (1): Main effects plots show the effects of different factors on *S. platensis* dry weight.

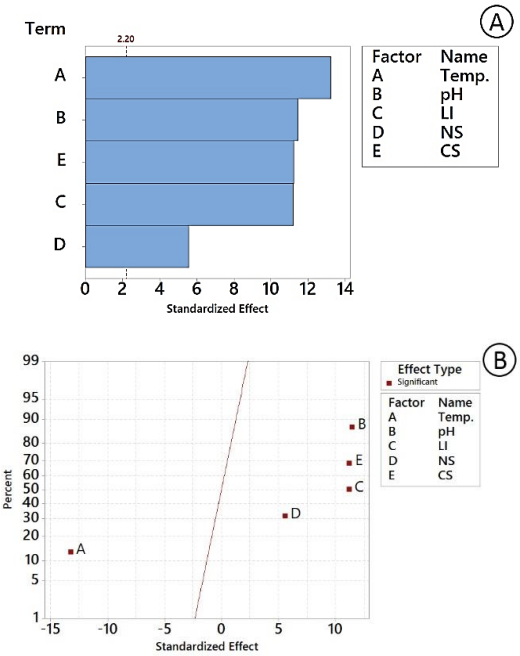


Fig. (2): The Pareto chart (A) and normal probability plot (B) show the standardised effects of different factors on *S. platensis* dry weight.

The biomass, total fatty acid, and total protein of the *S. platensis* were investigated at temperatures (20, 29, and 38) (C), light intensity (2600, 4300, 600) lux, pH (7, 9, 11), NaHCO3 concentrations (0,8.4, 16.8 g/l) and NaNO3 concentrations (0, 1.25, and 2.5 g/l). As shown in (Fig.1), when the temperature is 20 (C, the maximum *S. platensis* biomass, total fatty acid, and total protein were achieved.

3.2. Total protein content

As shown in Fig. 3A, the gradual increase in the nitrogen source compared to other factors led to a significant increase in the total protein content of *S. platensis* ($p < 0.05$). It is influential because it is the main constituent of the amino acid units that make up the protein and the intakes of the rational organism and, therefore, is of relative importance to the protein content of *S. platensis*. On the other hand, the effect of temperature was not significant. These results are close to the results of [22], who reported that nitrogen is an essential nutrient for the microalgal biomass.

The Pareto chart of the standardized effects for total protein (Fig. 3A) shows that the main effects of $NaNO_3$ concentration (D), $NaHCO_3$ concentration (E), light intensity (C), and pH (B) are extended beyond the reference line, indicating a significant effect of these terms at a level of $p \leq 0.05$, while the relative importance of each term is represented by the column length. Temperature (A) didn't exceed the reference line, indicating a non-significant effect. The main terms of $NaNO_3$ concentration (D), followed by $NaHCO_3$ concentration (E), light intensity (C), and pH (B) are far away from the fitted straight line, indicating a strong and significant impact on the total protein. Furthermore, their contribution had a positive effect due to their location on the right side of the graph. On the other hand, the temperature was non-significant.

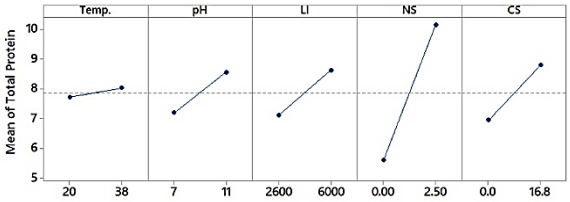
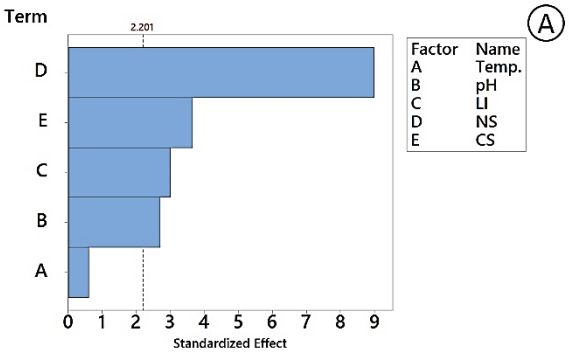


Fig. (3): Main effects plots show the effects of different factors on *S. platensis* protein content.

3.3. *Total fatty acids content*

As shown in (Fig. 5A) the gradual increase in the nitrogen source compared to other factors led to a significant increase in the total fatty acids content of *S. platensis*. On the other hand, the effect of PH was not significant. Pareto chart of the standardized effects for total fatty acids (Fig. 5A) exposed that the main effect of $NaNO_3$ concentration (D), light intensity (C), temperature (A), and $NaHCO_3$ concentration (E) are extended beyond the reference line and indicating a significant effect of these terms at level of $p \leq 0.05$, while the relative importance of each term is represented by the column length.



As shown in Fig. 5A, the gradual increase in the nitrogen source compared to other factors led to a significant increase in the total fatty acid content of *S. platensis*. On the other hand, the effect of PH was not significant. The Pareto chart of the standardized effects for total fatty acids (Fig. 5A) shows that the main effects of $NaNO_3$ concentration (D), light intensity (C), temperature (A), and $NaHCO_3$ concentration (E) are extended beyond the reference line, indicating a significant effect of these terms at a level of $p \leq 0.05$, while the relative importance of each term is represented by the column length.

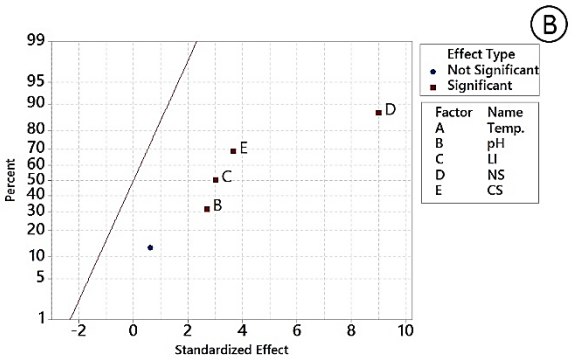


Fig. (4): Pareto chart (A) and normal probability plot (B) show the standardized effects of different factors on *S. platensis* total protein content.

pH (B) didn't exceed the reference line, indicating a non-significant effect. The main terms of NaNO3 concentration (D), followed by light intensity (C), temperature (A), and $NaHCO_3$ concentration (E), are far away from the fitted straight line, indicating a strong significant impact $p \leq 0.05$ on the total fatty acids. Furthermore, their contribution had a positive effect due to their location on the right side of the graph. (D, C, E), but temperature (A) had a negative effect due to its location on the left side of the graph. (A). On the other hand, the pH (B) was non-significant (Fig 5B).

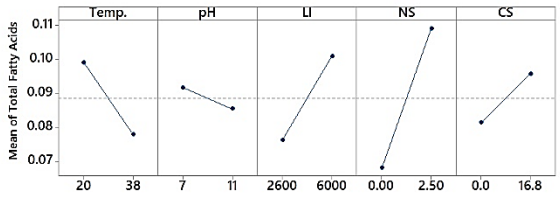


Fig. (5): Main effects plots show the effects of different factors on *S. platensis* total fatty acid content.

Because of the importance of *S. platensis* high protein, vitamin, carotenoids, and essential fatty acid content as a human food and pharmaceutical [23,24], many studies have been conducted to maximize the production of these vital biomolecules [25].Fig. (6): The Pareto chart (A) and normal probability plot (B) show the standardized effects of different factors on *S. platensis* total fatty acid content. Temperature, light intensity, nitrogen sources, and carbon sources are all known factors that have a significant impact on the biomass productivity and biochemical composition of *S. platensis* [26,27,28,29,30]. Temperature is undoubtedly the fundamental factor of all living organisms, which influences all biological activities. Temperature affects the biochemical composition of microalgae in addition to its influence on growth [31]. According to our results, 20 °C was shown to be the optimum temperature for biomass, protein, and fatty acid content. pH has a significant impact on the microalgae's metabolic processes. The physiology of the cell and biomass production of microalgae are significantly impacted by pH variation [32,33,34,35]. The optimal pH for biomass, protein, and fatty acid content was found to be 9 in agreement with [36,37]. Light is one of the major energy input sources for the photosynthesis of microalgae. It plays a central role in the cultivation of photosynthetic microorganisms [38]. [39] reported that light intensity is the major factor controlling the growth (final biomass concentration), lipid, and fatty acid yield of *S. platensis* species.

Photosynthesis is carried out in two stages: light reactions and dark reactions. In light reactions, cyanobacteria use light to break down water molecules. This reaction produces chemical energy, providing NADPH (nicotinamide adenine dinucleotide phosphate) and a highly energetic compound, ATP (adenine triphosphate). NADPH2 and TP are necessary for the assimilation of inorganic nutrients. Cyanobacteria assimilate CO2 and produce carbohydrates and lipids in dark reactions or enzymatic reactions [40]. Nitrogen is an essential element required for the synthesis of amino acids, proteins, and main and accessory pigments [41]. The nitrogen source in Zarrouk's medium is $NaNO_3$. In the present study, the control (2.5 g/l) exhibited high protein content while decreasing $NaNO_3$ led to a significant decrease in the proteins, fatty acids, and biomass. These findings agreed with the findings of [42], who reported that when algae were grown in control Zarrouk's medium, a high content of biomass and proteins was observed.[25] reported that the decrease in sodium nitrate led to a remarkable decrease in growth, biomass, and proteins. These results agree with the findings of [43]. Carbon is one of the most important elements that enter into the composition of all living organisms, and algae use it in the form of carbon dioxide for the process of photosynthesis. The carbonate and bicarbonate sources of carbon are best for the maximum biomass production of *S. platensis* [44]. The optimal sodium bicarbonate concentration as a carbon source was 16.8 for biomass, protein, and fatty acid content. The photosynthetic rate of *S. platensis* was higher in the medium containing higher HCO_3 [45].

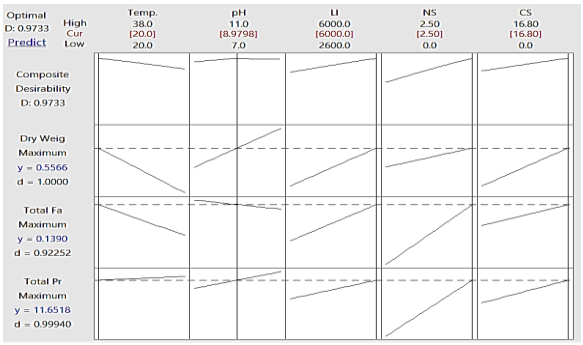


Fig. (7): The optimization curves show how the factors affect the predicted responses (y), including maximum protein content, low and high levels. The optimum factor settings (Cur) were predicted with a composite desirability (D) = 0.9733 (97.33%).

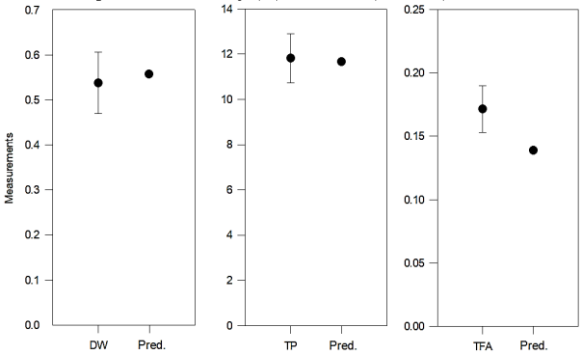


Fig. 8: Scatter plot, comparing the actual (n = 5) and predicted (Pred.) values of DW, TP, and TFA. Error bars represent standard deviations.

Conclusion

S. platensis, a filamentous blue-green microalga, is one of the best algal species for usage as a nutritional supplement. In this study, the response surface methodology was used to statistically maximize biomass production, total protein, and total fatty acid content. The dry weight of the *S. platensis* was determined, as well as the total protein and total fatty acid content of the culturing media. According to the results of the present study, it is recommended to grow *S. platensis* in the presence of $NaHCO_3$ at 16.8 g/l and $NaNO_3$ at 2.5 g/l at 20°C, pH 9, and LI 6000 lux to maximize biomass production, total protein, and total fatty acid content, which is one of the important nutrients that must be present in the daily diet of

humans. *S. platensis*, a filamentous blue-green microalga, is one of the best algal species for usage as a nutritional supplement. In this study, the response surface methodology was used to statistically maximize biomass production, total protein, and total fatty acid content. The dry weight of the *S. platensis* was determined, as well as the total protein and total fatty acid content of the culturing media. According to the results of the present study, it is recommended to grow *S. platensis* in the presence of $NaHCO_3$ at 16.8 g/l and $NaNO_3$ at 2.5 g/l at 20°C, pH 9, and LI 6000 lux to maximize biomass production, total protein, and total fatty acid content, which is one of the important nutrients that must be present in the daily diet of humans.

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