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Evaluation of Nutrient Dependent Growth of *Spirulina platensis* for Pigment-proteins Production

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ABSTRACT

Background: *Spirulina* is a multicellular, filamentous cyanobacterium, belonging to the *Phormidiaceae* family which appears as blue-green filaments composed of cylindrical cells arranged in unbranched helicoidal trichomes. It contains a wide spectrum of nutrients that include proteins with all essential amino acids, carbohydrates, vitamins, minerals, pigments, carotenoids and super antioxidants apart from trace elements.

Methods: The aim of the present study is to optimize the growth of cyanobacterium *i.e*., *Spirulina platensis* in selected media such as Zarrouk's modified medium, Zarrouk's medium, BG11 medium and F-2 medium. The growth analyses were recognized after 30 days. The temperature was maintained at 30±2°C under 12:12 hour light-dark cycles, light illuminated (4500 lux).

Result: The maximum biomass of 0.641 gm/ml was achieved in Zarrouk's modified medium. The inoculation of *S. platensis* in the F-2 medium showed the least growth of alga. The maximum concentration of phycocyanin content (0.19 mg/ml) and total carotenoid contents (5.99 µg/ml) were observed in Zarrouk's modified medium followed by Zarrouk's medium (0.12 mg/ml and 5.51 µg/ml) and minimum amounts were observed in F-2 medium (0.08 mg/ml and 3.08 µg/ml). According to the results, this study concluded that the growth and biomass of *Spirulina* with significant cell count and higher pigment proteins can be enhanced by using the naturally modified medium.

Key words: BG11 medium, Carotenoid, F-2 medium, Phycocyanin, *Spirulina platensis,* Zarrouk's medium, Zarrouk's modified medium.

Abbreviations: CPC-C- Phycocyanin, HPLC- High performance liquid chromatography, KNO_3- Potassium nitrate, $NaNO_3$- Sodium nitrate, OD- Optical density, PBP- Phycobiliproteins, PC- Phycocyanin.

INTRODUCTION

Cyanobacteria have agricultural, industrial, pharmaceuticals and biomedical research potential in terms of the production of natural products like pigments, vitamins, fatty acids, polysaccharides and different essential minerals (Rastogi and Sinha, 2009). Natural pigments [chlorophylls, carotenoids and phycobiliproteins (PBP)] play a specific role in metabolism and photosynthetic process of cyanobacteria (Mulders *et al*., 2014). People used *Spirulina* in food diets and remedies because it has significant components mainly vitamins and proteins (Benneman, 1988).

Spirulina platensis is a filamentous, multicellular and autotrophic cyanobacterium that has economic value. It resides in alkaline water. *S. platensis* recognized as the best bio-agent and is highly considered for experimental purposes by researchers (Moris *et al*., 2001; Kawata *et al*., 2004; Chen *et al*., 2006) due to having various phytochemical compounds in the highest amounts such as vitamins, polysaccharides, essential amino acids and fatty acids, minerals, phycocyanin (PC), carotenoids, (Cohen *et al*., 1987; Cohen and Vonshak, 1991) and protein (Umesh and Sheshagiri, 1984). Among the phycobiliproteins, phycocyanin was found in the maximum amount and allophycocyanin and phycoerythrin are present in a minimum concentration in *S. platensis*. Phycocyanin (PC) has bright blue color, which depends on the level of purity (Gualtieri and Barsanti, 2006). PC has more attention in research fields due to its significant values in various fields such as in cosmetic and food industries for pigmentation (Eriksen, 2008), fluorescence purposes and pharmaceutical applications like anticancer, anti-inflammatory, to protect nervous system and to scavenge free radicals (Kronick and Gilpin, 1986; Romay *et al*., 1998). Phycocyanin has been also used in immunochemical techniques and clinical industries (Sio *et al*., 2013; Romay

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et al., 2003). *Spirulina* spp. have been utilized as human food in many parts of the World.

The culture media play a vital role in the growth and development of different plant species. There are many different media available for the sustainable growth of plants in laboratory conditions. The selection of media depends on the plant species and its growth patterns in the natural environment. Some traditional media like Murashige and Skoog's Medium (1962) is used to develop in vitro culture of some plants like *Caralluma edulis* (Parihar, 2016, 2018; Parihar and Dwivedi, 2019), *Ceropegia bulbosa* (Parihar, 2017), *Glossonema varians* (Parihar, 2020; Parihar and Dwivedi, 2020), *Stevia rebaudiana* (Mathur *et al*., 2017). Zarrouk's medium was initially synthesized as a synthetic medium by Zarrouk in 1966 that is still utilized in the form of a standard media. Subsequently, several other media like CFTIR medium (Venkataraman *et al*., 1995), OFERR medium and Rao's medium (Singh, 2006), Revised medium (Raoof, 2006), Bangladesh medium (Khatum *et al*., 1994) have been used to observed highest *Spirulina* growth. Considering the various applications and cost of production of *S. platensis*, the present study focuses onto develop a modified culture media for better growth of *Spirulina platensis* for better production of pigment proteins.

MATERIALS AND METHODS

Collection of algal samples

The freshwater cyanobacteria were collected from Jal Mahal, Jaipur (Rajasthan) in a sterile plastic container. *S. platensis* was isolated from the serial dilution method. Cultures were raised in BG-11 Medium (Stanier *et al*., 1971). The initial OD (optical density) of the suspension culture was maintained at 0.3 at 750 nm and was allowed to grow in light intensity provided by cool-white fluorescent tubes of 50 µ mol photons/m^2/s following 12:12 hour, light and dark regime at 30°C ± 2°C (Hemlata and Fatma, 2009). Cultures flasks were shaken manually to allow air and nutrient circulation.

Culture media preparations

Spirulina platensis strain was cultivated in four different media to detect their higher biomass.

Zarrouk's agar media

The Zarrouk's medium was supplemented with potassium hydrogen phosphate (0.5 gm/l), potassium sulfate (1.0 gm/l), magnesium sulfate heptahydrate (0.2 gm/l), Iron (II) sulfate (0.01 g mL^{-1}), Ethylene diamine tetra-acetic acid (0.08 gm/l), Sodium bicarbonate (4.5 gm/l), Sodium nitrate (2.5 gm/l), Sodium chloride (1.0 gm/l) and Calcium chloride (0.04 gm/l) at saline pH 8.8 to 9.0 (Costa *et al*., 2000). The cultures were maintained in growth chamber under 30±2°C temperature, 4500 lux illumination and intermediate photoperiod (light and dark cycle for 12:12 h). Suspension cultures were manually shaken three times a day for better circulation of air and nutrients.

Modified Zarrouk's agar media

The Zarrouk's medium was supplemented by Potassium hydrogen phosphate (0.5 gm/l), Potassium sulfate (1.0 gm/l), Magnesium sulfate heptahydrate (0.3 gm/l), Iron (II) sulfate (0.03 gm/l), Ethylene diamine tetra-acetic acid (0.1 gm/l), Sodium bicarbonate (4.5 gm/l), Sodium nitrate (2.5 gm/l), Sodium chloride (1.0 gm/l) and Calcium chloride (0.04 gm/l) at saline pH 8.8 to 9.0 (Costa *et al*., 2000). The cultivation has been maintained in growth chamber under 30±2°C temperature, 4500 lux illumination and intermediate photoperiod (light and dark cycle for 12:12 h). Cultures were manually shaken for three times a day for passage of air and nutrients.

BG-11 media

The medium contains K_2HPO_4 0.04 gm/l, $NaNO_3$ 1.5 gm/l, $MgSO_4.7H_2O$ 0.075 gm/l, Citric acid 0.006 gm/l, $CaCl_2 \cdot 2H_2O$ 0.036 gm/l, Ferric ammonium citrate 0.006 gm/l, Na_2CO_3 0.001 gm/l and 0.02 gm/l EDTA (disodium salt). 1.0 ml/l trace metal A5 (pH 7.1) was mixed. Trace Metal Mix A5/l: The mixture of trace metal A5 contains H_3BO_3 2.86 gm/l, $ZnSO_4.7H_2O$ 0.222 gm/l, $MnCl_2 \cdot 4H_2O$ 1.81 gm/l, $NaMoO_4 \cdot 2H_2O$ 0.39 gm/l, Co $(NO_3)_2 \cdot 6H_2O$ 49.4 mg/l, $CuSO_4 \cdot 5H_2O$ 0.079 gm/l. The alga strain in BG11 medium was grown at 28±1°C with 2500 lux and under a light-dark regime of 12:12 h. Cultures were manually shaken thrice a day for better circulation of air and nutrients (Yoon *et al*., 2002; Robert and Andersen, 2005).

F-2 media

The F-2medium contains $NaNO_3$ 75 gm/l, $NaH_2PO_4.H_2O$ 5 gm/l, $Na_2SIO_3.9H_2O$ 30 gm/l, $FeCl.6H_2O$ 3.15 gm/l, $Na_2EDTA.2H_2O$ 4.35 gm/l, $CuSO_4$ 9.8 gm/l, $Na_2MO_4.2H_2O$ 6.3 gm/l, $ZnSO_4.7H_2O$ 22 gm/l, $COCl_2.6H_2O$ 10 gm/l and $MnCl_2.4H_2O$ 18 gm/l. Biotin and cyanocobalamin were mixed in F-2 medium as a trace. The alga strain in F-2 medium was grown at 281°C with 2500 lux and under a light-dark regime of 12:12 h. Manual shaking of cultures was done thrice a day for passage of air and nutrients (Guillard, 1975).

Cell counting

The cell count of *Spirulina* was done by hemocytometer (Andersen and Throndsen, 2004). The hemocytometer chamber was filled with *Spirulina* culture of each media (Zarrouk's agar media, modified Zarrouk's agar media, BG-11 media and F-2 media) by capillary action. The whole slide or a selected number of large squares was observed under the microscope for counting the significant cell number. The average cell number in one ml sample was calculated by using the following formula:

The average number of cells per ml = Average count per large square × 10,000

Analysis of secondary metabolites

Determination of Phycocyanin

The water-soluble pigments (phycobiliproteins) including C-phycocyanin (CPC), were extracted from the fresh algal

sample (1 gm) with 0.05M phosphate buffer (10 ml, pH 6.8). The absorbance (A) of the solution was measured at 620 and 650 nm (Goh *et al*., 2010) and the concentrations were calculated as follows:

$$CPC\ (mg/ml) = \frac{A_{620} - 0.72 \times A_{650}}{6.29}$$

Determination of total carotenoids

5 ml of homogenized *S. platensis* suspension culture was centrifuged at the speed of 3000 rpm for 5 minutes tentatively. The supernatant was discarded and the pellet was washed three times carefully with the help of deionized water. To the pellet, 3 ml of 85% acetone was added, followed by freezing and thawing process was repeated. The solution was centrifuged and the supernatant containing pigment was collected. To collect pure carotenoids, this extraction process was repeated continuously until colorless supernatant was obtained. The pooled fractions of supernatants were made up to a final known made up to 5 ml by 85% acetone. The optical density (OD) was measured by spectrophotometer at 450nm. 85% acetone had been taken as a blank. The total carotenoids concentration was measured by using the following formula (Saleh *et al*., 2011):

$$C = \frac{D \times VF}{\text{Average extinction coefficient (2500)}} \times 100$$

Whereas,
D = Absorbance at 450 nm.
F denotes = Dilution factor and V denotes volume of the extract.

RESULTS AND DISCUSSION

Optimization of the growth condition of *S. platensis*

The optimization of appropriate media for optimum cultivation of *S. platensis* was very important. Four different types of media *viz*. Zarrouk's modified medium, Zarrouk's medium, BG-11 and F-2 medium were to initiate culture of Spirulina (Fig 1). The maximum growth of culture was observed in Zarrouk's modified medium with 0.641 gm/ml, followed by 0.610 gm/ml on Zarrouk's medium as compared other medium (Table 1). Zarrouk's medium is also reported as appropriate medium for S. *platensis* growth by Devanathan and Ramanathan, 2013. In 2016, Rajasekaran *et al.* used modified Zarrouk's medium in which they added potassium nitrate (KNO_3) despite of sodium nitrate ($NaNO_3$) and they suggested that SP-6 and CCMB strains of *S. platensis* could be used for higher growth with Zarrouk's modified medium as compared with Zarrouk's medium. Michael *et al.* (2019) used cost-effective culture medium (LCMA) that exhibited *Spirulina* growth in large scale with maximum 0.75 gm/100 ml of dry weight, 0.99% chlorophyll level and maxim um absorbance (2.06 at day 15) than the Zarrouk's medium. Fanka *et al*., 2022 also evaluate the biomass production and biomolecule concentrations of *Spirulina* species in Zarrouk's medium, BG-11 and modified Zarrouk's medium, which has reduced nitrogen (40%), carbon (83%), magnesium (63%) and phosphorus (94%). In their study Zarrouk's modified medium gave maximum growth of *Spirulina* species (0.19 gm/l/d) as well as 21.6% carbohydrates which is 160% higher as compared to Zarrouk's medium. In the present study also, a modified Zarrouk's medium showed higher biomass production of *Spirulina platensis*in comparison to other medium.

Cell counting

The maximum number of cells of *S. platensis* was observed from Zarrouk's modified medium *i.e*. 4,00,000 average no. of cell/ml, followed by Zarrouk's medium with 2,00,000 average no. of cell/ml as shown in Table 2. The cell count 98000 average number of cells per ml was recognized from BG-11 Spirulina culture. The least average number of cells in one ml of *Spirulina* culture *i.e*. 92000 was found from F-2 medium. The results indicated that the Zarrouk's modified

Table 1: Biomass production of *Spirulina platensis* on different media.

Media	Biomass (gm/ml)
Zarrouk's modified medium	0.641±0.047
Zarrouk's medium	0.610±0.075
BG-11 medium	0.538±0.032
F-2 medium	0.399±0.029

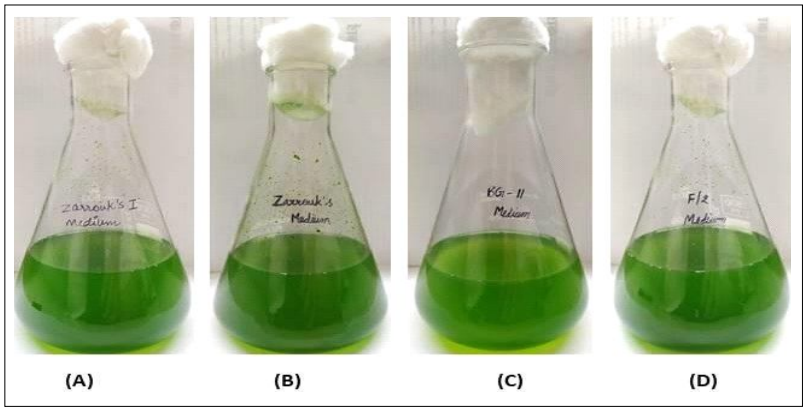

Fig 1: Growth of *Spirulina platensis* in (A); Zarrouk's modified medium, (B); Zarrouk's medium, (C); BG-11 medium, (D); F-2 medium.

medium was the optimum medium for significant growth of *S. platensis*. The similar results were obtained from Rajasekaran *et al.* (2016) in which Spirulina gave optimum growth in Zarrouk's modified medium. Abd El-Monem *et al.* (2021) reported Zarrouk's medium as the most prominent medium for better cultivation of *S. platensis*. On the other hand, Joshi *et al.* (2018) revealed that BG-11 medium is more effective to cultivate specific growth of Spirulina in comparison to Walne and CHU medium.

Analysis of pigment proteins

Phycocyanin

Among the phycobiliproteins, phycocyanin plays a vital role in numerous fields with highest economic value. Hence *S. platensis* attains more attention for large scale biomass production with simple procedures (Chofamba, 2021). Fig 2 showed the calculated data of the phycocyanin contents in the different medium of the *Spirulina platensis*. As compared to other *Spirulina* culture medium, the maximum phycocyanin level (0.19 mg/ml) was observed in Zarrouk's modified media of *S. platensis*. Thereafter 0.12 mg/ml and 0.1 mg/ml phycocyanin content was observed in Zarrouk's medium and BG-11 media, respectively. The lowest concentration (0.08 mg/ml) of phycocyanin was observed with F-2 media of *Spirulina*. Ali *et al.* (2014) reported high phycocyanin content (0.15 mg/ml) in ethyl acetate extract of *S. platensis* cultivated in Zarrouk's medium. UV spectrometer and Liquid chromatography–diode-array detection (LC-DAD) method were used by Park *et al*., 2018 to identify phycocyanin concentration from seven different *Spirulina* products with freeze-dried *S. platensis* trichomes cultures in which maximum phycocyanin level (251.2 mg/gm) was detected with *S. platensis* trichomes and 94.9-153.3 mg/gm average range in spirulina products. A study conducted by Chofamba, 2021 revealed that lowest illumination is appropriate to maximum PC level from *S. platensis* cultured on modified Zarrouk's medium.

Total carotenoids

Carotenoids is a significant natural pigment among the other pigments which is present in algae (Borowizka, 1988), few fauna and all vascular flora (Zeb and Mehmood, 2004). Carotenoids have yellow–orange–red color that is dissolved with hydrophobic solvents. It has vital applications in various fields such as food and cosmetics industry and therapeutic purposes especially for anti-oxidant properties that are responsible to increase large scale production of carotenoids (Borowizka, 1988). As similar to phycocyanin results in this research, maximum total carotenoid content was also observed with Zarrouk's modified media, followed by Zarrouk's medium containing *S. platensis* culture in comparison to other media. The total carotenoid content of *S. platensis* was 5.99 µg/ml with Zarrouk's modified media, 5.51 µg/ml with Zarrouk's medium, 4.48 µg/ml with BG-11, while F-2 medium contains minimum amount of carotenoid (3.08 µg/ml) as shown in Fig 3. Devanathan and Ramanathan, (2013) reported the highest carotenoid results in Zarrouk's medium (6.65 µg/ml) followed by seawater enriched medium (5.93 µg/ml). Total carotenoid content showed high correlation coefficients of 0.92 and 0.86 to chlorophyll a and CPC content, respectively. The total carotenoid content also exhibited greater positive correlations with antioxidant activities (Park *et al*., 2018). Ghaeni *et al*. (2014) evaluated zeaxanthin, astaxanthin, lutein, beta carotene and lycopene from *S. platensis* by using High-Performance Liquid Chromatography (HPLC) with amount of 6652 µg/ml, 0.21 µg/ml, 424 µg/ml, 7393 µg/ml and 741 µg/ml respectively. At last, they suggested in their study that Spirulina is a good source of carotenoids as a pro-vitamin A in organisms.

Table 2: Cell count of *Spirulina platensis* on different cultivation media.

Media	Cell count (average no. of cell/ml)
Zarrouk's modified medium	400000
Zarrouk's medium	200000
BG-11 medium	98000
F-2 medium	92000

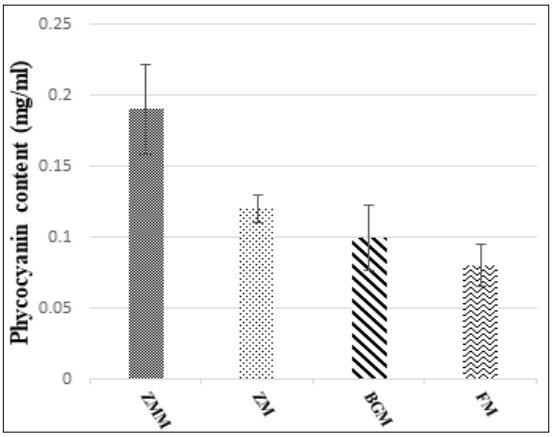

Fig 2: Phycocyanin content in different culture media of *S. platensis*.

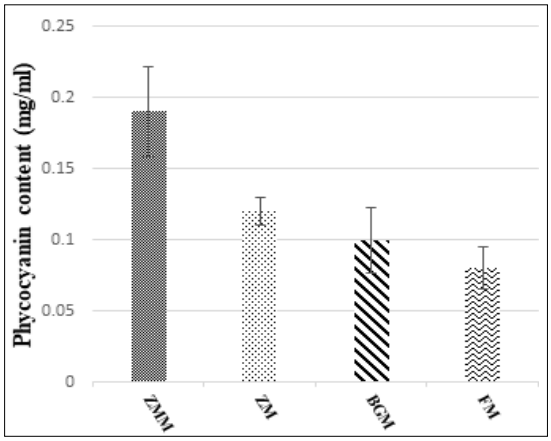

Fig 3: Carotenoid content in different culture media of *S. platensis*.

CONCLUSION

Spirulina is multicellular blue green algae, which have substantial popularity in various fields such as aquacultures, health sector and food industry. It is rich sources of micro and macronutrients, lipids, proteins, minerals, essential amino acids and anti-oxidant compounds. Spirulina have been proved as a safe food supplement as well as good source of nutraceutical and pharmaceuticals. In developing countries, it is highly used to cure malnutrition deficiency disorder. With traditional medium, there is always problem of contamination and yield of alga, therefore, it is important to develop a modified media to increase biomass production of Spirulina and reduce chances of contamination. The present study reveals that Zarrouk's modified medium has highest potential among other culture medium to grow *Spirulina platensis* at large scale. In spite of cultivation result, the significant concentration of phycocyanin and carotenoid in *Spirulina platensis* culture were also observed with Zarrouk's modified medium. Thus, the present study investigates that Zarrouk's modified medium could be considered for commercial cultivation of *Spirulina platensis* and its important protein pigments.

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Author contribution

Suman Parihar conceptualized the study; Neelam Solanki conducted all the experiments, Neelam Solanki, Preeti Soni and Gourav Chouhan prepared the manuscript, G.S. Shekhawat and G.P. Singh helped with manuscript corrections.

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Conflict of interest

Authors declare that there is no conflict of interest.

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