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Protein and amino acid profiling by the *Cyanobacterium Spirulina (Arthrospira) platensis* Strains in different Inorganic Formulations

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Abstract

Spirulina platensis is a microscopic, filamentous and alkalophilic fresh water microalga isolated from Jalmahal Lake, Jaipur (Rajasthan), India. The biochemical constituent of cyanobacteria depends on the nature of strains, nutrient composition, physiological state of the culture and the environment. To find out the best inorganic composition cultures were grown in five different defined inorganic medium and kept at the temperature of 25 ± 2°C, illuminated with white fluorescent lamps at a light intensity of 2,500 lux with photoperiod of 12 hours light/dark cycle. Maximum protein content was observed in Zarrouk's media. In SDS-PAGE profile total 12 polypeptides were observed in which three protein bands having molecular weight of 52.1 kDa, 105.2 kDa, and 110.0 kDa which has not been reported in cultures grown in Kratz and Myer medium. Total 17 amino acids were observed in chromatogram of HPLC and paper chromatography in cultures grown in Zarrouk's medium. Cysteine, histidine, methionine and serine were absent in CHU 10 medium and Kratz and Myer medium. Asparagine, glutamine and tryptophan were never observed in all inorganic formulations.

Keywords: Amino acid, HPLC, Protein, SDS-PAGE, *Spirulina*

1. Introduction

Cyanobacteria are photosynthetic microorganisms and one of the largest and most important groups of prokaryotes on Earth [1], which found in different terrestrial habitats with widely fluctuating environmental factors, such as nutrient availability, light intensity and quality, temperature, and water activity [2]. Cyanobacterium *Spirulina platensis* is still used in food supplements due to its excellent nutrient compounds and digestibility [3]. *Spirulina* contains unusually high amounts of protein, between 55 and 70 percent by dry weight, depending upon the source [4]. It is composed of complete protein, containing all essential amino acids, though with reduced amounts of methionine, cystine, and lysine, as compared to standard proteins such as that from meat, eggs, or milk. It is especially rich in all the essential amino acids, natural pigments, such as chlorophyll, β-carotene, and other natural phytochemicals. *Spirulina* is also rich in γ-linolenic acid (GLA), which has enormous pharmaceutical potential for reducing inflammation and alleviating the symptoms of premenstrual syndrome, heart diseases, Parkinson's disease and multiple sclerosis [5]. Among these, *Spirulina* is an economically well known filamentous cyanobacterium that is commercially produced as a source of human health food and cosmetic colorants [6].

The present investigation showed that different chemical composition in media induces different metabolic activities that are responsible for variation in protein expression. These various inorganic formulations either induced expression of some novel polypeptides or suppressed the expression of some existing polypeptides. In order to evaluate impact of various suggested media on the growth and pigments of *Spirulina platensis* five inorganic

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media differing in chemical composition and pH values were employed. This research paper reports the effect of different inorganic formulations on the protein and amino acid contents of *Spirulina platensis.*

2. Materials and Methods

Strain and growth medium:The experimental organism *Spirulina platensis* was isolated from Jalmahal Lake, Jaipur, Rajasthan. The cells were grown in Zarrouk's medium at 25 ± 2°C; pH was 10.0. In order to find out optimum culture medium, cultures were subjected to five different media of different chemical compositions and pH i.e. Zarrouk's medium, pH;10.2, CFTRI medium, pH 10.0, BG-11 medium, pH;7.1, CHU-10, pH;7.65, Kratz and Myer medium pH;7.31. Three sets of flasks for each medium were prepared. Each flask contained 70 ml of culture medium added with 30 ml healthy and profusely growing cultures and used for protein and amino acid estimation.

Protein profiling: The effect of various inorganic composition on polypeptide profile was determined by SDS-PAGE. Soluble proteins were extracted by homogenizing 0.5g of algal tissues with extraction buffer composing of 20 ml solution of 0.5 M Tris (pH 7.0) 2.5 ml., urea (4.0 g), SDS (0.5 g), glycerol (4.0 ml), B-mercaptoethanol (500 µl), dH_2O (10.0 ml); final pH 6.8) and centrifuged at 4°C for 20 min at 15,000 rpm. The supernatant was used as crude protein extract and protein quantity was measured following the method of [7]. The sample extracted from the algae (50 µl) was mixed with equal quantity of sample buffer [0.5 M Tris (pH 7.0) 2.5 ml, urea (9.6 g), SDS (1.0 g), glycerol (4.0 ml), B-mercaptoethanol (500 µl), dH_2O (5.0 ml).

The mixture were heat denatured in water bath at 100°C for 8 minutes and after that put on ice till loading. Protein extracts from all the treatments were resolved on 12 % SDS-polyacrylamide gel [8] and stained with 0.1 % Coomassie Brilliant Blue (R250) dye. In order to score and preserve the banding pattern, the gel was subjected to image scanning using BIO-RAD GS –700 imaging densitometer (USA) and the protein profiles were obtained for each variety. The presence of each band was scored as (+) plus and when absence as minus (-) (Table. 1).

Table 1. SDS-PAGE banding pattern showing presence and absence (+/-) of Spirulina platensis culture grown in different inorganic formulations.

M.W. (kDa)	Zarrouk's	CFTRI	BG-11	CHU-10	Kratz-Myer
14	+	+	+	+	+
18.4	+	+	+	+	+
28.4	+	+	+	+	+
32.2	+	+	+	+	+
45	+	+	+	+	+
52.1	+	+	+	-	-
55.4	+	+	+	+	+
60.3	+	+	+	+	+
88.1	+	+	+	+	+
105.2	+	+	+	+	-
110	+	+	+	+	-
123.5	+	+	+	+	+

Amino acids profiling: Amino acids extraction was carried out by taking 500 mg fresh material of each sample following the method suggested by Singh [9]. Quantitative estimation of amino acids was carried out following the method suggested by Lee and Takahashi [10]. The amino acids were estimated qualitatively by paper chromatography, employing the methods of Block *et al*. [11]. Amino acids were extracted according to the method proposed by Shad et al. [12], and separated by method recommended by Christian [13] using HPLC system (HP1050) with a UV detector at 254 nm. Identification of each amino acid peak was confirmed by comparison with the retention time of individual amino acids.

3. Observations and results

Protein profiling: In Zarrouk's media maximum growth of *Spirulina platensis was* observed. The quantity of the protein concentration determined through Bradford method was maximum in Zarrouk's medium i.e. 5.19 mg/ml and it is gradually decreased in CFTRI (4.88 mg/ml), BG-11 (4.71 mg/ml), CHU-10 (3.28 mg/ml) and in KM medium (3.12 mg/ml) (Graph: 1). Total 12 polypeptide species were observed in SDS-PAGE profiles in Zarrouk's medium, that were highest in number as well as in quantity as compare to other medium (Figure: 1, Table: 1). The gene expression of three polypeptides of 14.0 kDa, 18.4 kDa and 28.4 kDa were not affected by different inorganic compositions, so uniquely and highly expressed in all inorganic formulations. Two another polypeptide of 32.2 kDa and 45.0 were highly expressed upto 4 to 5mm only in Zarrouk's media (Graph: 2A).

The genotype of 18.4 kDa polypeptide was highly expressed upto 5mm level in the CFTRI medium as compare to other medium(Graph: 2B). The genotype of 52.1 kDa polypeptide was completely degraded in CHU-10 and KM inorganic composition. Total 9 polypeptides was observed in SDS-PAGE profile of KM medium (Table: 1). Unlike other medium two polypeptide of 105.2 kDa, and 110.0 kDa were uniquely and completely degraded in the nutrient limited KM medium (Figure: 1).

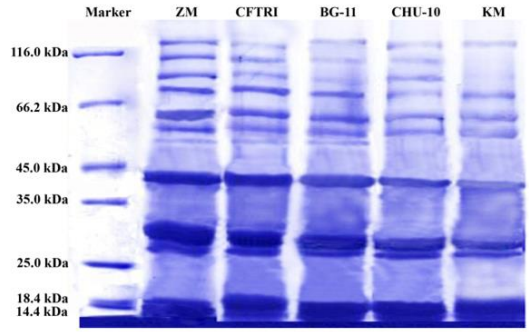
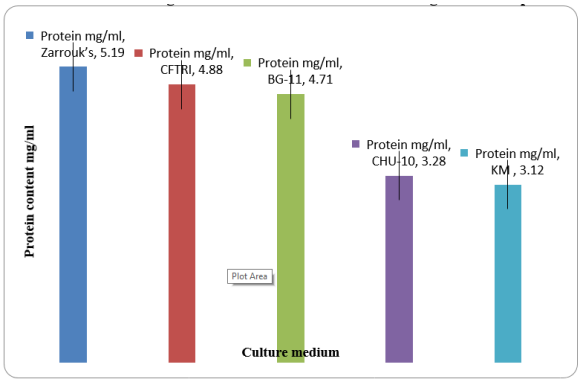


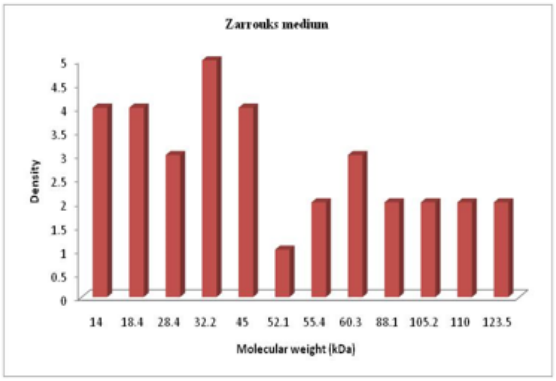
Figure 1. Coomassie blue-stained polypeptide profiles of extracted protein separated by SDS-PAGE. The proteins were extracted from *Spirulina plantensis* cells grown under different inorganic formulations i.e. Zarrouk`s medium (ZM), CFTRI medium, BG-11 medium, CHU-10 medium and Kratz and Myer mediu (KM)



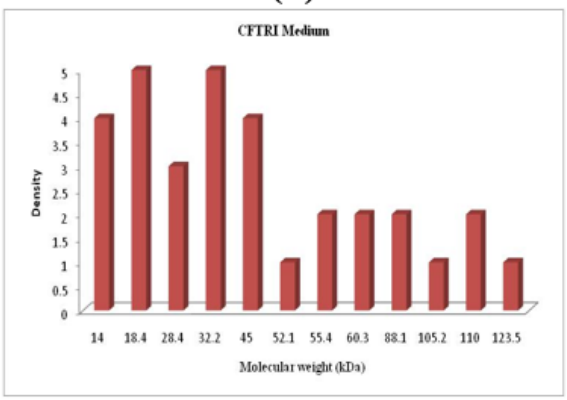
Graph 1. Total measured protein content of *Spirulina platensis* subjected to different inorganic conditions. Each bar is an average of three replicates.

The expression of some genotypes i.e. 55.4 kDa, 60.3 kDa, 88.1 kDa and 110.0 kDa was highly decreased or suppressed under the CHU-10 as well as KM medium (Graph: 2D, 2E). The genotype of 60.3 kDa polypeptide was induced and expressed upto 3mm level whereas two other polypeptides of 105.2 kDa and 123.5 kDa were expressed upto 2mm level in Zarrouk's medium that was highest as compare to all other inorganic compositions (Graph: 2A).

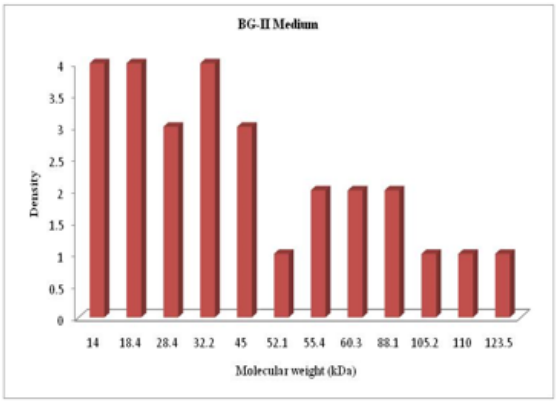
(A)



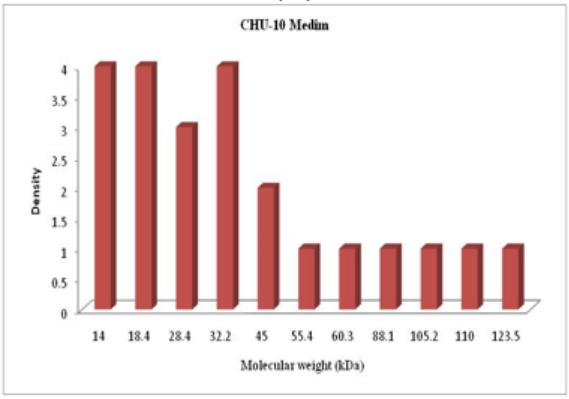
(B)

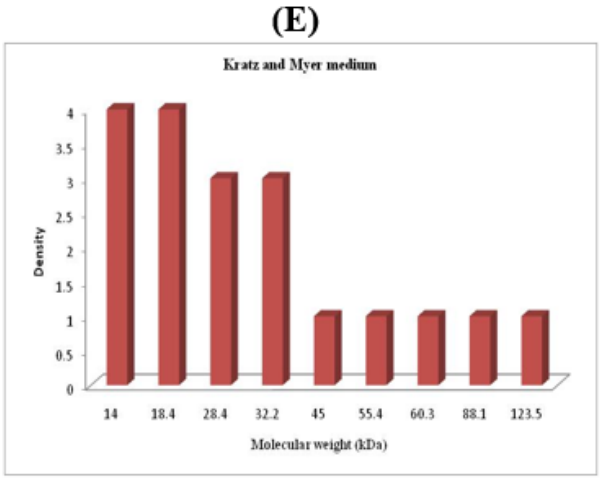


(C)



(D)





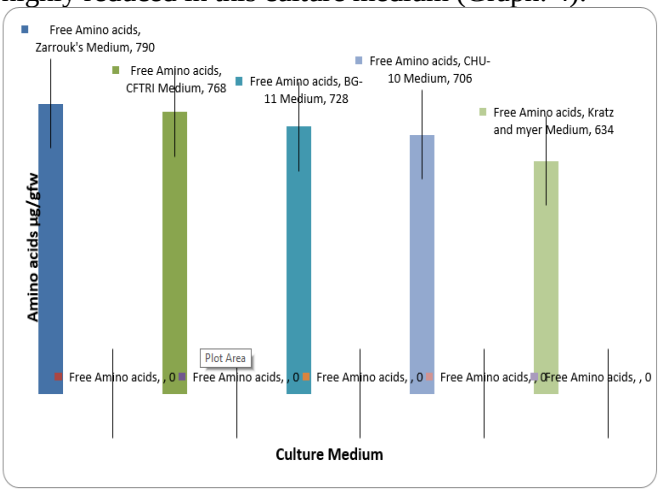
Graph 2. Densitometric analysis of SDS-PAGE banding pattern in Spirulina Platensis in different inorganic formulations.

Amino acid profiling: Quantity of free amino acids was maximum in Zarrouk's medium i.e. 790 µg/gfw and its gradually decreased in CFTRI (768 µg/gfw), BG-11 (728 µg/gfw), CHU-10 (706 µg/gfw) and in KM medium (634 µg/gfw) (Graph: 3). Total 17 amino acids were observed in HPLC chromatogram in cultures grown in Zarrouk's medium (Figure: 2). Among analyzed amino acids the most abundant amino acids were: aspartic acid, glutamic acid, glycine, alanine, proline, arginine, threonine, lysine, isoleucine, leucine, valine and tyrosine. Glutamic acid, aspartic acid and leucine peaks were significantly higher than other amino acids. Cysteine, methionine, serine and histidine were observed at low concentration than other amino acids. In the chromatogram of HPLC three amino acids i.e. asparagine, glutamine and tryptophan were never observed in Zarrouk's medium.

The paper chromatographic results showed that concentration of four amino acids i.e. glutamic acid, aspartic acid, leucine, alanine and valine were very high in cultures grown in Zarrouk's medium as well as CFTRI medium**.** Asparagine, glutamine and tryptophan were not observed in chromatogram of Zarrouk's medium as well as all the inorganic formulations (Table: 2). The concentration of cysteine, histidine, serine and methionine were lower as compared to other amino acids (Graph: 4).

The biosynthesis of glycine, alanine, leucine, phenylalanine, threonine, tyrosine and valine were slightly reduced in CFTRI inorganic formulation. The biosynthesis of some amino acids i.e. arginine, aspartic acid, glycine, leucine, isoleucine, proline and tyrosine were highly reduced and were observed in both free as well as protein bound form in the cultures grown in both CHU-10 and KM growth medium (Table: 2, Graph: 4).

Amino acids existing in free forms as well as in the fractions of bulk protein and peptides. Histidine was observed only in free form in both Zarrouk's and CFTRI medium but it was absent in all other mentioned inorganic formulations. Aspartic acid, glycine, arginine, threonine, leucine, methionine and cysteine were observed only in protein bound form while alanine, glutamic acid, isoleucine, phenylalanine, serine, tyrosine and valine were most prominent amino acids that present in both free as well as protein bound forms in Zarrouk's as well as CFTRI medium (Table: 2). Some protein bound amino acids i.e. glycine, leucine, methionine and threonine were also observed in free forms as well, due to fractionation of the protein or peptides in BG-11 medium, while many amino acids such as cysteine, histidine, methionine and serine were not observed under both CHU-10 and KM growth medium (Table: 2). The amino acids i.e. glutamic acid, aspartic acid, alanine, arginine, leucine, isoleucine, tyrosine, glycine, lysine and valine were highly reduced in this culture medium (Graph: 4).



Graph 3. Quantitative analysis of free amino acids of Spirulina platensis cultures grown in different inorganic formulations.

Table 2. Amino acid composition (free and bound) of *Spirulina platensis* grown under different inorganic formulations.

S. No.	Amino acids	Zarrouk's Medium		CFTRI Medium		BG-11 Medium		CHU-10 Medium		Kratz and Myer Medium	
		Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
1	L-Alanine	+	+	+	+	+	-	+	-	+	-
2	L-Arginine	-	+	-	+	-	+	+	+	+	+
3	L-Asparagine	-	-	-	-	-	-	-	-	-	-
4	L-Aspartic Acid	-	+	-	+	-	+	+	+	+	+
5	L-Cysteine*	-	+	-	+	+	-	-	-	-	-
6	L-Glutamine	-	-	-	-	-	-	-	-	-	-
7	Glycine	-	+	-	+	+	+	+	+	+	+
8	L-Glutamic Acid	+	+	+	+	+	+	+	-	+	-
9	L-Histidine	+	-	+	-	-	-	-	-	-	-
10	L-Isoleucine	+	+	+	+	+	+	+	+	+	+
11	L-Leucine	-	+	-	+	+	+	+	+	+	-
12	L-Lysine	+	+	+	+	+	+	+	-	+	-
13	L-Methionine	-	+	-	+	+	+	-	-	-	-
14	L-Phenylalanine	+	+	+	+	+	+	+	+	+	+
15	L- Proline	+	+	+	+	+	+	+	+	+	+
16	L-Serine	+	+	+	+	+	-	-	-	-	-
17	L-Threonine	-	+	-	+	+	+	+	+	+	+
18	L-Tryptophan	-	-	-	-	-	-	-	-	-	-
19	L-Tyrosine	+	+	+	+	+	+	+	+	+	+
20	L-Valine	+	+	+	+	+	+	+	-	+	-
	Total	17		17		16		13		13	

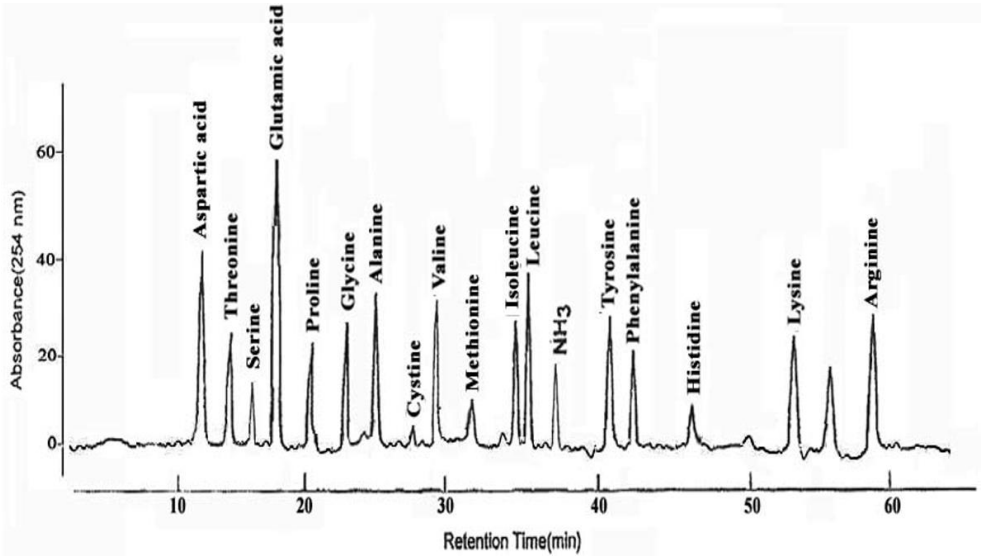
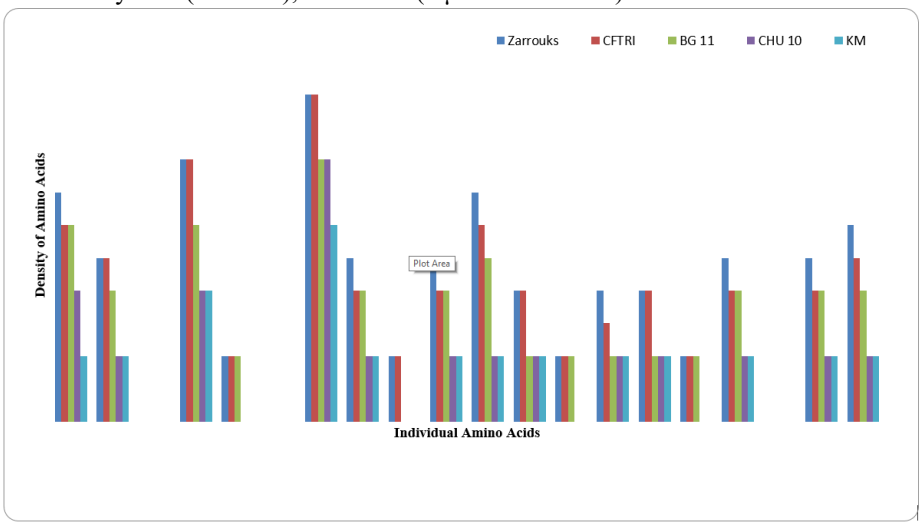


Figure 2. Chromatogram of amino acids extracted from *Spirulina platensis* culture, grown in Zarrouk's medium, resolved on a HPLC system (HP1050), ODS C18 (5 µm 4 × 250 mm) column with a UV detector at 254 nm.



Graph 4. Amino acid composition of Spirulina platensis cultures grown in different inorganic formulationsdetermined through paper chromatography. Asparagine (Asn), Glutamine (Gln) and Tryptophan (Trp) were not present.

4. Discussion

Considerable variation in the biochemical composition under various inorganic conditions can be observed in algae depending upon which nutrient is limited and to what degree. The availability of non-mineral nutrients, macronutrients, and micronutrients, greatly influence the biochemical composition of microalgae [14]. Generally, the growth rate and biochemical composition of algae is proportional to the uptake rate of the most limiting nutrient under optimal conditions of temperature and pH and is generally described by Michaelis-Menten equation [15].

Nitrogen and phosphate are two important macronutrients for growth and metabolism of algal cells. Studies have found that there is a co-limitation of primary production by both nitrogen and phosphorus [16]. Nitrogen is a fundamental element for the formation of amino acids, proteins and nucleic acids. Nitrogen is identified as a major limiting nutrient for biotic productivity [17, 18]. Yeesang and Cheirsilp [19] have reported loss of biomass when green alga, *Botrycoccus* spp. was exposed to nitrogen deficient conditions. Major effects of nitrogen deficiency in algal culture include the enhanced biosynthesis and accumulation of lipids [20] and triglycerides with a concomitant reduction in protein content [21]. This, in turn, results in a higher lipid/protein ratio [20] at the expense of growth rate [22]. Degradation of phycolbillisomes with nitrogen limitation has been demonstrated in the case of cyanobacteria and red alga [23].

Being an integral part of essential molecules such as ATP phosphate is another very important nutrient. Wanger *et.al* [24] investigated that under phosphate-limiting growth conditions some membrane proteins were induced in cyanobacterium *Anacystis nidulans*. Similar to the effects of nitrogen deficiency, phosphorus starvation reduces chlorophyll *a* and protein content thereby increasing the relative carbohydrate content in algal cells [25]. Carbon can be utilized in the form of CO_2, carbonate, or bicarbonate for autotrophic growth and in form of acetate or glucose for heterotrophic growth. The study with the cyanobacterium *Spirulina platensis* reported that elevated CO_2 concentrations decrease relative concentrations of proteins and pigments in the cells but increase carbohydrate content [26].

Mg is a constituent of chlorophyll and also a part of photosynthetic Mg-dependent enzymes [27]. Mg also can regulate the transport trough the Ca-channels of the cell membrane by improving their permeability [28]. Sulphur is essential for the synthesis of amino acids i.e. cysteine and methionine, secondary metabolic production and the synthesis of co-enzymes such as glutathione, thioredoxin and co-enzyme A. Several studies demonstrated that thylakoid membrane proteins were affected by salt stress. In *Spirulina platensis*, salt stress inactivated both PSII and PSI due to the changes in K/Na ratio [29] or decrease in the PS II electron transport by increasing the number of the QB-nonreducing reaction centers [30]. Moradi and Ismail [31] reported that chlorophyll contents reduced at higher salinities are due to decrease in photosynthetic rate because of salt osmotic and toxic ionic stress. Many previous studies reported that the cultivation under higher saline concentrations had lowered chlorophyll and protein contents [32].

Trace elements are present in algal cells in extremely small quantities but that are an essential component of phycophysiology. Iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), copper (Cu) and nickel (Ni) are the six most important trace metals required by algae for various metabolic functions [33]. Iron limitation also reduces cellular chlorophyll concentration [34]. Decrease in iron content reduces carotenoid composition [35] and results in partial loss of pigment [36].

Similarly, other studies showed that different environmental conditions are responsible for induction or inhibition of certain peptides expression and also increase or decrease level of the protein expression [37]. Appearance of this new protein band in the SDS-PAGE profile have been assigned to originate either from the degradation of larger molecular weight proteins or from de novo synthesis induced by nutrient starvation [38]. Aldehni *et al.*, [39] reported that overall reduction in the rate of protein synthesis in nutrient starvation. Subhashini *et al.* [40] observed significant variations in protein content among the four isolates of *Anabaena azollae*. Weber and Jung [41] demonstrated that changes in protein profiling and newly formed proteins might be helping cyanobacteria to tolerate adverse stress conditions.

Various environmental factors (inorganic composition, pH, photoperiods, light intensity and temperatures) that might affect the amino acid profile. The processes of formation of free amino acids and peptides are on different culture conditions varying in different inorganic composition, photoperiods and temperatures. A number of other groups have shown a seasonal variation of protein patterns present in algal tissue [42]. The nutritional quality of food can be determined by the content, proportion and availability of its amino acids, particularly for evaluation of a new protein resource [43].

The amino acids contents observed in free forms as well as in the fractions of bulk protein and peptides. Aspartic acid, glutamic acid, glycine, alanine, proline, arginine, threonine, lysine, isoleucine, leucine, glutamine, valine and tyrosine are most abundant in cyanobacterium *Spirulina platensis*. Cysteine, methionine and histidine were found in low concentrations in all experimental blue-green-algae. Glutamine, asparagine and tryptophan were not observed in the chromatogram of HPLC as well as paper chromatography. Similar work on amino acid profiling in *Spirulina platensis* was carried out by Aly and Amber [44]. Holm-Hansen *et al.* [45] reported an increase in amino acid content of *Chlorella pyrenoidosa* at the expense of sugar phosphates with addition of ammonium (nitrogen source) to the growing culture. In addition to their structural role in proteins, amino acids are the starting point for the synthesis of pyrimidines (aspartate), purines (glutamine and glycine), growth regulators (tryptophan, methionine), and many secondary metabolites. Free amino acids are the currency in nitrogen metabolism, with which nitrogen is transferred between cells and organs [46]. The proteinogenic amino acid proline functions as an osmolyte, radical scavenger, electron sink, stabilizer of macromolecules, and a cell wall component [47]. Increased levels of proline correlate with enhanced salinity tolerance [48]. Impact of mineral composition of the medium on the growth of cyanobacteria has been studied by the number of workers i.e. *Ankistrodesmus fusiformis* [49], *Spirulina labyrinthiformis* [50], *Chlorella* [51], *Dunaliella salina* [52].

In conclusion, in the present work, the optimal inorganic medium and pH for protein and amino acids biosynthesis were demonstrated for *Spirulina platensis.* Various inorganic formulations affect synthesis of metabolic end products synthesised by cyanobacteria are essential since they contribute to understanding the control of metabolic activities and optimising yields of metabolic end products of interest.

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Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human / or animal subjects (if exist) respect the specific regulation and standards.

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