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Feasibility of *Spirulina platensis* Production Using Optimized Beet Filter Cake Extract Medium on Large-Scale Raceway Open Pond

Sara Saad, Mervat Hosny Hussien, Heshmat Soliman Aldesuquy, Eladl Eltanahy*, Ghada Samir Abou-ElWafa

Botany Department, Faculty of Science, Mansoura University, Mansoura 35516, Egypt

***Corresponding author: eladl@mans.edu.eg**

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ABSTRACT

In Egypt, the beet sugar industry produces huge amounts of solid wastes called beet filter cake (BFC), which presents a problem for disposal, polluting the environment. On the other hand, the commercial production of cyanobacterium *Spirulina platensis* as an alternative protein source is costly. Therefore, this study intended to recycle BFC waste by exploiting it for the economical production of single-cell protein *Spirulina platensis* on a large scale using an open raceway pond. The cultivation was done in winter using two open ponds of 1000L capacity fitted with a paddle wheel for mixing, which was constructed in the Faculty of Science, Mansoura University, Egypt. One of them was for standard Zarrouk's medium (SZM), and the other was for an optimized beet filter cake extract (BFCE) medium. Environmental conditions, such as temperature and solar radiation, as well as growth parameters, such as optical density, photosynthetic activity, specific growth rate, and dry weight were monitored every two days. Experimental results showed that the temperature was 19.1- 21°C in SZM and 17.8- 21°C in the optimized BFCE, and the pH increased during the cultivation period to maximum values of 10.5 and 11.1 in SZM and optimized BFCE, respectively, which maintains alkaline conditions. The maximum dry weight values were 0.47 and 0.57g/ l in SZM and optimized BFCE medium at the end of the cultivation period, and there was no significant difference in the Fv/ Fm values in both media. Furthermore, no significant differences were observed in the protein content in both media, which was 55.11± 0.75 and 52.58± 1.31 in SZM and optimized BFCE medium. The cost of biomass produced from optimized BFCE medium is 2.6 times cheaper than that of Zarrouk's medium. Finally, we concluded that optimized BFCE can be used as a promising, cost-effective medium for the large-scale production of single-cell protein *Spirulina platensis*.

INTRODUCTION

There is an increasing request for healthy food and microbial high-quality protein sources other than conventional agricultural foods to fill the protein demand gap. Therefore, researchers have turned to microalgae not only since they are environmentally friendly and sustainable sources of protein but also due to their high-added-value products **(Williamson *et al.*, 2023)**. In particular, the cyanobacterium *Spirulina platensis* has long been consumed as a dietary supplement that provides a promising single-cell protein feedstock containing up to 60% protein

of its dry weight **(Barka & Blecker, 2016)** and a wide range of valuable products, such as essential amino acids, phycocyanin pigment, antioxidant, essential fatty acids vitamins and minerals **(Romay *et al.*, 2003; Costa *et al.*, 2019)**.

The first trials for the large-scale cultivation of *S. platensis* were in Mexico **(Ahsan *et al.*, 2008)**. In contrast, its mass production is currently in different countries, such as China, India, and Australia **(Chen *et al.*, 2016)**. The commercial production of *S. platensis* depends on critical factors, such as media composition, temperature, irradiance, pH, aeration, and mixing **(Thevarajah *et al.*, 2022)**. These factors, especially the media composition, may affect the overall production costs. It is well known that Zarrouk's medium is the standard medium used for cultivating *S. platensis*, which gives high biomass productivity and protein content due to its optimal nutrient concentrations **(Junior *et al.*, 2020)**. However, in large-scale cultivation, this standard medium is considered costly. Thus, modifying the medium composition or formulating an alternative medium using food processing wastes and wastewater is necessary to reduce the production cost while maintaining high biomass productivity and protein content.

Using *S. platensis* as a transformer of agricultural and industrial wastes into high biomass-based protein is a promising strategy that enhances the economic viability of the large-scale production of single proteins and waste management **(Ukaegbu-Obi, 2016; Thevarajah *et al.*, 2022)**. In Egypt, the beet sugar industry produces vast amounts of solid wastes called beet filter cake (BFC) that present a problem for disposal, polluting the environment. This solid waste contains organic carbon, calcium carbonate and some minerals, which can be helpful in the cultivation of *S. platensis* **(Asadi, 2006; Prado *et al.*, 2013)**. **Saad *et al.* (2023)** succeeded in the cultivation of *S. platensis* on different concentrations of beet filter cake extract (BFCE) and optimized the medium composition using central composite design (CCD) in laboratory scale with protein and biomass yields similar to that of the standard Zarrouk's medium.

In the outdoor open ponds, temperature and solar radiation are other factors that affect the growth and productivity of *S. platensis* as it is out of control. The maximum *S. platensis* growth temperature ranges from 30- 35°C, whereas the biomass yield reduces at temperatures below 17°C. Furthermore, solar radiation is a natural light resource that varies with seasonal and climate changes **(de Jesus *et al.*, 2018)**. Moreover, mixing the culture attained by the paddle wheel is necessary for uniform temperature, light, and CO_2 distribution, which enhances the biomass yield. The rotational motor speed should be optimized to maintain the viability of the cells and avoid filament breakage **(Soni *et al.*, 2019)**. The present study aimed to study the feasibility of the cultivation *S. platensis* on an optimized BFCE-supplemented medium in an open raceway pond to minimize the production costs of single-cell protein from *S. platensis* as alternative sources to compete in commercial applications.

MATERIALS AND METHODS

Culture scaling up

S. platensis isolate was scaled up from a 100ml stock culture volume using 1: 10 (inoculum: medium) ratio of 1L flask, then of a 10L flask. To prepare the final inoculum for outdoor cultivation, a vertical plastic photobioreactor with a total volume of 200L was used (Fig. 1). All cultivation steps were proceeded in standard Zarrouk's medium (SZM) **(Zarrouk, 1966)**

Fig. 1. The plastic vertical photobioreactors (200L) used as an inoculum for open pond cultivation

Raceway open pond

Based on the results from optimization experiments for BFCE **(Saad *et al.*, 2023)**, scaling up of *S. platensis* from the laboratory to an open raceway pond was carried out. The large-scale cultivation was performed in outdoor raceway ponds of 1000L capacity with 30cm depth, which was constructed in the Faculty of Science, Mansoura University, Egypt. The ponds are fitted with paddle wheels for mixing cultures, and the sunlight is the sole source of illumination. One of these ponds was determined as a control using SZM, and the other was used for the optimized BFCE medium. The chemical composition and corresponding price of each constituent of both media to assess the economic feasibility were represented in Table 1). Both ponds were inoculated with the same concentration of *S. platensis* inoculum (1: 10).

Table 1. The composition of Zarrouk's medium and optimized BFCE, and the corresponding price of each one for m^3

Component	Zarrouk's medium (g/l)	Optimized BFCE medium (g/l)	BFCE medium g/669.2ml +330.8 ml BFCE	Zarrouk's (Kg)/m^3	BFCE (Kg)/ m^3	Zarrouk's price $	BFCE price $
$NaNO_3$	2.5	2.5	1.675	2.5	1.675	1.625	1.08875
K_2HPO_4	0.5	0.5	0.335	0.5	0.335	0.7	0.469
K_2SO_4	1	1	0.67	1	0.67	0.55	0.3685
NaCl	1	1	0.67	1	0.67	0.07	0.0469
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.134	0.2	0.134	0.018	0.01206
$CaCl_2 \cdot 2H_2O$	0.04	0.04	0.0268	0.04	0.0268	0.0068	0.004556
$FeSO_4 \cdot 7H_2O$	0.01	0.01	0.0067	0.01	0.0067	0.001	0.00067
EDTA	0.08	0.08	0.0536	0.08	0.0536	0.12	0.0804
$NaHCO_3$	16.8	0.678	0.4543	16.8	0.45426	5.88	0.158991

Environmental parameters

In both ponds, pH, temperature, and sunlight intensity were measured using a digital pH meter, thermometer, and Lux meter, respectively.

Growth parameters

The *S. platensis* growth was assessed at two-day intervals by measuring optical density at 680nm using a spectrophotometer. The photosynthetic efficiency (Fv/ Fm) was evaluated by measuring the fluorescence in dark acclimated cells using a PAM fluorometer (AquaPen 101 C), and the dry weight measurements were carried out by taking 600ml from each pond, centrifuged at 4000rpm for 10 minutes, and subsequently lyophilized in a freeze drier for estimating the dry biomass weight (g/L). Moreover, the growth performance of *S.* platensis was measured by the following equations **(Wood *et al.*, 2005)**:

-Specific growth rate = (ln (N_f / N_i)/ t_f - t_i

-Doubling time = ln (2) / specific growth rate

-Division/day = specific growth rate/ln (2)

Where, N_f and N_i correspond to final and initial cell density, respectively, related to their specific final (t_f) and initial time (t_i) in days.

Harvesting of *S. platensis* biomass

After the cultivation period, the *S. platensis* biomass was harvested by filtration through 50µm nylon cloth filter, lyophilized in a freeze drier, and subsequently determined for protein content according to the method of **Lowry (1951)**, phycocyanin content according to the method described by **Bennett and Bogorad (1973)** and chlorophyll-a content according to the equations of **Jeffrey and Humphrey (1975)**.

Statistical analysis

Results of triplicate experiments of protein and photosynthetic pigments of SZM and optimized BFCE media were analyzed by Duncan's test with significance at $P \leq 0.05$ using Costate software.

RESULTS

Based on the optimization results and to validate that single-cell protein *S. platensis* could be produced economically using optimized BFCE, scaling up from laboratory to outdoor raceway ponds for 14 days was done. The cultivation stages of *S. platensis* are shown in Fig. 2**)**, from the cultivation to harvest and drying stage.

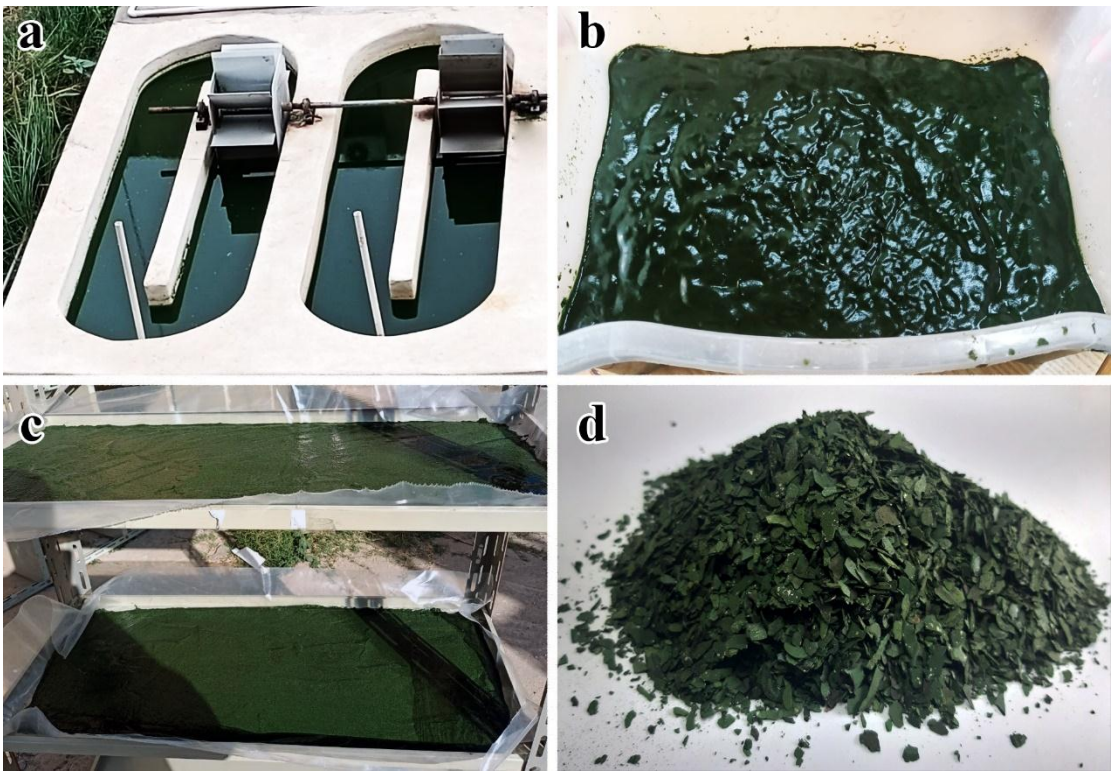

Fig. 2. Cultivation stages of *S. platensis* in Faculty of Science, Mansoura University, Egypt showing: **a)** Raceway open pond of SZM and optimized BFCE medium, **b)** Harvested fresh biomass, **c)** Air drying of biomass, and **d)** Dried biomass.

The minimum and maximum solar radiation values were 455.86 and 814.46µ mol m^{-2} $s^{-}1$ (Fig. 3). Moreover, this experiment was carried out from 13 November to 7 December 2022 as temperature ranged from 19.15 to 21.75°C with an average of 20.18°C in SZM, while the optimized BFCE temperature ranged from 17.8 to 21.55°C with an average of 19.81°C during the cultivation period (Fig. 4). Fig. 5) shows that initial pH values of the cultures after inoculation were close to 9.5, increasing rapidly after few days which affects positively on the dry weight progress, and the minimum and maximum pH values were 9.2 and 10.5 in SZM and 9.47 and 11.1 in optimized BFCE medium, with average values of 9.91 and 10.32, respectively, during the cultivation period, which are in the optimum pH range of *Spirulina*.

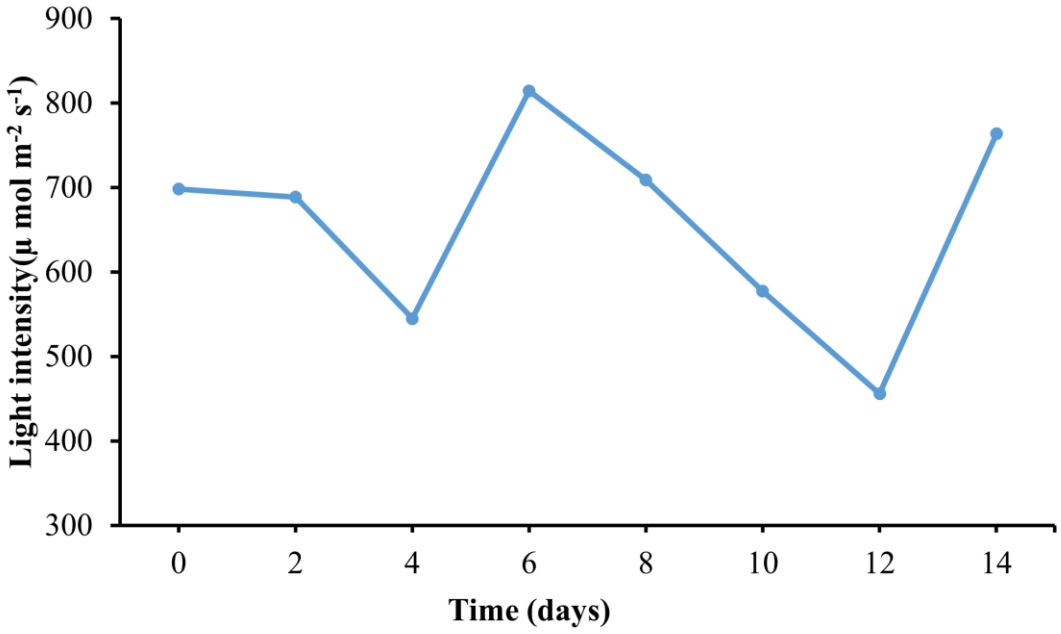

Fig. 3. Changes in light intensity during the 14 days of the experiment

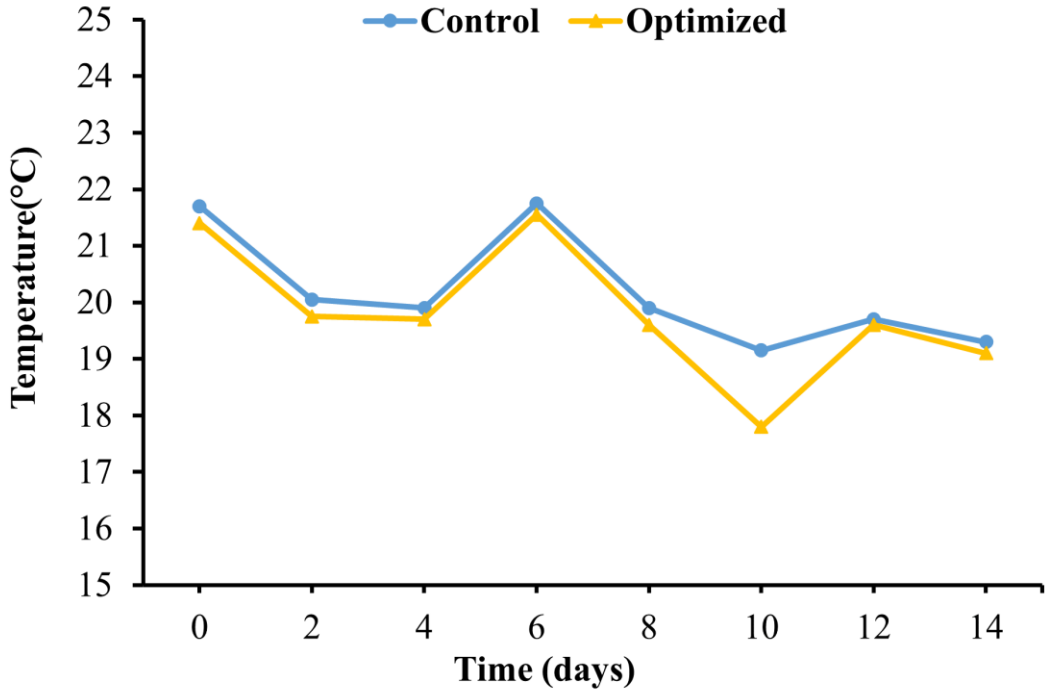

Fig. 4. Changes in the control and optimized outdoor culture temperatures during the 14 days of the experiment

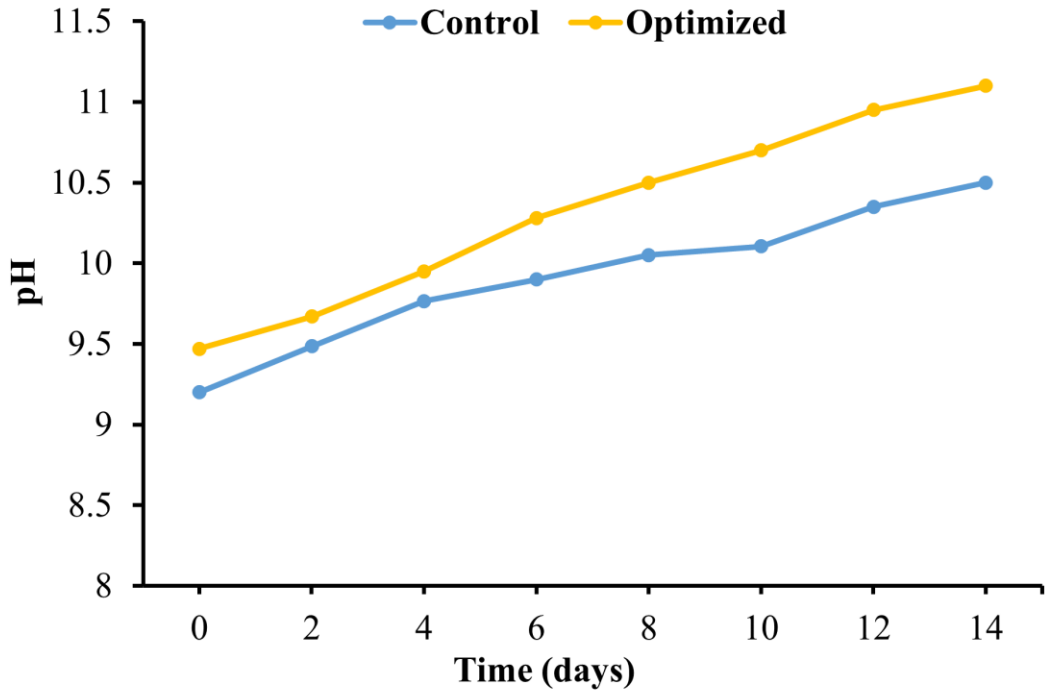

Fig. 5. Changes in the control and optimized outdoor culture pH during the 14 days of the experiment

As shown in Fig. 6), the Fv/ Fm values in SZM were low and constant during the adaption period, then recovery occurred, and the values became 0.42. Further, the Fv/ Fm values of optimized BFCE slightly increased after the adaption period until the 8th day, then a sudden decrease occurred in its values. However, a slight recovery occurred at the end of the experiment. As illustrated in Fig. 7), the optical density values of SZM were higher than the optimized BFCE. For SZM, it took two days in the lag phase and reached exponential growth within 12 days. Additionally, in optimized BFCE, OD values slightly increased after the 4th day until they reached the maximum OD on the 14th day of the cultivation. Moreover, the dry weight of both media was almost constant during the first two days of the cultivation. However, from the 4th day, the dry biomass increased until it reached the maximum values of 0.47 and 0.57g/ l in SZM and optimized BFCE media at the end of the cultivation period (Fig. 8). Moreover, the protein content was insignificantly different in both media, with values of 55.11± 0.75 and 52.58± 1.31 in SZM and optimized BFCE medium.

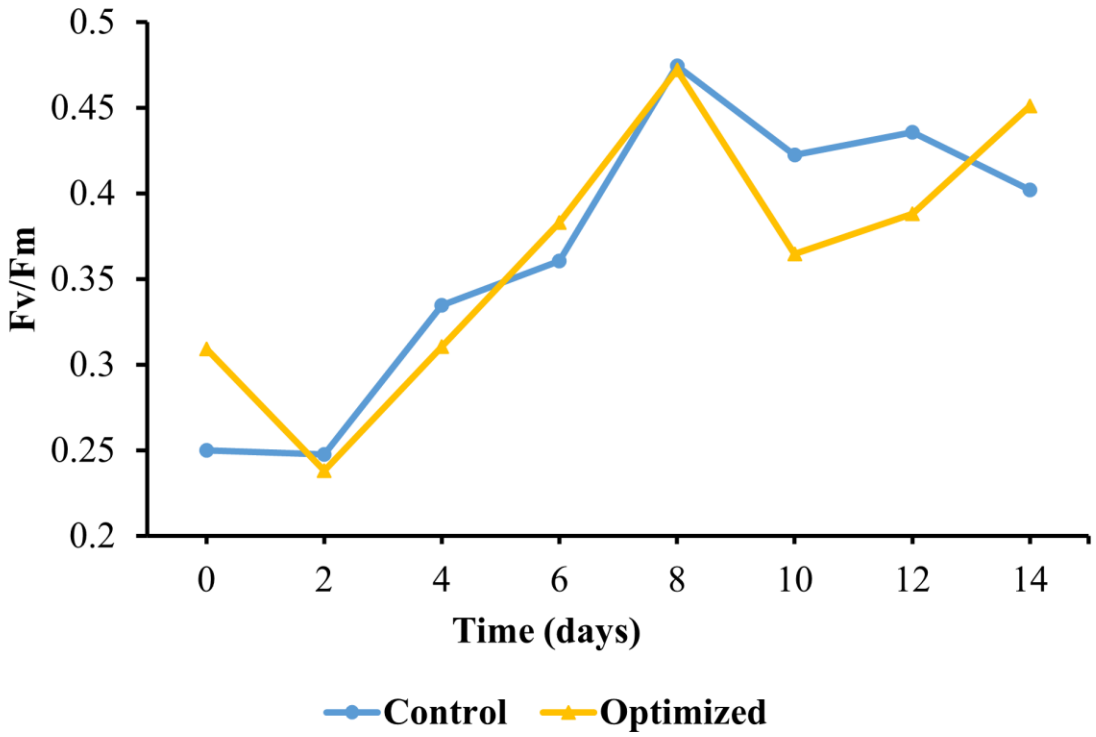

Fig. 6. Changes in the control and optimized outdoor culture photosynthesis activities (Fv/ Fm) during the 14 days of the experiment

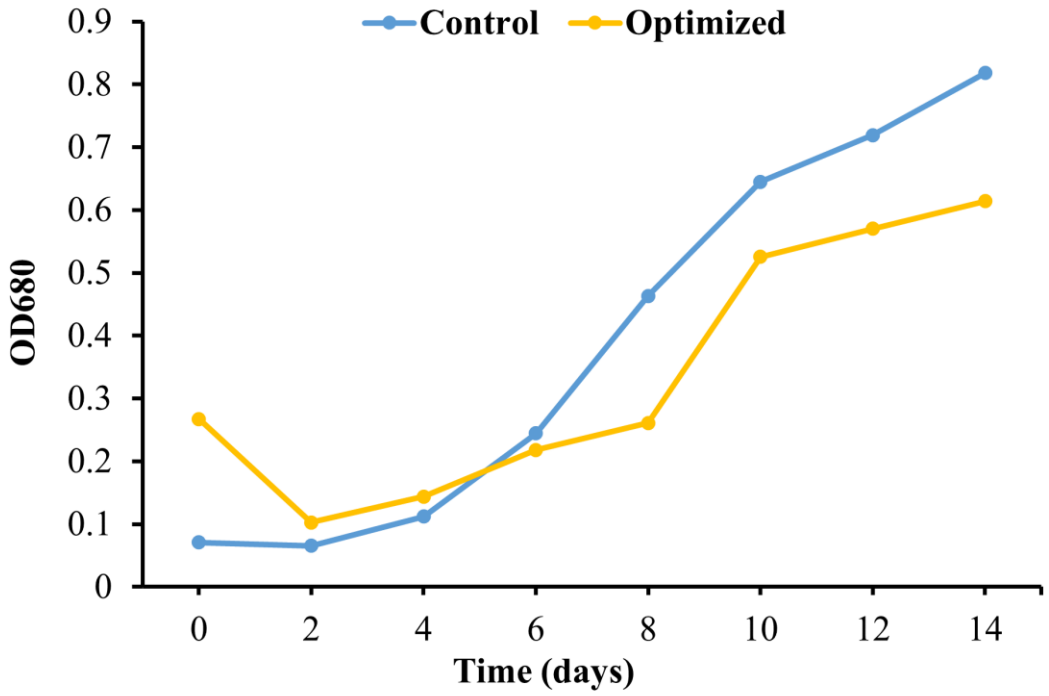

Fig. 7. Changes in the control and optimized outdoor culture optical density during the 14 days of the experiment

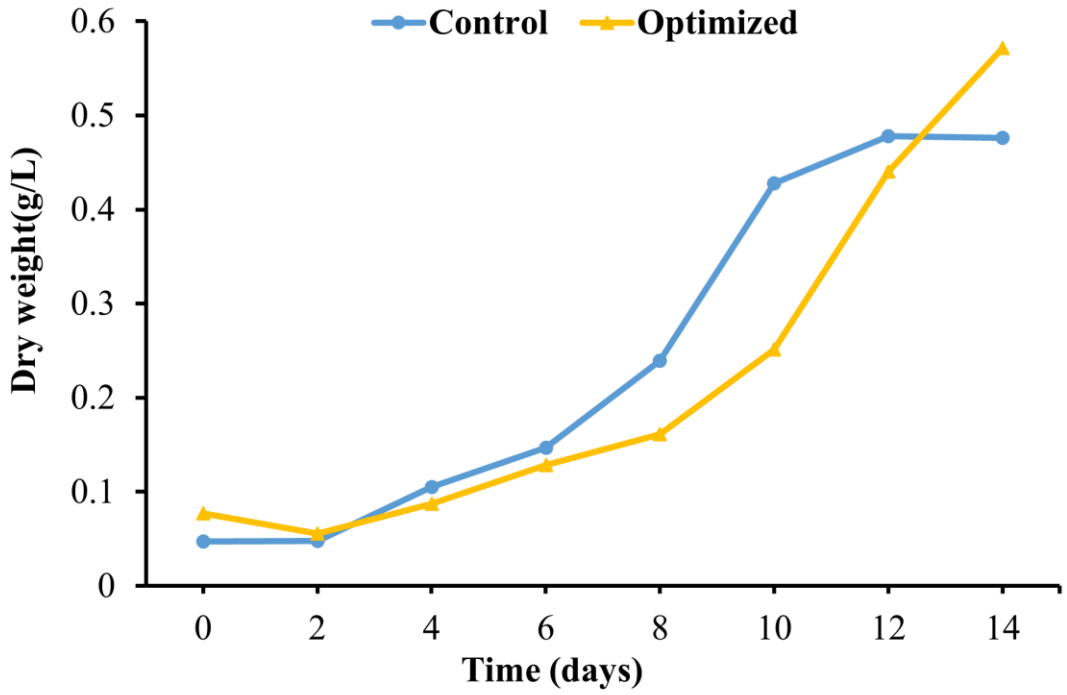

Fig. 8. Changes in the control and optimized outdoor culture dry weight during the 14 days of the experiment

As shown in Table 2), the maximum specific growth rate during the entire period of the experiment in SZM was 0.39 per day, the maximum divisions per day of 0.563 was achieved on the 6th day, having the lowest generation time of 42.62 hours, and these values decreased by the end of cultivation period. However, the maximum specific growth rate for the same period in the optimized BFCE was 0.35 per day, and the maximum divisions per day of 0.50 on the 10th day had the lowest generation time of 47.52 hours (Table 3).

Table 2. Growth parameters of *S. platensis* cultivated on Zarrouk's medium

Time (days)	Optical density (control)	Specific growth rate	Divisions per day	Generation time (days)	Generation time (hours)	Mean growth rate	No. of recycling
0	0.065						
2	0.071	0.04	0.058	17.241	413.784	0.04	0.116
4	0.112	0.228	0.329	3.04	72.96	0.134	1.316
6	0.2445	0.39	0.563	1.776	42.624	0.22	3.378
8	0.463	0.319	0.46	2.174	52.176	0.244	3.68
10	0.645	0.166	0.239	4.184	100.416	0.229	2.39
12	0.719	0.054	0.078	12.821	307.704	0.2	0.936
14	0.818	0.065	0.094	10.638	255.312	0.18	1.316

Table 3. Growth parameters of *S. platensis* cultivated on optimized BFCE

Time (days)	Optical density (optimized BFCE)	Specific growth rate	Divisions per day	Generation time (days)	Generation time (hours)	Mean growth rate	No. of recycling
0	0.267	0	0	0	0	0	0
2	0.1025	-0.479	-0.691	-1.447	-34.728	0.224	-1.382
4	0.144	0.17	0.245	4.082	97.968	0.197	0.98
6	0.218	0.207	0.299	3.344	80.256	0.2	1.794
8	0.261	0.09	0.13	7.692	184.608	0.173	1.04
10	0.5255	0.35	0.505	1.98	47.52	0.208	5.051
12	0.57	0.041	0.059	16.949	406.776	0.18	0.708
14	0.614	0.037	0.053	18.868	452.832	0.16	0.742

Moreover, the protein content in the optimized BFCE medium (52.58± 1.31%) was insignificant compared to SZM (55.11± 0.7%) as illustrated in Fig. 9). Additionally, the chlorophyll-a content difference was insignificant between the two media treatments. However, the phycocyanin content in the optimized BFCE (0.36± 0.035mg/ ml) was approximately half compared to the phycocyanin content in SZM (0.67± 0.036mg/ ml).

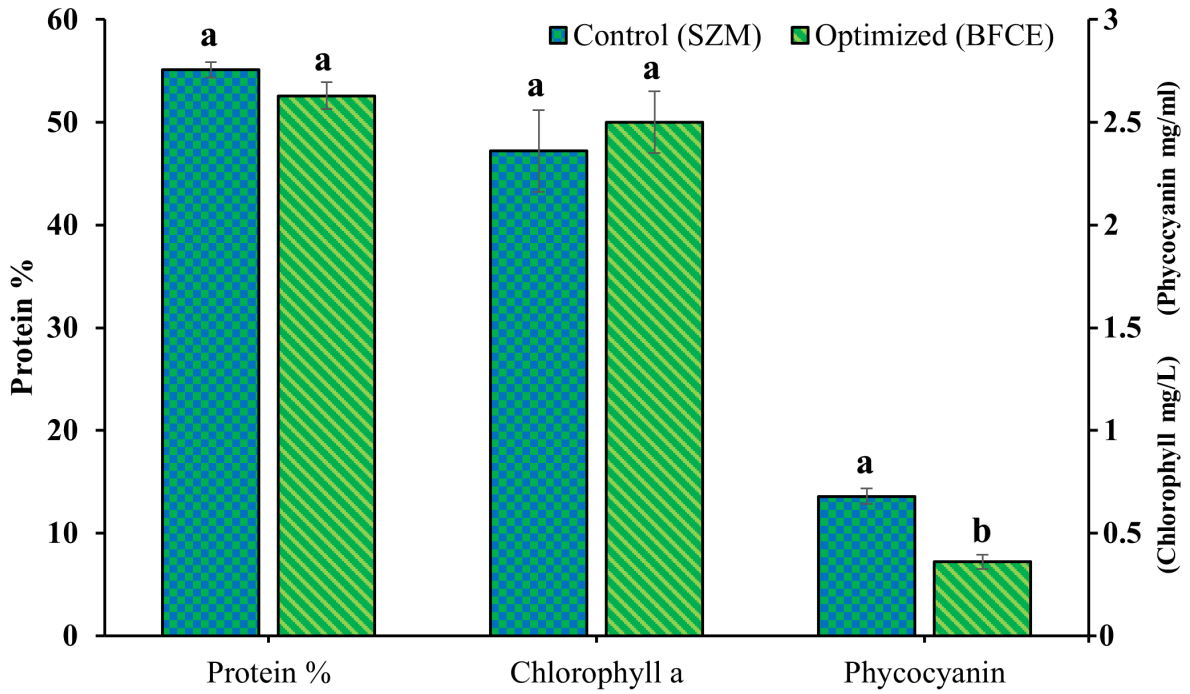

Fig. 9. The protein and photosynthetic pigment content of *Spirulina platensis* cultivated on standard Zarrouk's medium (SZM) and optimized BFCE

Media economical evaluation

Table 4) illustrates an economic analysis of both SZM and optimized BFCE media; the cost of Zarrouk's and optimized BFCE media was calculated based on batch culture mode considering 10^3L medium and the estimated cost to produce 1Kg of dry biomass. SZM showed a contribution to biomass cost of US$14.68/ kg, while optimized BFCE presented a cost of US$5.59/ kg. The cost of Zarrouk's medium presented a 10^3L around 75.14% that was higher than the optimized BFCE medium. The cost of biomass produced from optimized BFCE medium was 2.6 times cheaper than that of Zarrouk's medium.

Table 4. Economical evaluation of the used culture media for large-scale production of *S. platensis* (in US dollars)

Cost	Zarrouk's medium	Optimized BFCE	% Reduction
10^3L medium	8.97	2.22	75.14 %
Cost/ Kg of dry biomass	14.68	5.59	61.92

DISCUSSION

The commercial production of *S. platensis* is not a simple process that depends on different factors to meet the organism's requirements. Among these factors is the high cost of the standard nutrient medium that is used for its cultivation, which limits its commercial production **(Raoof, 2002)**. Therefore, formulation of a low-cost medium for the cultivation of single-cell protein *S. platensis* without affecting the dry weight or the protein content is one of the challenges in large-scale production. In our study, the large-scale production of *S. platensis* on optimized BFCE in comparison to SZM was performed in outdoor open ponds under uncontrolled environmental conditions where the temperature and intensity of solar radiation vary with seasonal climate changes. Certainly, the temperature is a limiting factor that affects the growth and productivity of *S. platensis*. It is well known that the optimum temperature for the growth of *Spirulina* is between 25- 35°C **(Soni *et al.*, 2019)**. However, **Richmond (2017)** mentioned that a temperature around 18°C is satisfactory for *Spirulina*'s outdoor growth, but a temperature lower than 12°C negatively impacts the culture. The temperature during this study ranged from 19.15 to 21.75°C, which is lower than the optimum. At the beginning of the cultivation, the growth rate was low, but it then slightly increased as the *Spirulina* adapted to changes of the environmental conditions.

In a similar study, the growth of three strains of *Spirulina* in outdoor ponds without any deterioration in the cultures during winter temperatures was successful **(Jiménez *et al.*, 2003)**. Light intensity is a critical factor in the production of cyanobacteria both in laboratory and in outdoor environments. Cyanobacteria are photosynthetic organisms that use light as an energy source in the photosynthesis process. It is well known that both low and high light intensity affect microbial growth, therefore the optimal light intensity must be employed to attain optimal growth **(RafiquI *et al.*, 2002)**. In outdoor open ponds, solar radiation is the sole energy source that is used by cyanobacteria or microalgae for growth, and the control of this factor is difficult. However, in the winter season, light is not a limiting factor for growth as the temperature is low and the effect of self-shading of *Spirulina* cells is also low **(Ahsan *et al.*, 2008)**.

The pH of the medium is one of the most important factors that control the growth of *Spirulina*. These alkaline conditions are essential to prevent contamination by other organisms. **Usharani *et al.* (2012)** stated that the optimum pH for the growth of *S. platensis* was 10, and this may be attributed to the fact that the maximum enzyme activity necessary for metabolism is at an alkaline pH. Moreover, the growth of *S. platensis* was evaluated in both growth media by measuring the optical density, photosynthetic activity, and cell dry weight. The photosynthetic activity using the chlorophyll fluorescence measurements can be used to assess any stressful factors that affect the photosynthetic system **(Lu *et al.*, 2000)**. The maximal quantum efficiency of PSII (Fv/ Fm) is the most used parameter, and their values changed in the stress condition **(Qi *et al.*, 2013)**. In the current study, at the beginning of the cultivation, the Fv/ Fm values were lower than the optimum values, and this may be assigned to the lag phase where the organism

adapted to the new environmental conditions, which was consistent with the optical density of organism and then the organism can adapt to the new environment and the value increased to 0.4 in both media, revealing that the newly optimized BFCE medium does not affect the photosynthetic activity of *Spirulina*. However, these values were lower than the optimum Fv/ Fm values of cyanobacteria (0.5), but still had little effect on biomass in the raceway open pond. Moreover, in optimized BFCE on the 8^{th} day, a sudden decrease was recorded in this value and it recovered again at the end of the cultivation period, which may be attributed to the limiting effect of the low temperature of this day (17.1°C) that negatively affected the photosynthetic activity **(Śliwińska-Wilczewska *et al.*, 2019)**. The growth of *S. platensis* was assessed every two days by measuring the optical density and dry weight biomass. At the first days, the growth was constant as the inoculum was transferred from closed photobioreactors of controlled conditions to an outdoor open pond under uncontrolled conditions, which represented a new environment. After adapting to the new environment, the growth began to increase until it reached maximum growth. However, the values of optical density in both media were different; the maximum OD was 0.8 in SZM, and 0.6 in optimized BFCE, which may be assigned to *S. platensis* growing mixotrophically in optimized BFCE, but in SZM, it depends primarily on photosynthesis. Additionally, the dry weight biomass reached to the maximum values of 0.47 and 0.57g/ l in SZM and optimized BFCE media at the end of cultivation period, revealing that the newly optimized medium positively affected the dry biomass. Further, *S. platensis* grown in SZM had a maximum specific growth rate on the 6^{th} day, but *S. platensis* grown in optimized BFCE had a maximum specific growth rate on the 10^{th} day, and the doubling time of *S. platensis* was less in SZM than optimized BFCE medium (Table 2, Table 3). Moreover, according to literature, *S. platensis* has high protein content (approximately 50- 70% of its dry weight) **(Belay *et al.*, 1996)**. In this experiment, no significant difference was detected between the protein content of both media. Additionally, the amino acids profile in both media was almost similar according to **Saad *et al.* (2023)**. Based on economic feasibility, the cost of medium components greatly affects the final production cost of *S. platensis*. Therefore, it is necessary to formulate a low cost-effective nutrient medium using inexpensive nutrients, especially from wastes that reduce the production cost without affecting the protein content. It was clear from the economic evaluation that SZM had the highest cost not only for medium components but also for dry biomass. The newly optimized BFCE medium reduced the cost by 75.14% to setting up a $1m^3$ medium compared to SZM, moreover the cost of biomass produced from optimized BFCE medium is 2.6 times cheaper than that of SZM. Inconsistent with **Raoof *et al.* (2006)**, who formulated a new cost-effective media called a revised medium that costs US$16.0 in comparison to SZM which costs US$79.5. They also revealed that, no significant difference in the protein profile in the new optimized medium and SZM. Based on the previous results, *S. platensis* has the potential to be produced using an optimized BFCE medium as a low-cost alternative to SZM without significantly affecting the protein content or amino acids.

CONCLUSION

In this study, *S. platensis* was cultivated by using SZM and optimized BFCE in the outdoor open pond to validate the feasibility of using optimized BFCE for the production of *S. platensis* without a significant effect on the protein content or amino acid profile. The maximum OD was 0.8 in SZM and 0.6 in optimized BFCE, and the maximum specific growth rate was on the 6^{th} day and 10^{th} day in SZM and optimized BFCE, respectively. The maximum dry weight was 0.47 and 0.57g/ l in SZM and optimized BFCE media at the end of the cultivation period. However, the protein content was 55.11± 0.75 and 52.58± 1.31 in SZM and optimized BFCE medium, respectively. Furthermore, the economic analysis of media showed that the preparation of 1000L of SZM could cost US$8.97, while optimized BFCE costs US$ 2.22. The cost of biomass produced from optimized BFCE medium is 2.6 times cheaper than that of SZM.

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