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# Influence of Anaerobically Digested Dairy Waste on Growth and Bio-Active Compounds of *Spirulina subsalsa* (Cyanobacteria) under Semi-Continuous Culture Conditions

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**The present communication deals with the standardization of suitable medium formulation along with anaerobically digested cow's urine (ADCU) for growth of *Spirulina subsalsa*. Growth was evaluated on the basis of photosynthetic and non-photosynthetic pigment. The results obtained from the study indicated that, SSM-1 and SSM-2 media are suitable for maximum synthesis of chlorophyll-*a* and carotenoids. The obtained results also indicated that SSM-5 medium is suitable for maximum synthesis of accessory light harvesting pigments phycobiliprotein, total carbohydrate, total protein and total lipid in *S. subsalsa*. From the study it could be concluded that all the five media combinations (*viz.* SSM-1, SSM-2, SSM-3, SSM-4 and SSM-5) would be suitable for mass cultivation of *S. subsalsa*. But among them, SSM-5 medium combination could be the most suitable medium.**

**Keywords:** **Cow urine, chlorophyll-*a*, carotenoids, phycobiliprotein, carbohydrate, protein, lipid**

## Introduction

Since the past few decades, Cyanobacteria (Blue-green Algae/Cyanoprokaryotes) are increasingly recognized as a prolific source of natural products and are well known for the production of a wide variety of bioactive compounds. Natural products from cyanobacteria were originally discovered through bioactivity-guided screening programs and revealed a truly fascinating variety of structures and inhibitory activities [1]. Recent reports on cyanobacteria also revealed that they have more than 300 nitrogen-containing secondary metabolites, represented by diverse structural types [2]. A majority of these secondary metabolites are biologically active and are products of either the non-ribosomal polypeptide (NRP) or the mixed polypeptide-NRP biosynthetic pathways [3]. The production of these low cost biologically active compounds could be used in food and pharmaceutical industries. Some marine forms of cyanobacteria are a source of potent neuro-toxins acting as an either blocker or activators [4].

The genus *Spirulina* Turpin ex Gomont of the Oscillatoriaceae family contains the group of filamentous cyanobacteria characterized by spiral-shaped chains of cells (trichomes) enclosed in a thin sheath. Now a days, *Spirulina* is marketed and consumed in many different

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countries such as Germany, Brazil, Chile, Spain, France, Canada, Belgium, Egypt, United States, Ireland, Argentina, Philippines, India, Africa and others, where public administration, sanitary organisms and associations have approved for human consumption [5]. Some of the best worldwide known *Spirulina* producing companies are: Earthrise Farms (USA), Cyanotech (USA), Hainan DIC Microalgae Co. Ltd (China), Marugappa Chettir Research Center (India), Genix (Cuba) and Solarium Biotechnology (Chile) [6].

The present work is focused on formulation and screening of low cost synthetic medium combinations along with anaerobically digested cow's urine (ADCU) for the growth in biomass and synthesis of primary photosynthetic pigment like chlorophyll, accessory light harvesting pigment like phycobiliprotein, photoprotective pigment like carotenoids in *S. subsalsa* Oersted ex Gomont. The work will also focus on the evaluation of natural compounds like total carbohydrate content, total protein content and total lipid content from *S. subsalsa* under the influence of different medium combinations.

## Materials and Methods

### Culture material

The experimental organism employed for the present study was isolated from logging waste water near Sobhapur, Meerut (U.P.), Bharat by repeated culturing and sub-culturing and deposited as *Spirulina subsalsa* in the Algal Germplasm Collection Centre, Department of Botany, Chaudhary Charan Singh University, Meerut, U.P., Bharat.

### Culturing and exponential growth of *S. subsalsa*

Experimental conditions were 28 ± 2℃ at light intensity of 138 µmol photons/$m^2$/$s^1$ for 14:10 hours light: dark regime. Under this condition *S. subsalsa* was cultured for ten days in SSM-1 medium [7] specialized for *Spirulina* cultivation (Table 1, 2) at pH 10.0 for the development and exponential growth of the culture organism. Exponentially growing 1ml of *S. subsalsa* was inoculated in 150 ml of culture flask containing 100 ml of modified medium specialized for *Spirulina* cultivation. All the culture flasks were shaken continuously on magnetic stirrer (LABQUEST-MHPS15P) for homogenous growth of the organism.

**Table 1. Chemical constituents of modified media specialized for *Spirulina* cultivation (SSM-1).**

| Chemicals | Amount (g/l) |
|---|---|
| $CaCl_2 \cdot 2H_2O$ | 0.036 |
| Citric Acid | 0.006 |
| $NaNO_3$ | 1.5 |
| $FeSO_4 \cdot 7H_2O$ | 0.006 |
| EDTA (Na) | 0.001 |
| $MgSO_4 \cdot 7H_2O$ | 0.075 |
| $K_2HPO_4$ | 0.04 |
| $Na_2CO_3$ | 0.02 |
| $NaHCO_3$ | 16 |
| Solution A5 (Micronutrients) | 1.0 ml/l |

**Table 2. Chemical constituents of Solution A5 (Micronutrients).**

| Chemicals | Amount (g/l) |
|---|---|
| $H_3BO_3$ | 2.86 |
| $MnCl_2 \cdot 4\ H_2O$ | 1.81 |
| $ZnSO_4 \cdot 7H_2O$ | 0.222 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.39 |
| $CuSO_4 \cdot 5H_2O$ | 0.079 |

**Table 3. Different combinations of synthetic medium for semi-continuous culturing of *Spirulina subsalsa*.**

| Variants | Nutrient constituents of the Medium |
|---|---|
| SSM-1 | Modified nutrient medium specialized for *Spirulina* cultivation (Table 1, 2) |
| SSM-2 | 6.25% ADCU + 93.75% SSM-1 |
| SSM-3 | 12.50% ADCU + 87.5% SSM-1 |
| SSM-4 | 25% ADCU + 75% SSM-1 |
| SSM-5 | 50% ADCU + 50% SSM-1 |

### Formulation of the synthetic nutrient media

For the experiment fresh Cow's urine was filtered and anaerobically digested for 21 days and four different synthetic media combinations SSM-2, SSM-3, SSM-4 and SSM-5 were formulated using 6.25%, 12.50%, 25% and 50% (ADCU) with 93.75%, 87.50%, 75% and 50% SSM-1 synthetic media respectively (Table 3). The culture flasks containing SSM-1 medium specialized for *Spirulina* cultivation is used as control while SSM-2, SSM-3, SSM-4 and SSM-5 are the variants.

### Experimental design and semi-continuous culturing of *S. subsalsa* in different nutrient containing medium

The experiment was conducted in a completely ran-

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domized design in triplicates in 150 ml conical flasks (Borosil) containing 100 ml of SSM-1, SSM-2, SSM-3, SSM-4 and SSM-5 nutrient media. Exponentially growing *S. subsalsa* was semi-continuously cultured at 28 ± 2 ℃ temperature under 138 µmol photons/$m^2$/s light for 14:10 hours light:dark regime for 30 days under the influence of five different medium combinations. Uniformly growing *S. subsalsa* were harvested every $10^{th}$ day replacing with 50% fresh SSM-1, SSM-2, SSM-3, SSM-4 and SSM-5 media for the observation of growth and synthesis of photosynthetic pigments like chlorophyll-*a*, synthesis of photo protective pigments like carotenoids, synthesis of secondary light harvesting pigments like phycobilins under semi-continuous culture conditions. Study was also conducted for the observation of value added compounds like total carbohydrates, total proteins and total lipid content after semi-continuous culturing under the influence of five different combinations.

#### Estimation of *Chlorophyll-a (Chl-a)*

Quantitative estimation of chlorophyll pigment is essential for assessment of growth and photosynthetic rates in cyanobacteria. *Chl-a* pigment is extractable completely in solvents like acetone/methanol and exhibits characteristics absorption at 663 nm and 645 nm. Extraction of *Chl-a* was done by the method followed by Sofia and Teresa [8] and the OD of the supernatant was measured at 663 nm in microprocessor UV-VIS spectrophotometer (Systronics 118) using methanol/acetone as blank. The whole experiment was done under subdued light condition to avoid photoreaction and loss of pigments.

#### Carotenoid estimation

Carotenoids include pigments like β-carotenes and xanthophylls soluble in polar solvents like acetone, exhibiting a characteristic absorption at 453 nm. The extraction of carotenoid pigment was done by the method followed by Jensen [9] in subdued light condition to avoid photoreaction and loss of pigments using microprocessor UV-VIS spectrophotometer against acetone blank.

#### Estimation of total protein

Estimation of total proteins in the cells was done by the method followed by Lowry *et al*. [10]. Total protein content in the *S. subsalsa* cells was calculated by reading the absorbance at 750 nm in a microprocessor based UV-VIS spectrophotometer (Systronics 118) against blank and a standard graph was prepared against BSA (Bovine Serum Albumin).

#### Phycobilins or phycobiliproteins estimation

Phycobilins namely phycocyanin (PC), phycoerythrin (PE), and allophycocyanin (APC) are water soluble pigments and extracted in phosphate buffer. Phycobilins or phycobiliproteins can be extracted by Bennett and Bogorad [11] method. The OD was measured at 562 nm, 615 nm and 652 nm in microprocessor UV-VIS spectrophotometer (Systronics 118) against phosphate buffer blank for C-phycocyanin, C-allophycocyanin and C-Phycoerythrin.

#### Estimation of total carbohydrate

Carbohydrate is an important component of storage and structural material in cyanobacteria. The carbohydrate exists as free sugars and polysaccharides. Total carbohydrate content in the *S. subsalsa* cells can be measured by hydrolyzing the polysaccharides into monosaccharide. Carbohydrates are first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms a green colored product with anthrone with an absorption maximum at 630 nm. The carbohydrate content in the cyanobacterial cells was determined by the method of Hedge and Hofreiter [12]. Total carbohydrate content was calculated by the absorbance at 630 nm in microprocessor UV-VIS spectrophotometer (Systronics 118).

#### Estimation of total lipid

Lipids are a diverse group of biological substances made up of primarily non-polar compounds (triglycerides, diglycerides, monoglycerides and sterols) and more polar compounds (free fatty acids, phospholipids and sphingolipids). They bind covalently to carbohydrates and proteins to form glycolipids and lipoproteins, respectively. Extraction of cyanobacterial lipid is an important key for biodiesel production based on microalgae. The Bligh and Dyer method [13] has been successfully applied for the determination of lipids from microalgae. This method is

one of the widely practised methods for lipid extraction.

### Statistical analysis of the data

The data obtained were subjected to statistical analysis of variance (ANOVA) by using completely randomized design using the method followed by Armstrong and Hilton [14]. The statistical analysis was carried out in Microsoft Office Excel 2007. Each mean was calculated from six different values. Standard deviation and standard error were calculated against the values obtained.

## Results

Synthesis of *Chl-a* by *S. subsalsa* under the influence of five different synthetic media (SSM-1, SSM-2, SSM-3, SSM-4 and SSM-5) was determined every 10$^{th}$ day over a period of 30 days. A slow but gradual decline in the synthesis of *Chl-a* was observed in all the culture flasks supplemented semi-continuously with all the synthetic media combinations after 1$^{st}$ harvesting except SSM-1. *S. subsalsa* growing under the influence of SSM-1 synthetic medium showed gradual increase in *Chl-a* synthesis till 20$^{th}$ day while decline in the content of *Chl-a* was observed on 30$^{th}$ day. All other flasks supplemented semi-continuously with SSM-2, SSM-3, SSM-4 and SSM-5 showed gradual increase in *Chl-a* pigment till 10 days after that a gradual decline was observed. Maximum *Chl-a* content was observed 7.16 µg/ml under the influence of SSM-2 medium after 10 days of semi-continuous culturing while minimum *Chl-a* content was observed 0.09 µg/ml under the influence of SSM-5 synthetic medium over a period of 30 days. A detailed result on the synthesis of *Chl-a* by *S. subsalsa* under the influence of five different media combinations is given in Fig. 1(A).

A gradual increase in the synthesis of photoprotective pigment carotenoid after semi-continuous supplement with five different media in the *S. subsalsa* cells was observed till 10 days in all the culture flasks, but after that a gradual decline was observed in all the flasks except SSM-1. Culture flasks supplemented semi-continuously with SSM-1 medium showed gradual increase in total carotenoid pigment content till 20 days while after that a decline phase in the content of total carotenoid pigment was observed. Maximum total carotenoid pigment content was observed 0.0385 µg/ml under the influence of SSM-1 synthetic medium while minimum total carotenoid content was observed 0.003 µg/ml in the culture flasks when cultured under the influence of SSM-5 synthetic medium over a period of 30 days. A detailed result on the synthesis of total carotenoid pigment by *S. subsalsa* under the influence of five different synthetic media is given in Fig. 1(B).

Culture flasks semi-continuously supplemented with SSM-1 medium showed gradual increase in c-phycocyanin pigment till 20$^{th}$ day while after that a decline phase was observed. While a gradual enhance in the c-phycocyanin content in other culture flasks semi-continuously supplemented with SSM-2, SSM-3, SSM-4 and SSM-5 synthetic media combinations was observed after 30 days. Maximum c-phycocyanin content was observed 0.017 µg/ml in the culture flasks supplemented with SSM-1 medium after 20 days while minimum c-phycocyanin content was observed 0.006 µg/ml when semi-continuously cultured under the influence of SSM-2 medium over a period of 30 days. A detailed result on the synthesis of c-phycocyanin pigment under the influence of SSM-1, SSM-2, SSM-3, SSM-4 and SSM-5 media is given in Fig. 1(C).

A drastic increase in the concentration of C-allophycocyanin in all the culture flasks was observed after 10 days while after that concentration of c-allophycocyanin declined in all the culture flasks supplemented with five different media combinations. Culture flasks semi-continuously supplemented with SSM-5 medium showed 0.097 µg/ml concentration of c-allophycocyanin after 10 days while minimum c-allophycocyanin content was observed 0.002 µg/ml when *S. subsalsa* was semi-continuously cultured under the influence of SSM-2 synthetic medium for 30 days. A detailed result on the synthesis of c-allophycocyanin pigment by *S. subsalsa* under the influence of all the five different media combinations is given in Fig. 1(D).

The concentration of red light absorbing pigment c-phycoerythrin increased in concentration in all the culture flasks supplemented with SSM-1, SSM-2, SSM-3, SSM-4 and SSM-5 media combinations. Maximum c-phycoerythrin content was observed 0.112 µg/ml in the culture flask semi-continuously cultured under the influence of SSM-5 medium after 30 days. Minimum c-phycoerythrin content was observed 0.053 µg/ml in the culture flask supplemented semi-continuously with SSM-1 syn-

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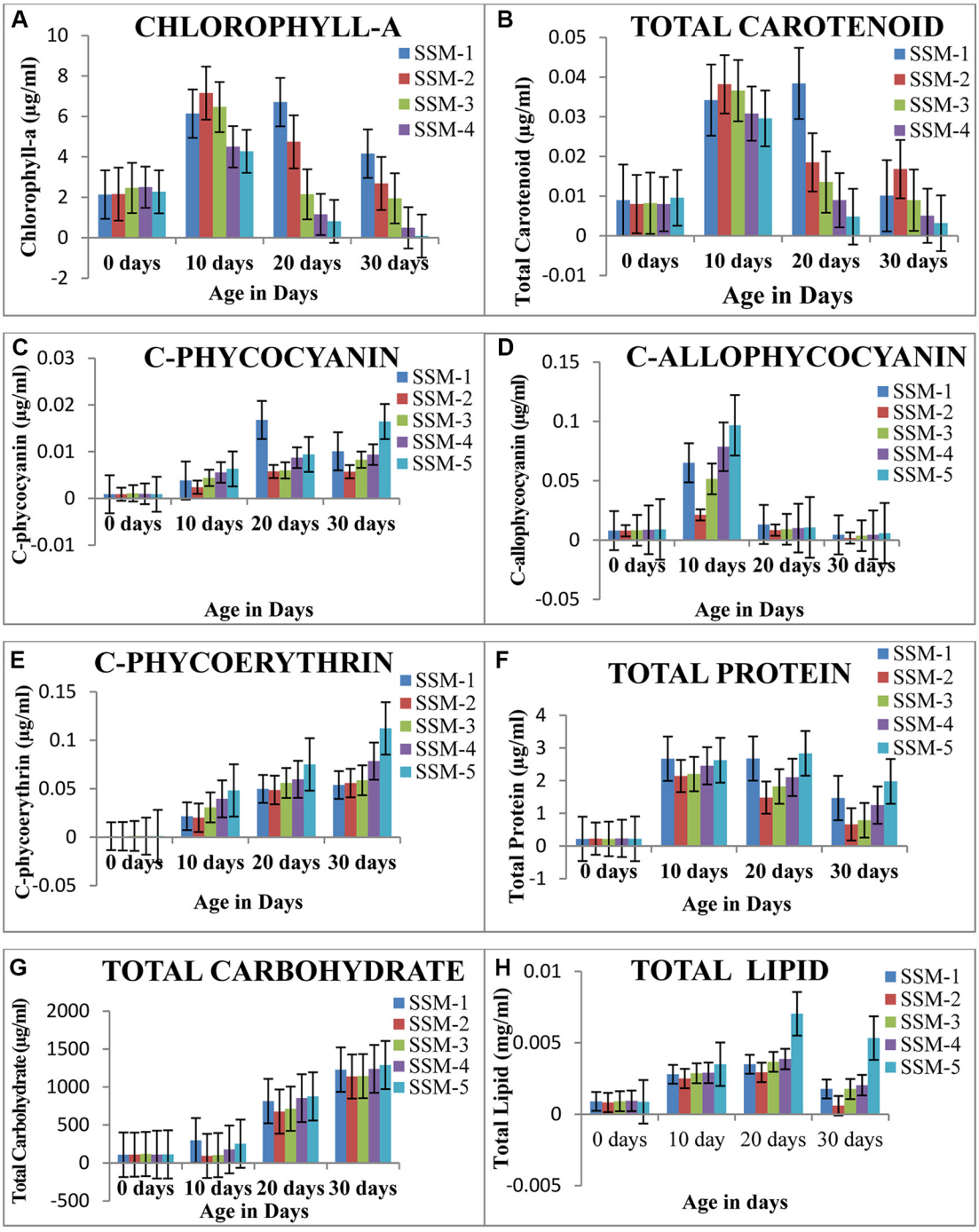


**Fig. 1. (A-H): Showing the effect of different synthetic nutrient medium in synthesis of (A) Chlorophyll-*a*, (B) Total carotenoids, (C) Total Carbohydrates, (D) C-Phycocyanin, (E) C-allophycocyanin, (F) C-phycoerythrin, (G) Total Protein and (H) Total Lipid in *S. subsalsa* grown by semi-continuous culturing for 30 days.**

thetic medium for 30 days. A detailed result on the synthesis of C-phycoerythrin pigment by *S. subsalsa* under the influence of five different medium combinations is given in Fig. 1(E).

Among the various blue green alga, *Spirulina* has drawn more attention because it shows an high nutri-

tional content characterized by a 70% protein content and by the presence of minerals, vitamins, amino acids, essential fatty acids etc. A gradual increasing in total protein content was observed till 2nd harvesting and then declining trend was observed in the culture flasks supplemented semi-continuously with SSM-1 and SSM-5 synthetic media while all other flasks supplemented with SSM-2, SSM-3 and SSM-4 synthetic media showed gradual decline in total protein content. Maximum total protein content was observed 2.83 µg/ml in the culture flask supplemented with SSM-5 medium after 20 days of semi-continuous culturing while minimum total protein content was observed 0.663 µg/ml in the culture flasks supplemented with SSM-2 medium after 3rd harvesting. A detailed result on the synthesis of total protein by *S. subsalsa* under the influence of five different synthetic media is illustrated in Fig. 1(F).

Micro-algae *S. subsalsa* synthesize and accumulate carbohydrates in their cells when they are exposed at various stressed situations. Carbohydrates accumulation in the cells is mainly due to the phosphorus starvation. A gradual increasing trend in the concentration of total carbohydrate content was observed in all the culture flasks supplemented with different media. Maximum carbohydrate content was observed 1290 µg/ml in the culture flasks semi-continuously supplemented with SSM-5 synthetic medium while minimum carbohydrate content was observed 1229 µg/ml in the culture flasks semi-continuously supplemented with SSM-1 medium after 30 days. A detailed result on the synthesis of total carbohydrate content by *S. subsalsa* under the influence of five different media is given in Fig. 1(G).

Cyanobacteria contain significant quantities of lipids and some of them are also rich in essential fatty acids such as linoleic and gamma linolenic acids. The lipid content in the cyanobacterial cells was determined under the influence of five different media over a period of 30 days harvesting semi-continuously every 10th day. A gradual increasing and then declining trend was observed in all the culture flasks supplemented semi-continuously with five different synthetic media. Maximum total lipid content was observed 0.007 mg/ml when *S. subsalsa* was semi-continuously cultured under the influence of SSM-5 synthetic medium for 20 days while minimum concentration of lipid was observed 0.0006 mg/ml in the culture flasks semi-continuously supplemented with SSM-2 synthetic medium for 30 days. A detailed result on synthesis of total lipid by *S. subsalsa* under the influence of five different medium is given in Fig. 1(H).

## Discussion

The blue green alga *Spirulina* has a long history of use as a safe functional food. It has been studied since many decades due to its health beneficial chemical constituents mainly includes high value protein, carbohydrates, essential amino acids, essential fatty acids, minerals constituents, vitamins, pigments [15–17] and major bioactive components like phycocyanin, allophycocyanin, sulfated polysaccharides, γ-linoleic acid (GLA), PUFAs etc [18, 19]. Phycocyanin is a biologically active secondary light harvesting pigment-protein complex of the family phycobiliprotein. It is a blue-light pigment having antioxidant, anti-inflamatory, and hepatoprotective properties and normally used in biotechnology, cosmetic, drug and food industry. Furthermore evidences on antiviral, antibacterial, anti-cancerous, anti-ageing, anti-inflammatory, immune-modulatory activities of *Spirulina* make it a potential microorganism responsible for improving human body functions [20, 21]. In view to this *Spirulina* has been commercially produced in large scale to cope up with this food supplement [22]. But large-scale production of *Spirulina* is costly due to its requirement of high value of chemical constituents and for this a large number of scientists formulated new and cheaper media formulations that could be beneficial for the growth and synthesis of pigments.

A comparative study of *S. platensis* ANS -1 was done with reference strain CAS-10 by Usharani *et al.* [23] in substrates diluted Zarrouk's medium [24] (control) and on rice mill waste water effluent (pH was adjusted to 9–11 by using sodium bicarbonate @ 800 mg/l and used as a medium. The well performed strain CAS 10 under *in vitro* condition was selected as efficient one. The growth of *S. platensis* was measured both in laboratory and outdoor condition by using the parameters *viz.*, optical density, population, dry weight, protein and chlorophyll content. The high growth and dry weight were recorded in 1/6 diluted Zarrouk's medium when compared to rice mill effluent medium. Maximum protein and chlorophyll content were noticed in 1/6 diluted Zarrouk's medium

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than rice mill effluent.

*S. platensis* cultivated in laboratory by Volkmann *et al.* [25] cultivated under controlled conditions (30℃, photoperiod of 12 hours light/dark provided by fluorescent lamps at a light intensity of 140 μmol photons/$m^2/s^1$ and constant bubbling air) in three different culture media and concluded that Paoletti medium prepared with desalinator wastewater would be most suitable for *S. platensis* cultivation. All essential amino acids, except lysine and tryptophan, were found in concentrations higher than those required by FAO.

A new medium RM6 for mass production of *Spirulina* sp. was prepared by Raoof *et al.* [26] formulated by incorporating selected nutrients of the standard Zarrouk's medium contains single super phosphate (1.25 g/litre), sodium nitrate (2.50 g/litre), muriate of potash (0.98 g/litre), sodium chloride (0.50 g/litre), magnesium sulfate (0.15 g/litre), calcium chloride (0.04 g/litre), and sodium bicarbonate (commercial grade) 8 g/litre and observed a maximum growth rate in terms of dry biomass, chlorophyll and proteins between 6 and 9 days of growth. From the observation they concluded that the revised medium to be highly economical, since it is five times cheaper than Zarrouk's medium. An attempt to culture *S. platensis* in human urine directly to achieve biomass production and $O_2$ evolution, for potential application to nutrient regeneration and air revitalization in life support system was done by Feng *et al.* [27]. The culture results have showed that *S. platensis* in diluted human urine was lighter than that in Zarrouk's medium.

An investigation on growth pattern of *S. platensis* in standard and modified media based on seawater-chemicals and seawater fertilizers was done by Bohra [28] and he observed that *S. platensis* have different specific growth characteristics in different media at same environmental parameters (temperature, pH and light intensity). Even though, *S. platensis* in standard media exhibited good growth patterns, biomass, protein content and chlorophyll content than other seawater-based media, the experiment discusses the feasibility of seawater based media.

Lignite fly ash (LFA) is the by-product of thermal power station. The Blue Green Alga *S. platensis* grows well at alkaline pH and the pH of the LFA is also alkaline. The LFA also contains an array of micronutrients and macronutrients which favours the growth of *Spirulina platensis*. In order to minimize the environmental pollution and recycle the LFA waste, Saranraj *et al.* [28] planned to utilize the LFA at different concentrations for the laboratory cultivation of *S. platensis*. *S. platensis* was cultivated in the conical flasks containing Zarrouk's medium alone [SP] and Zarrouk's medium with three different concentrations (0.5 g/l [SP - 1], 1.0 g/l [SP - 2] and 1.5 g/l [SP - 3]) of LFA supplementation. Among the four different supplementations used, SP-3 which contained 1.5 g LFA in one litre of Zarrouk's medium highly induced the growth and protein content of *S. platensis* when compared to other supplementations. The least growth and protein content was recorded in SP which contains Zarrouk's medium alone without LFA supplementation.

The influence of an agricultural liquid organic fertilizer on growth and biomass composition of *S. platensis* was studied by Ak [30]. The results obtained from his work showed that, liquid organic fertilizers + nitrate + phosphate + bicarbonate combination was at par with Zarrouk's medium depending on dry biomass or proteins content. From this he concluded that, the medium developed from liquid organic fertilizer along with nitrate, phosphate and bicarbonate combination is not only 5 times cheaper than the Zarrouk medium but also having a high productive alternative. Hence, the alternative organic nutrient is a cheaper source and could be economically beneficial for large scale cultivation of *Spirulina*.

Sarma *et al.* [7] studied the effect of cow's urine on the growth and synthesis of natural compounds on *Spirulina fusiformis* and revealed that addition of animal waste upto 12.5% boost the growth and synthesis of Chl-*a*, carotenoids, phycobilins, total carbohydrates and proteins.

## Conclusion

On the basis of the present investigation, it is concluded that SSM-1 medium combination along with ADCU could enhance the growth of *S. subsalsa* and enhance the synthesis of photosynthetic pigments, proteins, carbohydrates and lipids. From the results obtained in the present investigation it could be concluded that SSM-5 medium could be suitable for enhanced production of c-allophycocyanin, c-phycoerythrin, total protein,

carbohydrate and total lipid where as SSM-2 synthetic medium could maximize the production of Chl-*a* while SSM-1 medium could enhance the production of carotenoid pigment and c-phycocyanin in *S. subsalsa*.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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