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RESEARCH ARTICLE

Impact of Culturing Media on Biomass Production and Pigments Content of *Spirulina platensis*

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Abstract

Spirulina platensis is one of the most important microalgae that have high content of valuable pigments. In order to select the best medium for the biomass production of *S. platensis* and consequently pigments content; BG-11, modified BG-11, Zarrouk´s and SHU media were investigated. The maximum dry weight (4.87g l^{-1}) and filaments count (8.8 x10^7filament ml^{-1})were estimated when using Zarrouk´s medium, whereas modified BG-11 medium gave the maximum content of chlorophyll (147.43µg ml^{-1}) and carotenoids (139.88µg ml^{-1}). Regarding to phycobiliprotein pigments, *S. platensis* produced the highest amount of phycocyanin (55.37 µg ml^{-1}) and allophycocyanin (51.73µg ml^{-1}) in modified BG-11 medium, while the maximum content of phycoerythrin (44.13µg ml^{-1}) was observed in SHU medium.

Introduction

Spirulina is a photosynthetic, filamentous, spiral shaped, multicellular blue green microalgae. Since *Spirulina platensis* is reported to be easy in culturing, harvesting and drying process, it becomes the most common popular species in micro algal biotechnology studies. *S. platensis* is reviewed to have a high content of proteins, pigments, essential fatty acids, vitamins and minerals (Belay, 2008 and Mani *et al*., 2008).

In the meantime, *Spirulina* contains many photosynthetic pigments with concentrations exceeding 20% of dry weight, such as chlorophylls, beta-carotene, phycocyanins and xanthophylls, which may have beneficial and commercial values (Vonshak, 2002 and Koru, 2009). Chlorophyll is the most visible pigment in *Spirulina* which contributes between 6.8 to11g kg^{-1}. It releases ions when struck by the energy of sunlight. These free ions proceed to stimulate the biochemical reactions that form proteins, vitamins and sugars in *Spirulina* cultures (Rangel-Yagui *et al*., 2004).

Carotenoids are generally responsible for the red and yellow hues seen in nature and average between 3.4 to 4.0 g kg^{-1}. Beta-carotene accounts for 80% of the carotenoids present in *Spirulina*, which is convertible into vitamin A (Vonshak *et al*., 1996; Habib *et al*., 2008 and Theodore and Georgios, 2013). Xanthophyll presents in *Spirulina* at concentration of 1.0 g kg^{-1}, and its concentration depends upon the species and the environmental conditions. Xanthophylls are an important source of orange-yellow pigments. They are used in the poultry industry as feed additive to enhance the highly colored egg yolk, as well as meat and skin pigmentation that pleasing to consumer (Durand-chastel, 1980 and Richmond, 1988).

Phycobiliproteins are a small group of highly conserved chromoproteins that constitute the phycobilisomes, a macromolecular protein complex. The most common classes of phycobiliproteins are phycocyanin (PC), allophycocyanin (APC) and phycoerythrin (PE), which comprise about 20% of the cellular protein and are quantitatively the dominant pigments in *Spirulina* (Richmond, 1988). Phycocyanins are an important source of blue pigment for use in food coloring, they present in *Spirulina* at concentration between 30 to 220 g kg^{-1} (Fairchild and Glazer, 1994).

Many studies have been accomplished to study the effect of different environmental parameters on the biomass and pigment content of *S.platensise.g*. pH, temperature and light intensity (Pandey *et al*., 2010, Chauhan

and Pathak, 2010, Soundarapandian and Vasanthi, 2008). However, there are no available researches studied the impact of different media of *S. platensis* on the biomass and pigment contents at different incubation intervals. The objective of this study is to evaluate the effect of four media, BG-11, modified BG-11, Zarrouk's and SHU media under controlled conditions on the biomass and pigments content of *S. platensis*.

Materials and Methods

Microorganism

Spirulina platensis strain was isolated from Al-Khadra Lake, Wadi Al-Natroon, El-Baheira governorate, Egypt. Isolation and purification of *S. platensis* was performed by streaking plate method of Stein (1973) using BG-11 agar medium (Allen, 1973). Morphological identification of *S. platensis* was carried out using a phase contrast microscope (Carl Zeiss, Jena, Germany) according to Desikachary (1959), Prescott (1978) and Hindak (1988 and 1990).

Culture media

Four culture media were prepared for cultivation of *S. platensis*: BG-11 medium (Rippka *et al*., 1979), modified BG-11 medium (El-Sayed, 2004), Zarrouk's medium, ZM (Zarrouk, 1966) and synthetic human urine medium (SHU) (Gordon, 1982).

BG-11 medium is composed of 1.5 g $NaNO_3$; 0.004 g K_2HPO_4; 0.075 g $MgSO_4.7H_2O$; 0.036 g $CaCl_2.2H_2O$; 0.006 g citric acid; 0.02 mg Na_2CO_3; 0.001 g Na_2EDTA; 0.63 g ferric ammonium citrate and 1.0 ml trace elements (TE) in 1000 ml distilled water. TE (g/l) is combined of 2.86 g H_3BO_3; 1.81 g $MnCl_2.4H_2O$; 0.222 g ZnSO4.7H2O; 0.39 g $Na_2MoO_4.2H_2O$; 0.079g $CuSO_4.5H_2O$ and 0.0494 g $Co(NO3)_2.6H_2O$. After autoclaving and cooling, pH was adjusted to 7.1. Modified BG-11 medium is similar to BG-11 medium in its composition except using 0.53g urea (46.5%N)instead of 1.5g $NaNO_3$.

ZM medium is composed of 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 $MgSO_4{\cdot}7H_2O$; 0.01 g $FeSO_4{\cdot}7H_2O$ and 1.0 ml trace elements (TE) in 1000 ml distilled water. TE (g/l) isa combined of $H_3BO_3$2.86; $(NH_4)_6Mo_7O_{24}$ 0.02; $MnCl_2{\cdot}4H_2O$ 1.8; $CuSO_4$ 0.08 and $ZnSO_4{\cdot}7H_2O$ 0.22. The culture medium pH was adjusted to 8.2 by using 1 molar NaOH solution.

SHU medium is composed of 0.5 g $CaCl_2{\cdot}2H_2O$; 4.12 g K_2HPO_4; 0.47 g $MgCl_2{\cdot}H_2O$; 0.29 g KCl; 4.83 g NaCl; 1.55 g NH_4Cl; 2.37 g Na_2SO_4; 1.34 g urea; 1.0 g creatinine and 0.65 g sodium citrate (pH 6.8) in 1000 ml distilled water.

The optimum growth conditions (30±2°C and light intensity of 4.5 Klux m^{-2}provided by fluorescent lamps) were applied for this study according to Rafiqual *et al*. (2005), Soundarandian and Vasanthi (2008), Hemlata and Fatma (2009) and Chauhan and Pathak (2010). Suspension of *S. platensis* ($1.2x10^7$ filaments ml^{-1}) were prepared to inoculate the tested media. *S. platensis* was cultivated in 500 ml Erlenmeyer flasks containing 250 ml of representative media using shaking incubator (MP-7552, cv-cc power supply, hsiHefer, San Francisco). Experiments were initiated with 10% (v/v) of inoculum.

Growth evaluation

Dry weight

Biomass concentration of *S. platensis* in each medium was estimated at 5 days intervals during the 40 daysincubation period. Five milliliters of each culture media was taken and filtered through a pre-weighted Whatman sterile membrane filters (0.45µm cellulose acetate filter) and rinsed with 25 ml of acidified distilled water (pH 4) to release all salts and nutrients. After filtration, filter papers were left to dry for two hours at 105˚C and reweighted. Then, dry weight was calculated as g l^{-1} (AOAC, 2000).

Filaments count

Counting of *S. platensis* filaments at different intervals (5-40 days) was carried out using Sedgwick-rafter counting filament under phase contrast microscope (Carl Zeiss, Jena, Germany), as recommended by APHA (1975).

Pigments contents

Total chlorophyll and total carotenoids

Five milliliters of each culture was filtered into 0.47 µm membrane filter using millipore filtration system(Three milliliter of DEMSO 95% was added to the filtrated cells in 10 ml tube. The mixture was sonicated and incubated at 70 °C for 5 min in water bath. Then, it was centrifuged at 3500 rpm for 5 minutes. The optical density (OD) of the supernatant was measured at different wavelengths (468 and 666 nm) using spectrophotometer (Jenway, 6405 UV/vis) according to Seely *et al*. (1972).

The total chlorophyll and total carotenoids concentrations were calculated with the following equations:

Total chlorophyll (mg l^{-1}) = OD_{666} × D × F

Where, E_{666}= the reading at 666 nm, D= volume of extract/volume of sample, F= 11.3 (factor to equal the reduction in absorbance).

Total carotenoids (mg l^{-1}) = OD_{468} × D × F

Where, E_{468}= the reading at 468 nm, D= volume of extract/volume of sample, F= 4.5 (factor to equal the reduction in absorbance).

Phycobiliproteins

Cultures were sonicated for 40 seconds to break up filaments and release the water phycobiliprotein pigments, followed by centrifugation at 8000 rpm to remove filament debris (Moares *et al*., 2010). The optical density (OD) of the supernatant was measured at different wavelengths e.g. 562, 615 and 652 nm for phycoerythrin, phycocyanin and allophycocyanin, respectively. Phycobiliprotein concentration was calculated according to the following equations in µg ml^{-1} according to Bennett and Bogard (1973).

Phycocyanin (PC) = OD_{615}-0.474(OD_{652}) / 5.34

Allophycocyanin (APC) = OD_{652}-0.208 (OD_{615}) / 5.09

Phycoerythrin (PE) = OD_{562}-2.41(PC)-0.849(APC) / 9.62

Statistical analysis

Statistical significance was determined using Statistica Version 9 (StateSoft, Tulsa, Okla., USA). The means of *S. platensis* dry weight (g l^{-1} medium), filament counts, pigments concentration were determined by analysis of variance (ANOVA, one way analysis) ($p<0.05$). Fisher's LSD Method ($\alpha=0.05$) was applied to compare significant differences between different media and interval times.

Results and Discussion

Biomass production

The dry weights of *S. platensis* (g l^{-1}) for different incubation intervals in selected media were determined (Figure 1). In general, incubation for 30 days was the best for biomass production in all media ranging from 4.30 g l^{-1} in SHU medium to 4.87g l^{-1} in Zarrouk's medium. The higher biomass production recorded in Zarrouk's medium is most likely due to the high alkalinity, pH 8.2, of such medium (Pandey *et al*., 2010). In this respect, Soundarandian and Vasanthi (2008) reported that the maximum growth of *S. platensis* was observed at a pH 10. They mentioned that the reason could be attributed to optimal activity of all the enzymes needed for photosynthesis and respiration at such pH.

The growth of *S. Platensis* started to decline after 30 days of incubation. This could be attributed to the increase of death rate over the growth rate. Most of the previous works measured the biomass of *S. platensis* after 15 and 25 days and none of them used extra time to follow the biomass depletion (Olivera *et al*., 1999, Volkman *et al*., 2008, Murugan and Radham, 2010& Kumer *et al*, 2011). While, in the current study the growth of *S. platensis* was estimated until getting a noticeable decrease in the dry weight after 30 days. The behavior of biomass production in different media was confirmed by filament counts estimation (Figure 2). Similar trend of that results obtained in dry weight, was noticed. The highest filament count (8.8 x 10^7 filament ml^{-1}) was recorded after 30 days of cultivation in Zarrouk's medium, followed by 7.2 x 10^7, 7.0 x 10^7, 6.2 x 10^7 filament ml^{-1} in modified BG-11, BG-11 and SHU media, respectively.

Pigments contents

Total chlorophyll

Figure 3 shows the concentration of total chlorophyll accumulation in *S. platensis* grown in different media. In general, incubation for 25 and 30 days resulted in maximum production of chlorophyll depending on the applied medium. The cultivation in modified BG-11 medium for 30 days gave the best yield of total chlorophyll (147.43 µg ml^{-1}); while in BG-11 medium the chlorophyll concentration decreased to 110.76 µg ml^{-1} at the optimum incubation time (25 days). This might be due to the composition of modified BG-11 medium since it contains urea as a source of nitrogen instead of $NaNO_3$ in BG-11. This results is confirming the observations of Danesi *et al*. (2011) who reported that maximum total chlorophyll content was achieved using urea while using potassium nitrate gave lower amounts. Regarding the influence of other tested culturing media Zarrouk's and SHU, on chlorophyll accumulation in *S. platensis*, no differences were observed.

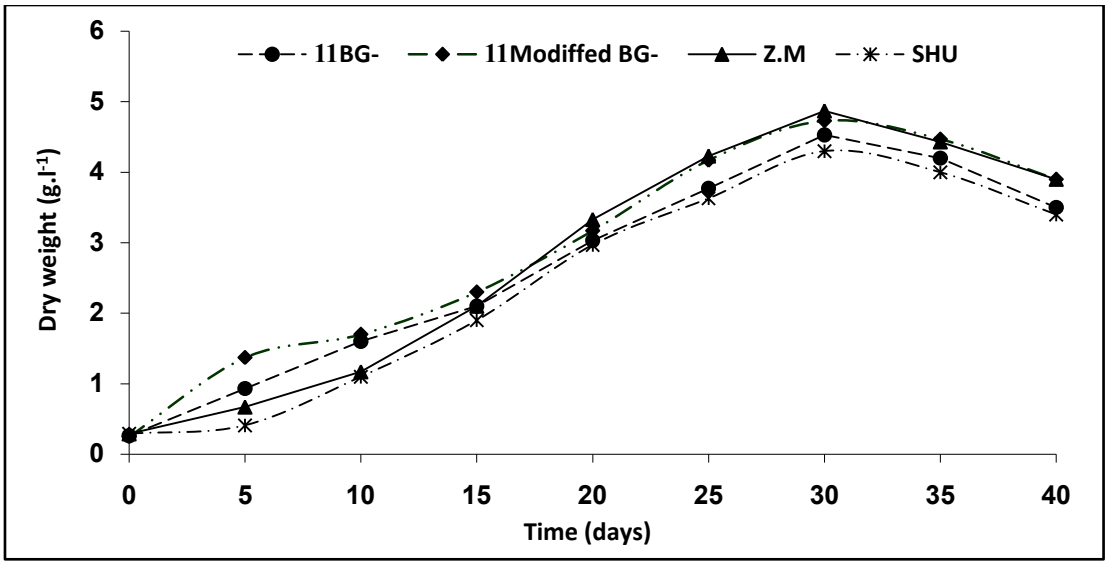

Fig. (1) Growth curve of *Spirulina platensis* (g l^{-1}) in different media.

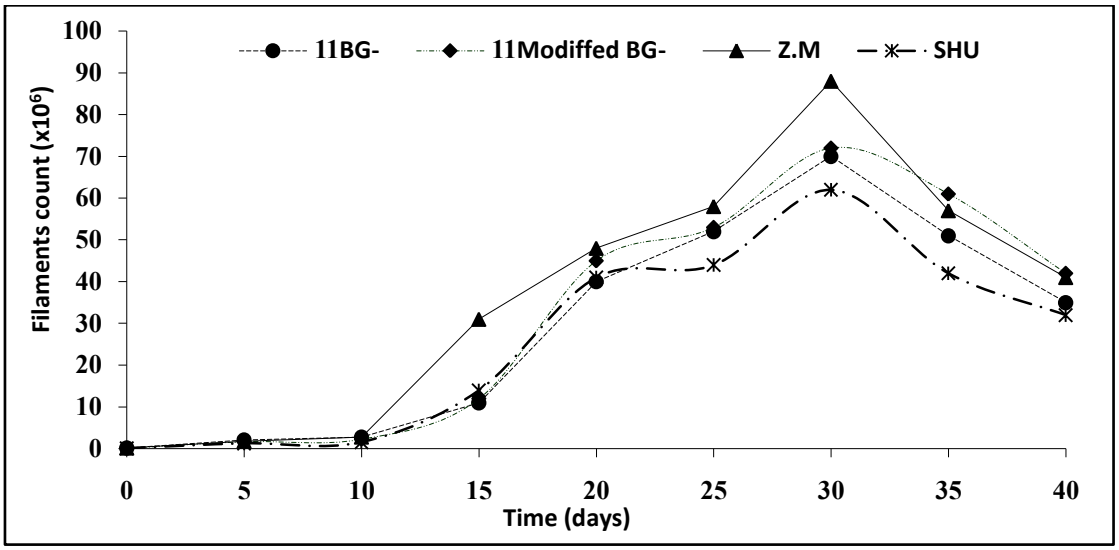

Fig. (2) Filaments count (filamentml^{-1})of *Spirulina platensis* in different media.

Total carotenoids

Concerning the influence of medium type on carotenoids concentrations, it was found that similar trend of total chlorophyll content profile was recorded (Fig. 4). The highest total carotenoid concentration was recorded in modified BG-11 medium (139.88 µg ml^{-1}) after 30 day of inoculation. Carotenoids content in other media were almost the same with an average of around 115 µg ml^{-1} at their optimum incubation time. This may indicate a strong relation between both chlorophyll and carotenoids contents. Such correlation could be attributed to that the carotenoids protect chlorophyll molecules against photo destruction and oxidation by molecular oxygen (Krinsky, 1979). Similarly, Vonshak (1997) reported that there was a positive correlation between chlorophyll and carotenoids content of *S. platensis* and the incubation period up to 30 day at 35˚C.

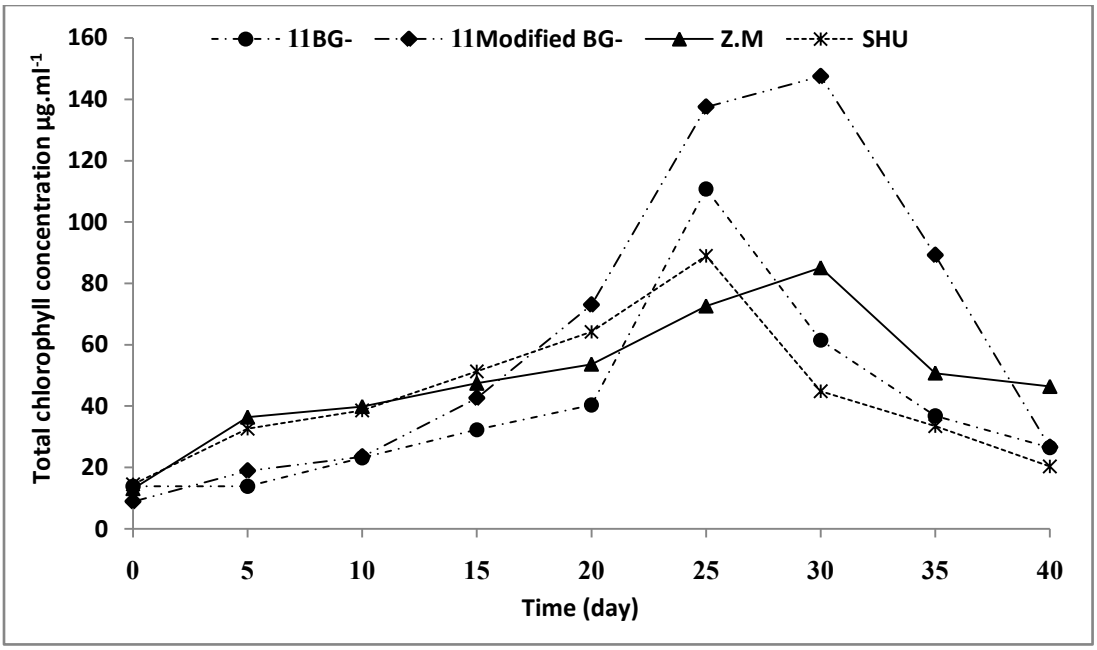

Fig. (3) Total chlorophyll content in *Spirulina platensis* grown in different media.

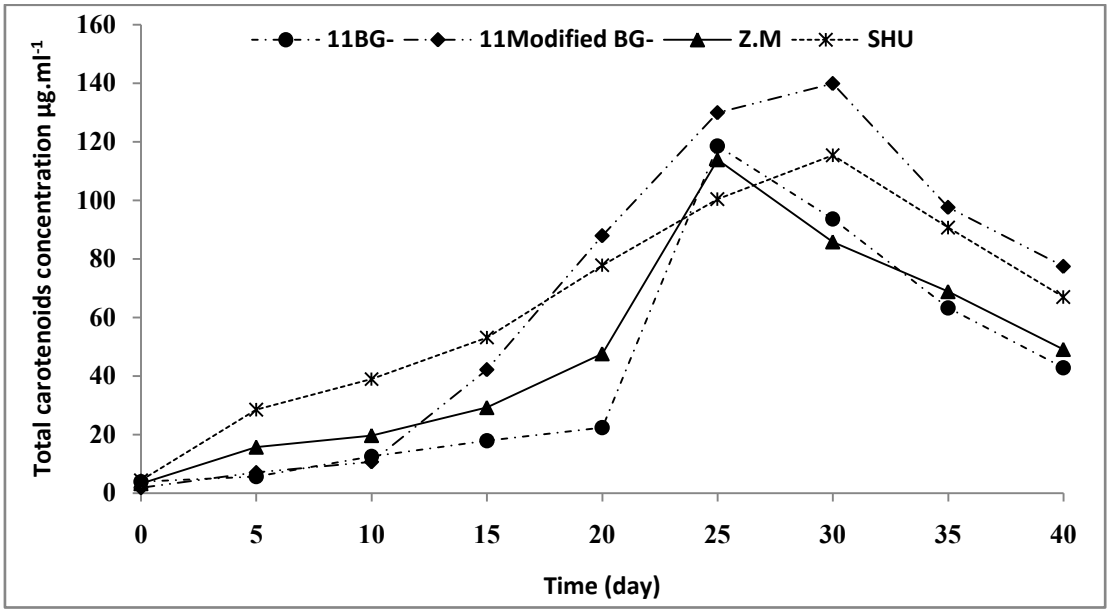

Fig. (4) Total carotenoids content profile of *Spirulina platensis* in different media.

Phycobiliproteins

Figure 5 indicates the behavior of *S. platensis* grown in different media regarding phycocyanin content. In contrast to the obtained results of chlorophyll and carotenoids contents, 20 days incubation was the optimum one for phycocyanin content in *Spirulina* (50.6-55.4 µg ml^{-1}) in any of the tested media. At that time, there was no significant difference in its content when grown in BG11, modified BG-11 and Z.M. Where, SHU media showed the lowest amount of phycocyanin (50.63 µg ml^{-1}). Several studies evaluated the impact of different sources of nitrogen on phycocyanin content in *S. platensis*. Abd El-Baky (2003) found that decreasing the nitrogen concentration in the nutrient media led to a decrease in phycocyanin content. Also, Chouhan *et al*. (2013) found that the addition of different nitrogen source in the culture increased both phycocyanin and phycoerythrin contents. As mentioned before in this study, the modified BG-11 contained urea in addition to ferric ammonium citrate. This considered an

advantage as it recorded the highest content of phycocyanin (55.4 µg ml^{-1}). This observation could be interpreted by the finding of Garcia-Ferandez and Diez, 2004) who reported that in case of presence of several nitrogen sources in culture media of several cyanobacterial species, cyanobacteria might prefer the reduced one e.g. amino acids, urea and ammonium.

The concentrations of allophycocyanin in *S. platensis* in different media after incubation for 40 days are illustrated in Figure 6. Result indicated that, no significant differences were observed at the maximum production of allophycocyanin (average of 51 µg ml^{-1}) after 20 days compared to BG-11 and modified BG-11. Also, the maximum production was very close (average of 51.5 µg ml^{-1}) after 25 days in both SHU and Zarrouk's media. Similar amounts of the pigment were obtained in SHU medium after 20 and 25 days as they 51.03 and 52.9 µg ml^{-1}, respectively. This means that *S. platensis*, in all media except Zarrouk's, can be harvested after 20 days for the maximum content of allophycocyanin. As mentioned before, there are no available studies regarding the allophycocyanin production in different culturing media from *S. platensis*. However, Saleh *et al*. (2011) found that the highest amount was observed after 15 days of incubation in Zarrouk's medium.

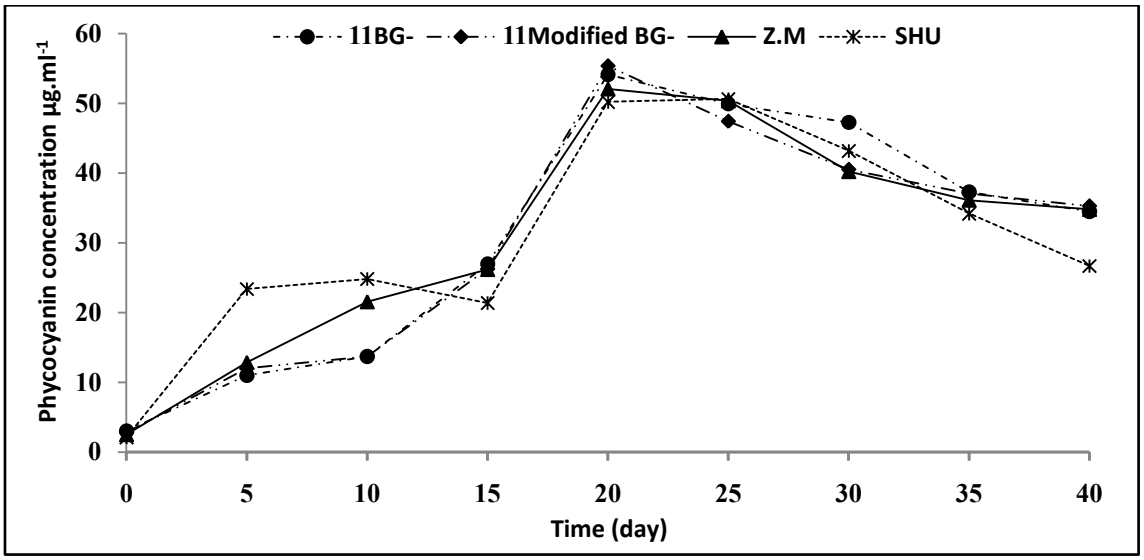

Fig. (5) Phycocyanin content of *Spirulina platensis* in different media

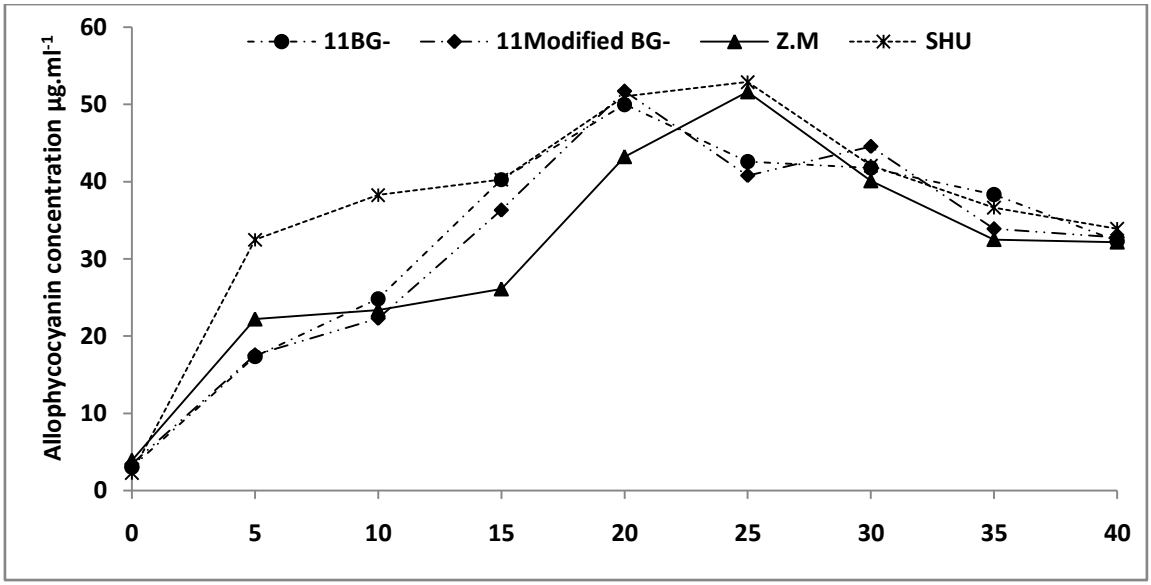

Fig. (6) Allophycocyanin content of *Spirulina platensis* in different media.

Figure 7 shows the contents of phycoerythrin in *S. platensis* in different media. Since no significant difference ($P<0.05$) was observed in pigment content in SHU after 25 and 30 days. So, the cultivation for 25 days was chosen as an optimum period to yield the maximum production in all media (39.6-44.1 $\mu g.ml^{-1}$). No significant effect ($P<0.05$) on the pigment amount was noticed by changing the medium at 25 days. Results of present study are similar to that obtained by Saleh *et al*. (2011) who reported that the maximum production of phycoerythrin content in Zarrouk's medium was obtained after 25 days.

Table 1 shows statistical effects of media the type, incubation time and their two way interactions on dry weight, filament count and pigment contents. Incubation time factor had the main significant impact followed by medium type. The only factor observed no significance effect was medium type on phycocyanin content in *S. platensis*.

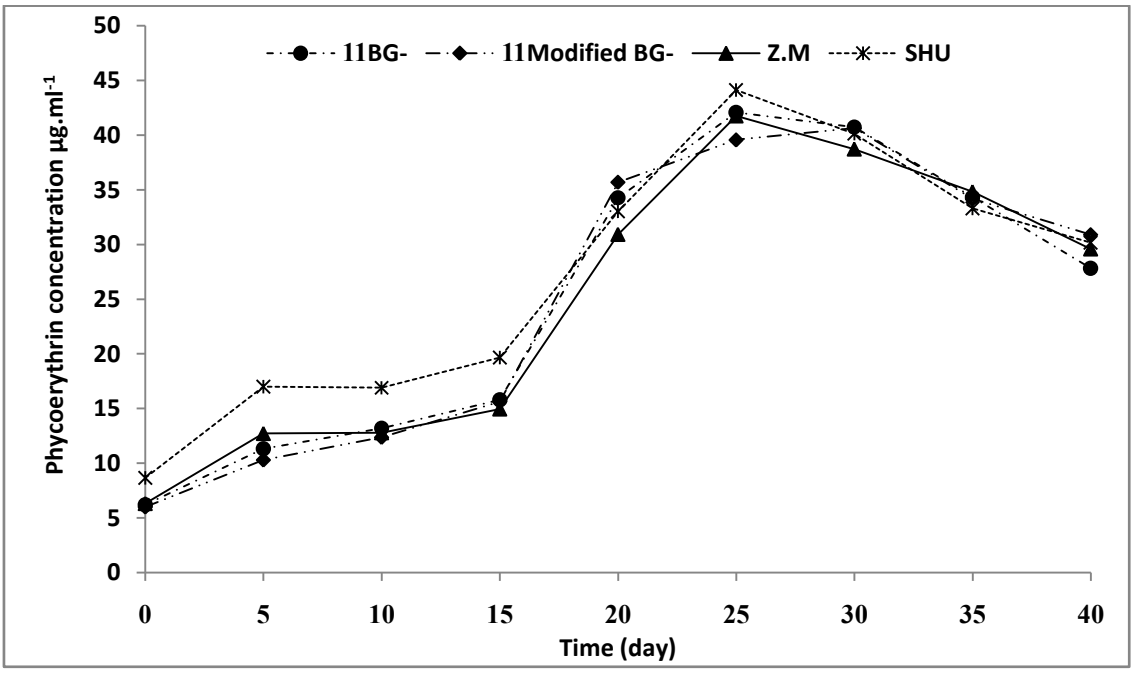

Fig. (7) Phycoerythrin profile of *Spirulina platensis* in different media.

Table 1. Analysis of variance of the effect of different media of *Spirulina platensis* on the dry weight (g l^{-1}), filaments count (filaments ml^{-1}) and its pigments content (µg ml^{-1} media) for several incubation time (5-40 days).

Dry weight					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	783.5984	1	783.5984	18244.04	0.000000
Media type	3.0729	3	1.0243	23.85	0.000000
Time (day)	242.5037	8	30.3130	705.76	0.000000
Media x Time	2.2226	24	0.0926	2.16	0.006622
Error	3.0925	72	0.0430		
Filaments count					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	1.057377E+17	1	1.057377E+17	64147.15	0.00
Media type	1.821826E+15	3	6.072752E+14	368.41	0.00
Time (day)	6.807600E+16	8	8.509500E+15	5162.40	0.00
Media x Time	2.400160E+15	24	1.000067E+14	60.67	0.00
Error	1.186820E+14	72	1.648361E+12		
Chlorophyll					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	251022.7	1	251022.7	18430.47	0.00
Media type	8562.3	3	2854.1	209.55	0.00
Time (day)	74698.2	8	9337.3	685.56	0.00
Media x Time	31583.0	24	1316.0	96.62	0.00
Error	980.6	72	13.6		
Carotenoides					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	328463.5	1	328463.5	30879.48	0.00
Media type	11207.9	3	3736.0	351.22	0.00
Time (day)	157393.7	8	19674.2	1849.61	0.00
Media x Time	12884.6	24	536.9	50.47	0.00
Error	765.9	72	10.6		
Phycocyanin					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	101212.6	1	101212.6	17771.89	0.000000
Media type	22.9	3	7.6	1.34	0.267366
Time (day)	26995.8	8	3374.5	592.52	0.000000
Media x Time	952.9	24	39.7	6.97	0.000000
Error	410.0	72	5.7		
Allophycocyanin					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	115771.3	1	115771.3	26004.11	0.000000
Media type	587.3	3	195.8	43.97	0.000000
Time (day)	19010.6	8	2376.3	533.76	0.000000
Media x Time	1343.7	24	56.0	12.58	0.000000
Error	320.5	72	4.5		
Phycoerythrin					
Effect	**SS**	**DF**	**MS**	**F**	**P**
Intercept	70033.33	1	70033.33	15790.07	0.000000
Media type	86.65	3	28.88	6.51	0.000584
Time (day)	16358.25	8	2044.78	461.03	0.000000
Media x Time	180.33	24	7.51	1.69	0.045338
Error	319.34	72	4.44		

SS: sum of squares, DF: degree of freedom, MS: mean square, P: probability at confidence 0.95.

Conclusion

The present study revealed the importance of the impact of cultivating media and incubation period on the biomass and pigments production of *S. platensis.* It could be considered as one of the few works followed the biomass production and pigment contents in different media during long period of incubation (40 days). The incubation of *S. platensis* for 30 days was an optimum period to yield the maximum biomass production. Zarrouk's medium was the best for biomass production of *S. platensis* due to its high alkalinity (pH 8.2). Generally, the highest levels of studied pigments were noticed in all media between 20-30 days incubation times. Modified BG-11 showed the maximum productivity of total chlorophyll, carotenoids and phycobiliprotiens pigments. Finally, it could be concluded that the optimum medium and cultivation period could be chosen depending on the required final product either biomass or pigment.

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