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REPLACEMENT OF SODIUM BICARBONATE AND MICRONUTRIENTS IN KOSARIC MEDIUM WITH BANANA LEAF ASH EXTRACT FOR CULTURE OF *SPIRULINA PLATENSIS*

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Abstract

Spirulina platensis is one of the most promising microalgae for culture due to its high nutritional values. The main constraint to its production is the high cost of culture media. Therefore, an experiment was conducted to evaluate the growth performance of *S. platensis* with the aim of replacing sodium bicarbonate ($NaHCO_3$) and mineral nutrients in Kosaric medium (KM) with banana leaf ash extract. KM was used as control medium in treatment T_1. In treatments T_2 to T_5, 50% of $NaHCO_3$ in KM was replaced with banana leaf ash extract (BLAE). In addition, micronutrients in KM were reduced to 75%, 50% and 25% in treatments T_3 to T_5, respectively. The values of different physical and chemical parameters (temperature, pH, dissolved oxygen and light intensity) of the culture media were within the suitable range for *S. platensis* culture. Optical density in treatments T_2 and T_3 were similar to that was in treatment T_1. Maximum cell dry weight (0.65 gL^{-1}) was observed in treatment T_1 and it was similar to the treatments T_2 and T_3 where 50% of $NaHCO_3$ in KM was replaced with banana leaf ash extract with 100% and 75% micronutrients supply, respectively. The similar trend was found in chlorophyll *a* content. It was evident that 50% of $NaHCO_3$ in KM can be replaced with BLAE for the culture of *S. platensis,* in addition micronutrients in KM can be reduced to 75%.

Keywords: Microalgae, growth performance, cell dry weight, chlorophyll.

Introduction

Spirulina platensis is a multicellular, filamentous, free floating cyanobacterium or photosynthetic blue green algae. They are very small and microscopic and 300 to 500 μm in length. They flourish very well in alkaline, saline waters where the pH is too high 9-11 for most other species to thrive in. The blue-green algae, *S. platensis* is commercially produced in some tropical and subtropical climatic regions of the world (Venkataraman and Becker, 1985). It is one of the most promising microalgae for culture due to its high nutritional values (Babadzhanov *et al.,* 2004). *S. platensis* is gaining great interest for its high nutritional contents such as vitamins, minerals, polyunsaturated fatty acids, carotenoids and other pigments that have antioxidant activity (Lin *et al.,* 2007). These blue-green algae contain 50-70% protein, 10-12% carbohydrate (in dry condition), 6% fat, 7% minerals, (average range 2.76–3.00% of total weight) and lot of vitamins (Habib *et al.,* 2008). Beside high level of protein, *S.*

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platensis is an important source of essential fatty acids, phycocyanin and chlorophyll (Moraes *et al.,* 2011). Pre-clinical and clinical studies suggest that it has certain therapeutic effects, such as reduction in cholesterol, protection against some cancers, enhancement of the immune system, increase of intestinal lactobacilli, reduction of nephrotoxicity by heavy metals and drugs, radiation protection, reduction of hyper-lipidemia and obesity (Khan and Bhadouria, 2005; Hirahashi *et al.,* 2012). In earlier 1967, *S. platensis* was established as a "wonderful future food source" by the International Association of Applied Microbiology (Sasson, 1997). It has been considered as ''Food of the future'' and an ideal food for astronauts by NASA (Joshi *et al.,* 2013).

Kosaric medium (KM) is the most commonly used medium for *Spirulina* culture. However, it is expensive due to high ingredients cost. Thus, for mass production of *S. platensis*, particularly in developing countries there is a need to find out an effective, cheaper and readily available alternative media. Different efforts have been made to reduce the production cost of *S. platensis* by replacing nutrients in KM with commercial grade fertilizers (Madkour *et al*., 2012; Rizal *et al.,* 2017) or by replacing KM with organic wastes (Toyub *et al.,* 2011; Akhter *et al*., 2012; Jain and Singh, 2013). In KM, the ingredient used in large amount is $NaHCO_3$ which is used as a source of carbon and also to increase the pH of medium.

Banana (*Musa sapientum*) plant contains high amounts of base metals, and its ash extract is highly alkaline. Banana leaf ash extract also contain high levels of potassium, sodium and calcium (Anhwange *et al.,* 2008). These elements impart alkalinity properties on the ash and its solution (Tiisekwa *et al.,* 1999). Banana leaf can be used as cheap source for nutrients and pH in KM. It also can play as a significant source of micronutrients for *S. platensis* culture. The aim of the present work is to study the growth performance of *S. platensis* in KM supplemented with banana leaf ash extract as a source of alkalinity and micronutrients in order to minimize the production cost.

Materials and Methods

Preparation of Kosaric medium

Kosaric medium (KM) is widely used as the standard medium for *S. platensis* culture, which was used as the control medium in the present experiment. The prepared KM was sterilized at 121°C for a period of 6 hours with moist heat by autoclave and cooled for a period of 24 hours. The composition of KM is shown in Table 1.

For the preparation of KM, the amount of ingredients from no. 1 to 8 mentioned in Table 1 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.

Preparation of banana leaf ash extract

For preparation of banana leaf ash extract (BLAE), banana leaf was collected from the banana garden of BSMRAU. Then leaves were sun dried primarily and then dried in an oven at 40°C for overnight. Dried banana leaf was then burned in muffle furnace at 550°C for 6 hours. After cooling, 26 g of banana leaf ash

Table 1. Composition of Kosaric medium for *S. platensis* culture

Sl. No.	Chemicals/compounds	Concentration in stock solution
1	$NaHCO_3$	9.00 gL^{-1}
2	K_2HPO_4	0.25 gL^{-1}
3	$NaNO_3$	1.25 gL^{-1}
4	K_2SO_4	0.50 gL^{-1}
5	NaCl	0.50 gL^{-1}
6	$MgSO_4.7H_2O$	0.10 gL^{-1}
7	$CaCl_2$	0.02 gL^{-1}
8	$FeSO_4.2H_2O$	0.005 gL^{-1}
9	Micronutrient solution	0.5 mlL^{-1}
	Composition of micronutrient solution	
	i) H_3BO_3	2.86 gL^{-1}
	ii) $MnCl_2.4H_2O$	1.81 gL^{-1}
	iii) $ZnSO_4.7H_2O$	0.22 gL^{-1}
	iv) $CuSO_4.5H_2O$	0.08 gL^{-1}
	v) MoO_3	0.01 gL^{-1}
	vi) $CoCl_2.6H_2O$	0.01 gL^{-1}

was dissolved in 260 ml distilled water. After 7 days, the solution was filtered first with fine mesh to remove undissolved materials and then with 0.45 micrometer size filter paper to get a clear solution.

Experimental culture of *S. platensis*

The experiment was conducted in completely randomized design with five treatments. Kosaric medium (KM) was used as control medium in treatment T_1. In treatments T_2 to T_5, 50% of sodium bicarbonate in KM was replaced with banana leaf ash extract (BLAE) contained minerals (mg/L) as follow: Na 272.0, K 38.0, Ca 143.0, Mg 12.3, P 175.7, and Fe 11.5. From the KM 50% sodium bicarbonate was reduced and same amount of BLAE was added. In addition, micronutrients in KM were reduced to 75%, 50% and 25% in treatments T_3 to treatments T_5, respectively. When it was found that the pH value of one medium 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 become stable at 9.0. Pure stock of sample of *S. platensis* was collected from the Department of Aquaculture of Bangladesh Agricultural University. After collecting, the stock was maintained in KM in Live Food Culture Laboratory of the Department of Aquaculture, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University.

Experimental culture of *S. platensis* was conducted in 15 conical flask (1.0 L size) with 5 treatments and 3 replications for each treatment. *S. platensis* were inoculated into each culture flask to produce a culture containing 10% suspension (OD at 620 nm=0.20) (Habib *et al.,* 1996). 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). Six sub-samplings were performed at every four day for each flask to observe the cell density, optical density (OD) and physico-chemical properties.

Estimation of *S. platensis* cell dry weight

Sample containing 40 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 hours at 70°C and weighed prior to the filtration. When the sample was being filtered it was washed with 20 ml acidified water (pH = 4.0) in order to remove insoluble salts. After that the filter paper was put in a glass Petri dish and kept in the oven at 70ºC for overnight. For cooling, Petri dish was put into desiccators for 20 minutes and then filter paper was weighed. Dry weight of *S. platensis* was done following the formula (Clesceri *et al.,* 1989):

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

Where, W= Cell dry weight in gL^{-1}; FFW= Final filter paper weight in g; and IFW= Initial filter paper weight in g.

Estimation of chlorophyll *a*

Spirulina platensis sample, were collected in order to estimate chlorophyll *a* content. 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). This filtered sample 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. Then the test tube was wrapped with foil papers to inhibit the contact of light. The wrapped test tubes were kept into a refrigerator overnight. Then the refrigerated sample was 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 using UV spectrophotometer (DR 5000, USA). A blank with 100% acetone was run simultaneously. Chlorophyll *a* content was calculated by the following formula (Clesceri *et al.,* 1989):

Chlorophyll *a* (mgL^{-1}) = 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 a UV spectrophotometer (DR 5000, USA). The sample of *S. platensis* grown in different treatments were taken in cuvette and placed in spectrophotometer. Then the OD of the samples were recorded.

Determination of physico-chemical properties of the culture media

The Physico-chemical parameters of the culture media were measured at four days interval up to the experiment completion by the procedures given by Clesceri *et al.* (1989). Water temperature (ºC), light intensity ($luxm^{-2}s^{-1}$), dissolved oxygen (mg/L) and pH of the culture media was measured on the sampling day by an alcoholic thermometer, a lux-meter (LX-9621, China), a

dissolved oxygen meter (HQ40d multi, USA) and an electric pH meter (sensION^{TM}+ PH3, USA), respectively.

Statistical analysis

One way analysis of variance (ANOVA) of means of all physico-chemical parameters, cell weight, chlorophyll *a* content and optical density in 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. All statistical analysis were carried out using Microsoft Excel program (version 2013) and statistical software Statistix 10.

Results and Discussion

Physico-chemical parameters

The mean values of temperature in all treatments ranged from 28.22±0.12ºC to 31.53±0.12ºC. No significant difference (P>0.05) was observed in the temperature of different treatments in a sampling day. Chowdhury (2005) suggested that the optimum temperature for *Spirulina* culture is 25-35^{0}C. A temperature range of 28-35^{0}C was the best for the production of *S. platensis* also suggested by Torzillo and Vonshak (1994).

In this study, DO level for *S. platensis* was ranged from 3.11 mgL^{-1} to 4.83 mgL^{-1}. The DO level ranged from 3.1 mgL^{-1} to 5.5 mgL^{-1} suitable for *spirulina* culture, also reported by some researchers (Alam, 2002; Chowdhury, 2005). As artificial aeration was continuously provided, therefore, maintaining optimum DO level was never become a limiting factor in this experiment.

The light intensity recorded near different media found more or less similar during culture period. The range of light intensity was from 2200 to 2220 luxm^{-2}s^{-1} in this experiment. There was no significant difference (*P>0.05*) in the light intensity of different treatments. Kebede and Ahlgren (1996) observed that *S. platensis* grown in modified Zarrouk's medium and exposed to a range of light intensities (2000-2500 luxm^{-2}s^{-1}) showed a maximum growth. Alam (2002) also recorded the light intensity from 2100 to 2250 lux which is also similar to the findings of this experiment. He also reported that the *S. platensis* was saturated at a level of 25-30 K luxm^{-2}s^{-1}. In the present study, light intensity ranges were more or less similar to the optimum range of light intensity for *S. platensis* culture.

The most important chemical factor of the medium is pH for culturing *S. platensis*. Maintaining pH of over 9.5 is suitable for *S. platensis* culture in order to avoid contamination by other algae (Becker, 1984). In this study, the pH was ranged from 8.2 to 10.5, which was favorable for the growth of *S. platensis*. Initially the pH was set to 9.0 but gradually pH increase to 10.5 due to the shift of the bicarbonate-carbonate equilibrium towards the carbonate with the progress of culture period and pH decreased during the last phase of the culture period. Joshi *et al*. (2013) also reported that optimum pH level for *S. platensis* culture was 8.0-10.0. The increasing trend of pH (Fig. 1) up to the stationary phase favored the growth of *S. platensis.* However, no significant difference (*P>0.05*) was observed in the pH of different treatments in a sampling day. The decreasing trend of pH at the death phase might be occurred due to dead cells and other organic loads.

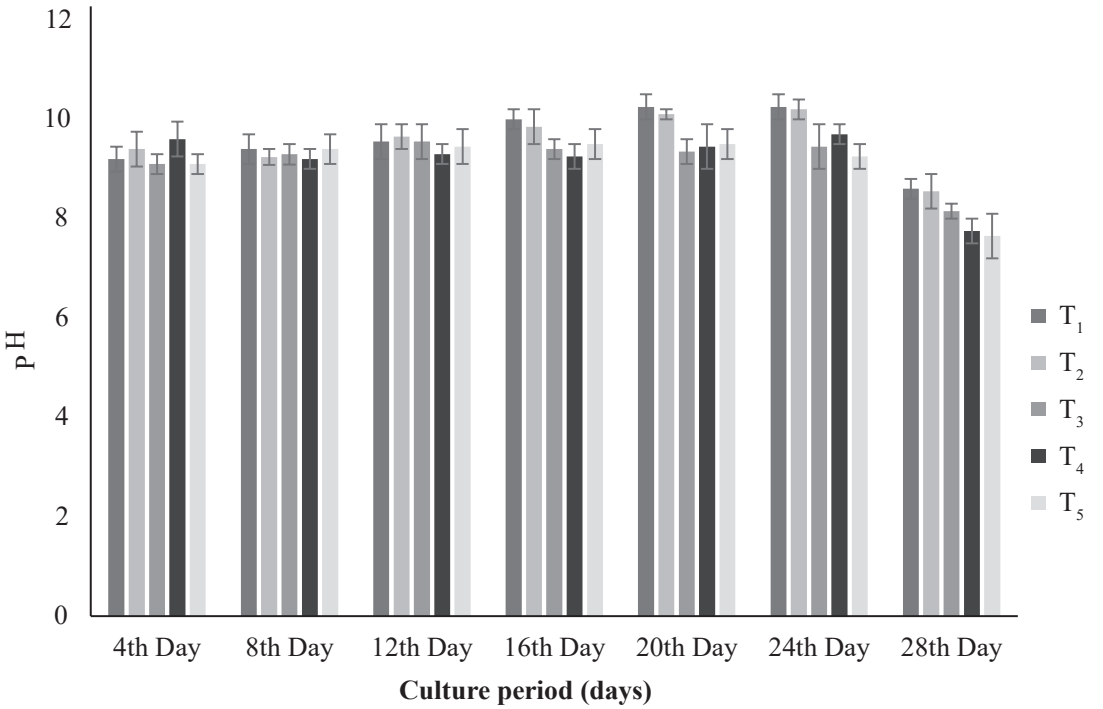

Fig. 1. pH of culture media under different treatments during the experimental period.

Cell dry weight

The mean cell dry weight in different treatments during the experimental period is presented in Fig. 2. The initial inoculum rate of *S. platensis* in all the treatments was 0.01±0.01 gL^{-1}. At the end of 28th day of experiment, the highest cell dry weight was recorded in treatments T_1. Cell dry weight in treatments T_2 was similar to treatment T_1, which indicated that 50% of $NaHCO_3$ in KM can be replaced with banana leaf ash extract. Cell dry weight in treatment T_3 was similar to treatment T_1 indicated that in addition to 50% reduction of NaHCO3, micronutrient can be reduced to 75% without affecting the cell dry weight of *S. platensis*. Cell dry weight in treatments T_4 and treatments T_5 was significantly lower than the treatment T_1, which indicated that further reduction of micronutrient in KM can reduce *S. platensis* growth.

Replacement of nutrients in media with low-cost ingredients is important to minimize the production cost of *S. platensis*.

Rizal *et al.* (2017) was able to replace 50% sodium nitrate-nitrogen in Km with urea-nitrogen without compromising the growth. Rahman (2003) observed that 7.2 mgL^{-1}banana leaf ash with 0.4 mgL^{-1} JSP and 0.2 g urea was suitable for culture of *S. platensis*. The findings of that study was similar to the result of the present study which indicate the possibility of using banana leaf ash extract in KM to lowering the cost of medium for *S. platensis* culture.

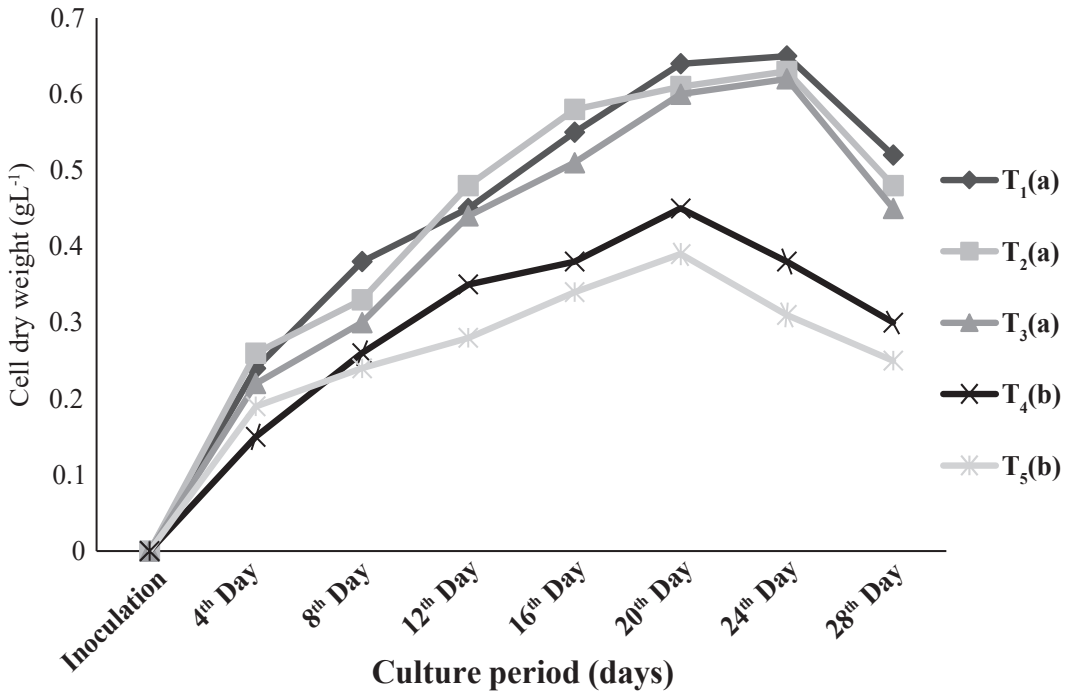

Fig. 2. Cell dry weight (gL^{-1}) of *S. platensis* under different treatments during the experimental period. Means with different letters are significantly different *(P<0.05)*.

Optical density

The mean values of optical density (OD) of *S. platensis* cultured under different treatments are presented Table 2. The mean values of OD in all treatments ranged from 0.29±0.10 mgL^{-1} to 0.81±0.10 mgL^{-1}. The variation of OD in different treatments was started from 16th day of culture. At the end of the experiment, maximum OD was found in treatment T_1 where KM was used as culture medium. Optical density in treatments T_2 and T_3 was similar to treatments T_1, which indicated that 50% of $NaHCO_3$ in KM can be replaced with banana leaf ash extract. In addition, mineral content can be reduced to 75% without affecting the OD. On the other hand, OD in treatments T_4 and treatments T_5 was lower than the treatments T_1, which indicated that replacement of 50% of $NaHCO_3$ with banana leaf ash extract and reducing micronutrient to less than 50% in KM can reduce optical density.

Replacement of sodium bicarbonate with BLAE and up to 75% micro nutrient might create a medium which was quite similar to that of KM and did not alter the culture medium constituent that made favorable growth condition for *S. platensis*. Therefore, difference between optical density in treatment T_1, T_2 and treatment T_3 was not significant (*P>0.05*). In treatment T_4 and T_5, the optical density was negatively affected. This might be due to use of low percentage of micronutrient. The result showed that 50% and 25% micronutrients are not suitable and favorable for optical density of *S. platensis*.

Table 2. Optical density (mgL^{-1}) of *S. platensis* under different treatments during the experimental period

Treatment	Optical density (mg/L) on sampling day						
	4th	8th	12th	16th	18th	24th	28th
T_1	0.33±0.10[a]	0.44±0.10[a]	0.58±0.13[a]	0.68±0.06[a]	0.72±0.10[a]	0.81±0.10[a]	0.69±0.12[a]
T_2	0.32±0.10[a]	0.42±0.11[a]	0.52±0.12[a]	0.65±0.11[a]	0.71±0.10[a]	0.80±0.13[a]	0.65±0.12[a]
T_3	0.31±0.12[a]	0.41±0.12[a]	0.48±0.11[a]	0.60±0.08[a]	0.69±0.11[a]	0.79±0.10[a]	0.60±0.11[a]
T_4	0.29±0.10[a]	0.40±0.11[a]	0.43±0.13[a]	0.55±0.13[b]	0.58±0.13[b]	0.59±0.11[b]	0.53±0.10[b]
T_5	0.30±0.11[a]	0.41±0.11[a]	0.42±0.10[a]	0.52±0.12[b]	0.54±0.11[b]	0.56±0.12[b]	0.49±0.12[b]

*Means with different letters in sampling day differ significantly ($p<0.05$).

These findings agree with the findings of Akter *et al.* (2012) who studied the growth response of *S. platensis* in papaya skin extract media, where maximum optical density of 0.31 mgL^{-1} was observed in $0.6gL^{-1}$ papaya skin extract medium. Rizal *et al.* (2017) conducted an experiment to investigate the effect of replacement of sodium nitrate in KM with urea on growth performance of *S. platensis,* where the values of optical density (OD) in different treatments ranged from 0.29±0.10 $mgL^{-1}$to 0.81±0.10 mgL^{-1}. Similar range of optical density was also observed by other researchers (Karim, 2004; Chowdhury, 2005) which is also supported the present findings of this experiment.

Chlorophyll a content

The mean chlorophyll *a* contents in different treatments after 28 days experimental period are presented in Figure 3. The mean values of chlorophyll *a* content in different treatments ranged from 6.4 $mgL^{-1}$to 8.02 mgL^{-1}.

Toyub *et al.* (2011) found that *S. platensis* cultured in different concentrations of papaya skin powder media produce chlorophyll *a* content ranging from 3.57 to 8.15 mgL^{-1}. Similar kind of results was also obtained by Islam (2004) using molasses as a nutrient medium for the culture of *S. platensis* where the maximum chlorophyll *a* was 10.09 mgL^{-1}. Karim (2004) produced *S. platensis* using fertilized factory effluents and found chlorophyll *a* 5.02 mgL^{-1} as maximum. Dey (2004) produced *S. platensis* using mustard oil cake medium and found maximum chlorophyll *a* content of 10.13 mgL^{-1}. These previous findings support the present result of chlorophyll *a* content of *S. platensis*.

At the end of the experiment, maximum chlorophyll *a* content was found in treatment T_1, where KM was used as culture medium. Chlorophyll *a* content in treatments T_2, T_3 and T_4 was similar to treatments T_1, which indicated that 50% of $NaHCO_3$ in KM can be replaced with banana leaf ash extract. In addition, 50% micronutrient in medium can be reduced without affecting chlorophyll *a* content of *S. platensis*. On the other hand, chlorophyll *a* content in treatments T_5 was significantly lower than the other treatments, which indicated that replacement of 50% of $NaHCO_3$ with BLAE and with only 25% micronutrients supplement in KM can adversely affect the chlorophyll *a* content of *S. platensis*.

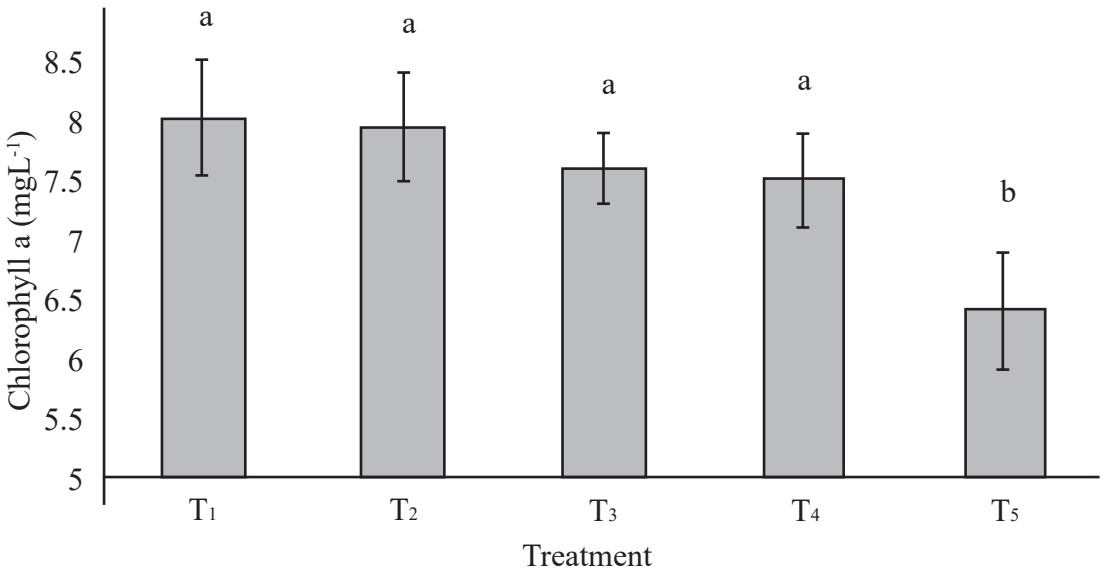

Fig. 3. Chlorophyll a content (mgL^{-1}) of different culture media at the end of 28th days of experimental period. Means with different letters are significantly different from each other.

Table 3. Cost of nutrient media for *S. platensis* culture

Medium	Ingredients	Amount (gL^{-1})	Price (Tk)	Total (Tk)
T_1 (Kosaric Medium)	$NaHCO_3$	9.00	22.50	30.00
	Other ingredients	-	7.50	
T_2,T_3,T_4 and T_5 (50% $NaHCO_3$ was replaced with BLAE)	$NaHCO_3$	4.50 g	11.25	18.75
	BLAE	4.50 g	0	
	Other ingredients	-	7.50	

Cost of nutrient media for *S. platensis* culture

A simple cost analysis of nutrient media for *S. platensis* culture is presented in Table 3. The main ingredient of the KM is $NaHCO_3$, which attribute bulk of the cost for the preparation. To prepare 1.0 litre of KM, 9.0 g of $NaHCO_3$ was required which valued approximately Tk. 22.50 and the total cost of KM was approximately Tk.30.00. On the other hand, with 50% banana leaf ash, the cost of 4.5 g $NaHCO_3$ was only Tk.11.25 and other ingredients cost Tk. 7.50 then the total cost to prepare 1.0 litre of medium was Tk. 18.75.

It was observed that the growth rate and chlorophyll *a* content of *S. platensis* cultured with medium containing 50% of sodium bicarbonate with BLAE was similar to that cultured with but the production cost of 50% a banana leaf ash extract supplemented media was significantly lower than KM.

Conclusion

Finally, on the basis of cell dry weight, optical density and chlorophyll *a* content value, it was evident that 50% of $NaHCO_3$ in KM can be replaced with banana leaf ash extract, in addition micronutrient in KM can be reduced to 75% without affecting the production of *S. platensis*.

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