E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2017; 5(3): 403-408

www.fisheriesjournal.com
Received: 21-03-2017
Accepted: 22-04-2017

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Replacement of sodium nitrate in Kosaric medium with urea for culture of *Spirulina platensis*

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Abstract
Spirulina platensis is an cyanobacteria, which is an important feed additives for human, animal and fishes. The major purpose of the present study was to optimize the production of *S. platensis* with lowering the production cost by using urea as a source of nitrogen. An experiment was conducted for 18 days to evaluate the growth performance of *S. platensis* with the aim of replacing sodium nitrate in kosaric medium with urea. Kosaric medium was taken as a standard culture medium in treatment T_1. In treatments T_2, T_3, T_4 and T_5 sodium nitrate-nitrogen in kosaric medium was replaced with urea-nitrogen at 25%, 50%, 75% and 100%, respectively. The mean values of temperature and pH in different treatments ranged from 29.10±0.10 ºC to 31.90±0.12 ºC and 9.10±0.40 to 9.35±0.32, respectively. The values of light intensity ranged from 2220 lux/m^2/s to 2295 lux/m^2/s. All the parameters were within a suitable range of *S. platensis* culture. Maximum optical density, cell dry weight and chlorophyll *a* was observed in treatment 3, where 50% sodium nitrate-nitrogen in kosaric medium was replaced by urea-nitrogen. This indicated that supply of nitrogen both from sodium nitrate and urea was better for *S. platensis* culture. Further addition of urea decreases the growth of *S. platensis*. Therefore, 50% of sodium nitrate-nitrogen in Kosaric medium can be replaced by urea-nitrogen, which will reduce the culture cost of *S. platensis*.

Keywords: *Spirulina platensis*, nitrogen, sodium nitrate, urea, replacement

Introduction
Spirulina spp. are multicellular blue-green algae. They are very small and microscopic and 300 to 500 µm in length. They flourish very well in alkaline waters where the pH is too high (9-11) for most other species to thrive in (Melack, 1979) [20]. These blue-green algae contain 50-70% protein, 10-12% carbohydrate, 6% fat, 7% minerals and a lot of vitamins (Habib *et al*. 2008) [12]. Beside high level of protein, *Spirulina* is an important source of essential fatty acids, phycocyanin and chlorophyll *a* (Spolaore *et al*. 2006; Moraes *et al*. 2011) [29, 21]. Its protein is complete containing all essential amino acids, though with reduced amounts of methionine, cysteine, and lysine when compared to the proteins of meat, eggs, and milk. It is, however, superior to typical plant protein, such as that from legumes (Babadzhanov *et al.* 2004; Khan and Bhaduria, 2005) [3, 17]. The cell wall of *Spirulina* consists of polysaccharide which has digestibility of 86%, and could be easily absorbed by human body. *Spirulina* species not only contributes to human health but also it plays considerable role as used as animal and fish feed. This alga is being widely studied, not only for nutritional reasons but also for its reported medicinal properties (Kim *et al.* 1998; Subhashini *et al.* 2004) [18, 30] and antimicrobial activities (Demule *et al.* 1996; Ozdemir *et al.* 2004) [8, 22].
Many factors are important for the production of *S. platensis* at large scale, of which most important factors are nutrient availability, temperature and light. The earliest attempts to culture algae started about more than a century ago with solutions of a few inorganic salts. A common feature of these media was their high contents of nutrients, particularly nitrogen, phosphorus and potassium (Rodhe, 1978) [27]. The commercial production of *S. platensis* can be made cost effective by reducing the input cost with cheap and readily available materials without sacrificing the production efficiency.
Kosaric medium (KM) is the most commonly used medium for *S. platensis* culture (Zarouk, 1996; Phang and Chu, 1999) [33, 24]. However, it is expensive and not readily available in Bangladesh. Thus, for mass production of *S. platensis*, particularly in developing countries

there is a need to find a way to reduce the cost of culture. In Kosaric medium sodium nitrate is used as a source of nitrogen (N_2), which is very important for *S. platensis* culture. Several studies have demonstrated the feasibility of replacing this conventional nitrogen source with low-cost alternatives such as urea, ammonium sulfate and ammonium chloride (Bezerra *et al*. 2008; Matsudo *et al*. 2009; Ferreira *et al*. 2010; Avila-Leon *et al*. 2012) [4, 19, 9, 2]. Urea is a very common fertilizer that is used as a cheap and rich source of N_2 for plant cultivation (Pathak, 2015) [23]. The production cost of *S. platensis* culture will be minimized if urea can be used as cheap source of N_2 in Kosaric medium. The present study was aimed to replace sodium nitrate-nitrogen ($NaNO_3$-N) of Kosaric medium by urea-nitrogen for *S. platensis* culture.

Materials and Methods

Experimental design

The experiment was carried out for eighteen days in the Live Food Culture Laboratory of the Department of Aquaculture, Faculty of Fisheries, Bangabandhu Sheihk Mujibur Rahman Agricultural University.

The experiment was conducted in completely randomized design with five treatments. Kosaric medium was used as a control medium for treatment T_1. In treatments T_2 to T_5, sodium nitrate of ($NaNO_3$) of Kosaric media was replaced with urea as shown in the Table 1.

Table 1: Experimental design of *S. platensis* culture

Treatment	Culture Media	Amount of $NaNO_3$ (g/L)	Amount of Urea (g/L)	Replication	Duration of Culture
T_1	KM* with 100%$NaNO_3$-N	1.25	0	3	18 Days
T_2	75% $NaNO_3$-N+ 25% Urea-N in KM	0.938	0.195		
T_3	50% $NaNO_3$-N+ 50% Urea-N in KM	0.625	0.390		
T_4	25% $NaNO_3$-N+ 75% Urea-N in KM	0.313	0.585		
T_5	100% Urea-N in KM	0	0.780		

*KM = Kosaric medium.

Collection and maintenance of *S. platensis*

Pure stock sample of *S. platensis* was collected from Department of Aquaculture, Bangladesh Agricultural University, Mymensingh. After collecting the stock, it was maintained in the Live Food Culture Laboratory of the Department of Aquaculture, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University. Pure stock culture of *S. platensis* was maintained in Kosaric medium (KM), modified after Zarrouk (1996). Growth of *S. platensis* was monitored at every alternative day and was checked under microscope to confirm its purity.

Preparation of Kosaric medium

Kosaric medium is widely used as the standard medium for *S. platensis* culture. The composition of Kosaric medium is shown in Table 2.

Table 2: Composition of Kosaric medium for *S. platensis* culture

SL. No.	Chemicals/compounds	Concentration in stock solution
1	$NaHCO_3$	9.00 g/L
2	K_2HPO_4	0.25 g/L
3	$NaNO_3$	1.25 g/L
4	K_2SO_4	0.50 g/L
5	NaCl	0.50 g/L
6	$MgSO_4.7H_2O$	0.10 g/L
7	$CaCl_2$	0.02 g/L
8	$FeSO_4.2\ H_2O$	0.005 g/L
9	Micronutrient solution*	0.5 ml/L

*Composition of micronutrient solution (g/L): i) H_3BO_3 2.86; ii) $MnCl_2.4H_2O$ 1.81; iii) $ZnSO_4.7H_2O$ 0.22; iv) $CuSO_4.5H_2O$ 0.08; MoO_3 0.01; $CoCl_2.6H_2O$ 0.01.

For the preparation of Kosaric medium, the above mentioned amount (table 2) of chemicals from no. 1 to 8 was weighted by the help of electric balance and took in a 1.0 L conical flask. Then 0.5 ml micronutrient solution was pipetted in the flask and distilled water was added to make the volume 1.0 L.

pH adjustment of all media

Prior to culture initiation of *S. platensis* pH of all media were adjusted at 9.0 by incorporating either 0.1 N HCl or 0.1 N NaOH depending on pH condition of the media. When it was found that the pH value of one media was above 9.0 then 0.1 N HCl was added and when the pH value was found below 9.0, 0.1 N NaOH was added until pH becomes stable at 9.0.

Experimental culture of *S. platensis*

Five treatments with three replication were used to culture microalgae *S. platensis* in 1.0 L conical flask. *S. platensis* were inoculated into each culture flask to produce a culture containing 10% suspension (OD at 620 nm=0.20) (Habib, 1998) [11]. The flasks were kept under fluorescent light (TFC, FL-40, SD/38 day light) in light: dark (12h: 12h) conditions in Live Food Culture Laboratory. These culture flasks were continuously aerated using electric aerators (Sobo, Aquarium pump SB-348A). Samplings were performed at every three alternative days for each flask to observe the optical density (OD), physico-chemical properties, dry cell weight and chlorophyll *a* content.

Estimation of *S. platensis* cell weight (dry basis)

Sample containing 50 ml *S. platensis* suspension was filtered through a Whatman GF/C filter paper of 0.45 µm mesh size and 47 mm diameter, which was dried in an oven for 24 hrs at 70 °C and weighed prior to the filtration. When the sample was being filtered it was washed with 20 ml acidified water (pH = 4) in order to remove insoluble salts. After that the filter papers were put in a glass Petridis and kept in the oven

at 70 ºC for overnight. For cooling, Petridis were put into desiccators for 20 minutes and then filter papers were weighed. Dry weight of *S. platensis* was calculated using following formula.

$$W = \frac{FFW - IFW}{\text{Amount of sample taken filtration (ml)}} \times 100$$

Where,
W= Cell dry weight in g/L; FFW= Final filter weight in g; and IFW= Initial filter weight in g.

Estimation of chlorophyll *a*

S. platensis sample were collected in order to estimate chlorophyll *a* content. Collected sample (10 ml) was filtered with an electric filtration unit using filter papers (Whatman GF/C of 0.45 µm mesh size and 47 mm diameter). These filtered samples together with filter paper was taken into a test tube and ground with a glass rod and finally mixed with 10 ml of 100% redistilled acetone. Each of the test tubes was wrapped with foil papers to inhibit the contact of light. The wrapped test tubes were kept into a refrigerator overnight. Then the refrigerated samples were homogenized for 2 minutes followed by centrifugation at 4000 rpm for 10 minutes. After centrifugation the supernatant was isolated and taken for chlorophyll *a* determination. Optical densities of the samples were determined at 664 nm, 647 nm and 630 nm by using UV spectrophotometer (DR 5000). A blank with 100% acetone was run simultaneously. Chlorophyll *a* was calculated using the following formula (Habib, 1998) [11]:
Chlorophyll *a* (mg/L) = 11.85 (OD 664) - 1.54 (OD 647) - 0.08 (OD 630)

Measurement of optical density

Optical density was measured during the time of sampling at 620 nm, by using UV spectrophotometer (DR 5000). The sample of *S. platensis* grown in different treatments were taken in puvet and placed in spectrophotometer. Then the OD of the samples was recorded.

Determination of physico-chemical properties of the culture media

Water temperature (ºC) of the culture media was measured on the sampling day by a Maximum- Minimum thermometer (push button reset). Light intensity (lux/m^2/s) was measured during sampling days by using a lux-meter (LX-9621). Dissolved oxygen (mg/L) of the culture media was measured by using a dissolved oxygen meter (HQ40d multi) (Figure 5). pH of the culture media was measured by an electric pH meter (sensION^{TM}+ PH_3).

3.7 Statistical analysis

One way analysis of variance (ANOVA) in completely randomized design of mean of cell weight, chlorophyll *a* content, and optical density of *S. platensis* cultured under different treatments was done to find out whether there was any significant difference among treatment means, while LSD test was used to compare the treatment means (Hofmann, 2008).

Results and Discussion

Physico-Chemical Parameters

The mean values of temperature in different treatments during the experimental period ranged from 29.10±0.10 ºC to 31.90±0.12 ºC. Optimum temperature for *S. platensis* culture is 25-35^{0}C (Chowdhury, 2005) [7]. A temperature range of 28-35 ºC was the best for the production of *S. platensis* (Torzillo and Vonshak, 1994) [32].

The mean values of pH in different treatments ranged from 9.10±0.40 to 9.35±0.40. It is reported that optimum pH leads to increased growth, CO_2 uptake and amino acid content. Reported optimum pH level for *S. platensis* culture was 8.0-10.0 (Joshi *et al*. 2013) [14]. Chowdhury (2005) [7] observed pH 8.5-10.0 as the best for the production of *S. platensis*. During the culture period dissolved oxygen level ranged from 4.90±0.10 mg/l to 5.80±0.22mg/l. Different researchers reported optimum dissolved oxygen level for *S. platensis* ranged between 3.1 mg/l to 5.5 mg/l (Chowdhury, 2005; Rahman, 2005; Chen and Lee, 2012) [7, 26, 5]. As artificial aeration was continuously provided in the present experiment, dissolved oxygen level was never become a problem.

The light intensity recorded near different treatments found more or less similar during culture period. The range of light intensity was 2220 to 2295 lux/m^2/s in the present experiment. Kebede and Ahlgren (1996) [16] observed that *S. platensis* grown in modified Zarrouk's medium and exposed to a range of light intensities (2000-2500 lux/m^2/s) showed a maximum specific growth rate. Zarrouk (1996) [33] is the pioneer in detailed study on the response of *S. platensis* to light. In a simple experiment, he reached a conclusion that the highest growth of *S. platensis* was obtained at a light intensity of 2500-3000 Lux/m^2/s.

It is evident from the results of the present experiment that temperature, light intensity, dissolved oxygen and pH were not varied significantly among the treatments and were within suitable range for *S. platensis* culture.

Optical Density

The mean optical density (OD) in different treatments during the experimental period is presented in Table 3. The variation in mean values of optical density in different treatments was started from the 6th day of culture. The exponential phase of the culture was found up to 15th day from the beginning and after 15th days the optical density found to decline. The highest optical density was recorded on the 15th day in treatment T_3. At the end of 18 days experimental period the significantly highest OD was observed in treatment T_3, where 50% of N_2 in Kosaric media was supplied from urea. At the end of 18 days experimental period, OD in T_3 was significantly higher than that of control treatment T_1 and treatment T_2. However, high levels of urea in treatment T_4 and T_5 decrease the OD.

Higher OD might be occurred due to presence of more favorable N_2 which enhanced growth in treatment 3 than other treatments. Tanticharoen *et al*. (1993) [31] reported that the addition of sodium nitrate ($NaNO_3$) and urea as nitrogen fertilizer in waste water from the stabilization ponds of starch factory, raised optical density, which was higher than, where urea was not used as nitrogen source. Islam (2004) [13] conducted an experiment to observe the effect of normal molasses on growth of *S. platensis* and reported that the addition of urea in normal molasses medium increased the OD of the culture.

Table 3: Optical density (mg/l) of *S. platensis* under different treatments during the experimental period

Sampling Day	Optical Density				
	T_1	T_2	T_3	T_4	T_5
3rd day	0.41±0.10	0.40±0.12	0.43±0.10	0.41±0.13	0.42±0.10
6th day	0.57±0.14 a	0.48±0.13 b	0.68±0.10 a	0.44±0.10 b	0.45±0.11 b
9th day	0.69±0.12 a	0.54±0.12 b	0.75±0.12 a	0.52±0.05b	0.51±0.12 b
12th day	0.71±0.11 a	0.57±0.10 b	0.82±0.15 a	0.61±0.10 b	0.55±0.05b
15th day	0.72±0.12 ab	0.61±0.10 b	0.90±0.10 a	0.66±0.11 b	0.59±0.10 b
18th day	0.63±0.11 b	0.59±0.10 b	0.80±0.13 a	0.61±0.10 b	0.57±0.10 c

*Means (±SD) in a sampling day bearing different letters are significantly different (P<0.05).

Cell Dry Weight

The mean cell dry weight in different treatments during the experimental period is presented in Figure 1. The initial inoculum rate of *S. platensis* in all the treatments was 0.01 g/l. Cell dry weight in all treatments increased with the progress of culture and attained peak at the 15 days of culture, then growth tends to decrease. At the end of 18 days culture period, maximum cell dry weight was obtained in treatment T_3, where 50% of N_2 in Kosaric medium was replaced with N_2 from urea. Which indicated that a combination of N_2 both from $NaNO_3$ and urea is better for production of *S. platensis* compared to N_2 from $NaNO_3$ alone. However, more that 50% replacement of N_2 in Kosaric media with urea decreased the cell dry weights in treatments T_4 and T_5, where 75% and 100% N_2 in Kosaric medium were replaced with N_2 from urea, respectively.

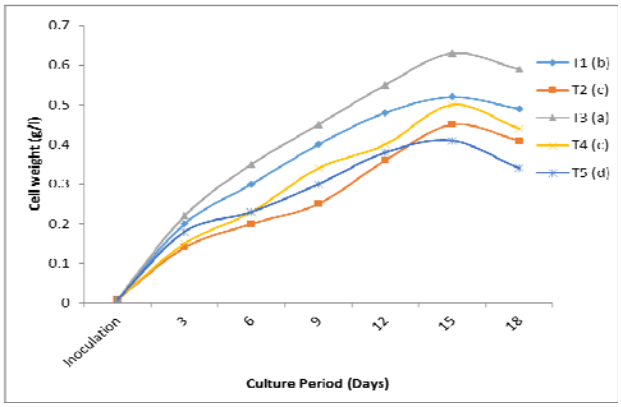

Fig 1: Cell dry weight (g/l) of *S. platensis* in different treatments during the experimental period. Means with different letters are significantly different (P<0.05).

Higher inoculation of urea in treatment T_4 and treatment T_5 decreased the growth of *Spirulina platensis*. Guo (2000) [10] observed that high concentration of urea caused the death of *S. platensis*. Alam (2002) [1] conducted an experiment to evaluate the organic nutrients of press mud medium as standard for growing *S. platensis* in the laboratory. He observed that addition of 0.2 g/L urea to press mud medium gave the maximum production of *S. platensis*. Chirasuwan *et al*. (2000) [6] also found that urea can be used as an important source of N_2 for production of *S. platensis*, however, production was lowest when 100% N_2 was supplied from urea. Ruprecht *et al*. (1998) [28] found from their experiment that growth was reduced due to excessive use of urea in plankton culture. They concluded that urea increases growth performance up to a certain level or concentration or dose beyond that concentration urea reduces growth performance of plankton. Pathak (2015) [23] reported that in case of plant culture, excessive use of urea reduces growth performance compared to other fertilizers.

Chlorophyll *a* Content

The mean chlorophyll *a* content in different treatments during the experimental period is presented in Figure 2. The highest and the lowest chlorophyll *a* content was observed in treatment T_3 and treatment T_5, respectively, at the end of the experiment. A combination of N_2 from both $NaNO_3$ and urea increase the chlorophyll *a* content of *S. platensis* in treatment T_3, which was better than that of the control treatment T_1, where source of N_2 was $NaNO_3$ alone.

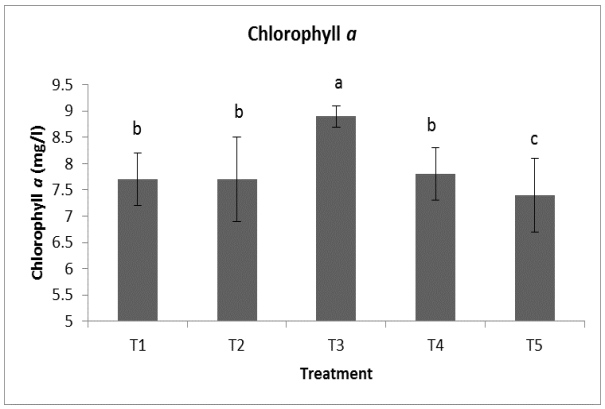

Fig 2: Chlorophyll *a* content (mg/l) of different treatments at the end of 18 days experiment period. Means with different letters differ significantly (P<0.05).

Karim (2004) [15] produces *Spirulina platensis* using fertilized factory effluents medium and found maximum chlorophyll *a* was 5.02 mg/l where fertilizer factory effluents along with urea as a nitrogen source was used. Pulz *et al*. (1992) conducted an experiment where algal species *S. platensis* was cultured in three concentration of soybean meal medium (SMM) supplemented with 0.2g/L urea and observed positive effect of urea on chlorophyll *a* content of *S. platensis.*

Conclusion

From the present study, it was observed that maximum optical density, cell dry weight and chlorophyll *a* were observed in treatment T_3, where 50% sodium nitrate-nitrogen in Kosaric medium was replaced by urea-nitrogen. Further addition of urea decreases the growth of *S. platensis*. Therefore, 50% of sodium nitrate-nitrogen in Kosaric medium can be replaced by urea-nitrogen, which will increase the production but reduce the culture cost of *S. platensis*.

Acknowledgement

Fund for Sub-Project 'Establishment of a Live Food Culture Laboratory in the department of Aquaculture CP3080' under 'Higher Education Quality Enhancement Project (HEQEP) is gratefully acknowledged. Thanks to Professor Dr. M.A.B. Habib, Department of Aquaculture, Bangladesh Agricultural University for providing *S. platensis* stock culture.

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