Development of Low-Cost Growing Media for *Spirulina* using Alternative Carbon Sources

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Abstract

Spirulina, a blue-green microalgae is grown commercially throughout the world due to the high protein content and non-toxic features of the algal biomass. The Zarrouk's medium which contains Na_2CO_3 and $NaHCO_3$ as the carbon sources is known to be the standard medium for *Spirulina* cultivation. Higher purchasing cost of the carbon sources is recognised to be a limiting factor for large-scale cultivation. Low-cost alternative sources of carbon which can replace Na_2CO_3 and $NaHCO_3$ in the Zarrouk's medium has a great economic impact on the commercial production. This study aimed at assessing the growth of *Spirulina* in different alternative sources of carbon under different concentrations. Table sugar, cassava flour, sweet potato flour and taro flour were used as alternative sources. The Zarrouk's medium was substituted with different levels (25%, 50%, 75% and 100%) of the solutions prepared with the alternative carbon sources. The algae were cultured at room temperature for 16 days under illumination of 4,000 Lux. The growth was assessed as optical density (OD) using a spectrophotometer at 560 nm and then converted to dry weight (g L^{-1}).

According to the results, 100% carbon in the Zarrouk's medium could be replaced by taro flour and 50% carbon could be replaced by cassava flour. A significantly higher dry weight (1.033 g L^{-1}) was recorded from taro flour at 100% replacement level at the end of 16-day incubation. Therefore $NaHCO_3$ and Na_2CO_3 in the Zarrouk's medium could completely be replaced by taro flour solution which substantially reduces the cost of production as well. Cassava flour solution could also be used as an effective replacement while mixing with Zarrouk's medium into the ratio 1:1. Table sugar and sweet potato flour are found to be poor sources of carbon to replace the $NaHCO_3$ and Na_2CO_3 in the Zarrouk's medium. The results could be further confirmed by assessing the growth of *Spirulina* under different supplementary levels of taro and cassava flour.

Keywords: carbon sources, growing media, low-cost, Spirulina, Zarrouk's medium

1. Introduction

Microalgae, a group of fast-growing unicellular or simple multicellular microorganisms, offer several advantages, including higher photosynthetic efficiency, higher growth rates and higher biomass production compared to other energy crops (Li et al., 2008; Gouveia et al., 2009). *Spirulina* is a multi-cellular, filamentous and unbranched blue-green microalgae naturally grown in alkaline waters in warm regions. The species has the ability to colonise in wide range of environments which are not suitable for many other living organisms (Madkour et al., 2012). Although *Spirulina* possess a simple prokaryotic cell structure, the absence of plant cell wall, photosynthetic ability and glycogen-containing cellular membrane are similarities to the bacteria, plant and animal kingdoms respectively (Usharani et al., 2014).

Spirulina has been named as "World's Future Food" due to its high protein content, non-toxic features and ability to synthesis quality concentrated foods more efficiently (Kantha, 2014). The species is widely applicable in the field of agriculture, food science, perfumeries, pharmaceutical and medical science thus grown commercially at large scale. Microalgae generally need suitable climatic composition also (Palaniswamy and Veluchamy, 2018). Bright sunlight throughout the year is considered to be ideal

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for *Spirulina* cultivation. The growth is reported to be badly affected by fluctuation of solar radiation, temperature, rain, and wind. As *Spirulina* thrive well at a temperature range of 30-35^{o} C, illumination of 20-30 lux and pH over 8.5, tropical countries are recognised for large-scale cultivation (Usharani et al., 2012).

Photosynthesis is the main carbon-fixation route in *Spirulina*, however when cultivate without external carbon source, low productivity (typically less than 1.0 g L^{-1}) is frequently reported (Chen et al., 2006), which constrains the commercialisation (Golmakani et al., 2012). Glucose is currently the conventional carbon source for mixotrophic cultivation of *Spirulina*. However, using less expensive carbon sources such as ethanol and acetic acid is particularly attractive from the economical point of view (Cohen et al., 2005). In order to produce food-grade *Spirulina*, both compounds can be obtained at highest possible purity at a low cost. Also, they have no residual non-fermentable components. On the other hand, unlike glucose, ethanol, and acetic acid are toxic to most cells (even to those of microalgae) when used at high concentrations (Golmakani et al., 2012). The commercial cultivation of *Spirulina* is affected by the high production cost. A variety of nutrients which include carbon, nitrogen, potassium and phosphorus are needed to ensure optimum growth. Carbon is among the main nutrients needed by *Spirulina*, thus the cost of the carbon source accounts for about 15-25% of the total production cost (Madkour et al., 2012). The higher purchasing cost of $NaHCO_3$ and Na_2CO_3, the main source of carbon in Zarrouk's media limits the commercial scale production of *Spirulina* (Habib, 2008).

Despite the fact that the hot tropical climatic conditions available in Sri Lanka, *Spirulina* cultivation is still found to be in the experimental stage due to higher cost of production and low availability of bulk inorganic nutrients. However, there are possibilities to use alternative sources to substitute chemical nutrients in the growing media. The present investigation was carried out to identify possible low-cost alternative source of carbon which could substitute $NaHCO_3$ and Na_2CO_3 and thereby enhance the commercial production. The aim of the study was implemented by replacing the carbon source of the standard media with locally available alternatives sources such as: Table sugar, Cassava flour, Sweet potato flour and Taro flour.

2. Materials and Methods

2.1 Microorganism

The experiments were conducted at the Research Laboratory of the Department of Crop Science, Faculty of Agriculture, University of Ruhuna during the period of June to December 2018. The growth medium for the experiment was prepared according to Zarrouk (1966). The microalgae *Spirulina* maintained in the culture collection of the Faculty of Agriculture, University of Ruhuna was used to prepare the mother stock in conical flask (2 L) containing Zarrouk's medium (standard) and maintained at 32±2^{o} C, pH 8.5 with continuous illumination using 35 W fluorescent tubes.

2.2 Culture medium

Four experiments were conducted separately with replacing the carbon sources of $NaHCO_3$ and Na_2CO_3 in the Zarrouk's medium to develop a new low-cost medium using locally available alternative carbon sources: table sugar (C-42.12%), cassava flour (C-38%), sweet potato flour (C-20%) and taro flour (C-34.6%). The total amount of alternative carbon sources needed to prepare 1 L of growth medium by replacing $NaHCO_3$ (27.22 g L^{-1}) and Na_2CO_3 (8.06 g L^{-1}) were calculated as 11.20, 12.40, 23.56 and 13.61 g respectively.

Cassava, sweet potato and taro tubers were sliced and kept in an oven at 55^{o} C. Dried slices were milled using a universal millto to obtain powders which were then sieved using a 1 mm mesh to get the fine powder. Two liters of control solution (Zarrouk's medium) and alternative medium (table sugar, cassava, sweet potato and taro) were prepared separately for the experiment. The alternative carbon solutions were boiled at 100^{o} C to hydrolyse the carbon compounds. All the other chemical

ingredients except $NaHCO_3$ and Na_2CO_3 were added to the alternative solutions as in the standard Zarrouk's medium. Four different concentrations of alternative solutions (100%, 75%, 50% and 25% volume basis) were prepared by mixing with Zarrouk's medium. The Zarrouk's medium without any alternative carbon source served as the control (Table 1).

The culture medium (200 mL) was transferred to conical flasks (250 mL) and exponentially grown algal culture (20 mL) from the mother stock was inoculated separately into each conical flask. The Completely Randomized Design (CRD) was used with three replicates for the experiments. All the activities were carried out at room temperature. The pH of the culture media was adjusted to 8.5 and cultured at 33^o C under illumination of 4,000 Lux. The cultures were agitated daily by gentle hand shaking. The glass wares used in the experiments were first rinsed with water properly and finally rinsed with distilled water. After draining out the water, the glass wares were sterilised in an autoclave.

2.3 Growth of Spirulina

The biomass concentration (growth) was monitored by measuring the optical density values of the culture medium. The readings were recorded at three-day intervals up to sixteen days (6 readings). A sample (5 mL) of culture medium was taken via a micropipette and the optical density value was measured at 560 nm using a Spectrophotometer (model 7,305). Growth readings were taken 1st, 4th, 7th, 10th, 13th and 16th days after inoculation.

2.4 Statistical analysis and calculating dry weights

The data were analysed using one-way analysis of variance (ANOVA) with the help of statistical analysis system (SAS) version 9.1. Mean separation was done by Duncan's Multiple Range Test (DMRT).The optical density values were converted into dry weights (DW) using the equation 1 (r=0.9927) (Arunakumara and Xuecheng, 2007).

$$DW\ (g\ L^{-1}) = 0.207 + 0.960 \times OD\ value \quad (1)$$

where: OD is the relevant optical density value.

Table 1: Composition of the growth medium.

Experiment	Treatment	Zarrouk's medium (control)		Alternative carbon solution	
Table sugar (01) Cassava flour (02) S. potato flour (03) Taro flour (04)	T1	100%	(200 mL)	0%	(0 mL)
	T2	75%	(150 mL)	25%	(50 mL)
	T3	50%	(100 mL)	50%	(100 mL)
	T4	25%	(50 mL)	75%	(150 mL)
	T5	0%	(0 mL)	100%	(200 mL)

3. Results and Discussion

Despite no significant difference in OD values is found among different treatments at the time of inoculation, gradual increase in algal growth is reported as incubation progressed.

3.1 Table sugar as an alternative carbon source

At the end of 16-day incubation, the highest dry weight (0.747 g L^{-1}) was recorded from 25% replacement level (T2), while the lowest (0.419 g L^{-1}) was recorded from 100% replacement level (T5). The other replacement levels exhibited significantly ($Pr<0.05$) lower dry weights compared to the control (T1). The results further revealed that the dry weights of *Spirulina* increased with the incubation period. As T2 has resulted in the highest growth, 25% of the carbon in Zarrouk's medium could be replaced by the table sugar (Table 2).

An axenic culture of *Spirulina* grown on a mineral medium enriched with 1% peptone was reported a higher growth rate than the one grown on the photoautotrophic medium (Golmakani et al., 2012). Marquez et al. (1995, 1999) reported that in the presence of glucose *Spirulina* was able to grow

heterotrophically in aerobic dark conditions as well as mixotrophically in the light. Soundarapandian and Vasanthi (2008) investigated the effect of different carbon sources (D-glucose, mannitol, $(NH_4)_2CO_3$, sucrose and urea) on the growth of *Spirulina* and found that the biomass production and biochemical composition varied with the source of carbon. They observed significantly higher growth in Zarrouk's (Na_2CO_3) compared to the other replacements. The molasses, a by-product of the sugar industry containing 50% of sugar was proved to be a potential substrate for *Spirulina* (Lee and Kim, 2001). Andrade and Costa (2007) studied the effect of different concentration (0.25, 0.5, 0.75 g L^{-1}) of molasses using 0.15 g L^{-1} initial *Spirulina* inoculums and reported that the algal biomass increased up to the maximum productivity on the 3rd day of the incubation followed by a gradual decrease as incubation progressed. According to Joardan (2006), 500 g of sugar were needed to produce 1 kg of *Spirulina* biomass. He then suggested that cheaper sugarcane juice be used in the commercial cultivation process. The present study revealed that the dry weights of all the replacement levels were more or less same to the control solution until the 7th day of incubation and then a steady increase. In this study, no death of organisms is recorded as in previous reports (Soundarapandian and Vasanthi, 2008). However, the higher replacement levels of table sugar didn't support the growth of *Spirulina* possibly due to the nature of carbon in the medium.

Table 2: Effect of different levels of table sugar as an alternative carbon source on the growth of *Spirulina* measured as dry weights (g L^{-1}).

Level	1st day	4th day	7th day	10th day	13th day	16th day
T1 (Control)	0.273a	0.267d	0.279b	0.427ab	0.431b	0.732b
T2	0.271a	0.284a	0.287a	0.438a	0.715a	0.747a
T3	0.273a	0.279b	0.279b	0.412b	0.481c	0.520c
T4	0.272a	0.273c	0.257c	0.310c	0.355e	0.482d
T5	0.272a	0.273c	0.249d	0.301d	0.422d	0.419e

Means with same letters are not significantly different (Pr<0.05)

3.2 Cassava flour as an alternative carbon source

The highest dry weight (1.174 g L^{-1}) at the end of the 16-day incubation was recorded from 50% replacement level (T3) followed by 25% replacement level (T2), both of which are significantly (Pr<0.05) higher than the other replacement levels. Considerable reductions in dry weights were recorded from 75% (T4) and 100% (T5) replacement levels implying that cassava flour alone could not be used to substitute carbon source in Zarrouk's medium (Table 3).

Table 3: Effect of different levels of cassava flour as an alternative carbon source on the growth of *Spirulina* measured as dry weights (g L^{-1}).

Level	1st day	4th day	7th day	10th day	13th day	16th day
T1 (Control)	0.413a	0.426e	0.608d	0.713c	0.807c	1.124b
T2	0.424a	0.803c	0.858c	0.977b	1.079a	1.170a
T3	0.417a	0.789d	0.979a	1.114a	1.123a	1.174a
T4	0.424a	0.923a	0.944b	0.952b	0.916bc	0.900d
T5	0.434a	0.825b	0.941b	1.053b	0.948b	0.939c

Means with same letters are not significantly different (Pr<0.05)

Wei et al. (2009) conducted an experiment to determine the cell growth of *Chlorella protothecoides* under different concentrations of cassava starch and glucose solutions and concluded that the cassava starch is a good source of carbon to replace the carbon source in the standard medium. According to them, cassava starch contains glucose and α-amylase and glucoamylase optimised the efficiency of cassava starch utilisation resulting in higher biomass and total lipids. They further stated that use of cassava starch is more economical than the use of glucose. As explained by Agwa et al. (2014), cassava waste could effectively be used as a source of essential nutrients required for the microalgae cultivation. They formulated the growth media with both cassava effluent and peel and the growth characteristics were monitored as cell density (at 600 nm), lipid productivity and as dry matter.

The microalgae growth was increased in all the samples indicating that cassava waste is a good substrate for algal growth. They observed deep green coloration in the samples on 4th day followed by a slight reduction from the 6th day. According to them, cassava peel could produce more lipid than the effluent. The highest dry weight and lipid production were recoded as 5.71 g L^{-1} and 40.7 mg L^{-1} respectively. In the present study, *Spirulina* was grown in different concentrations of cassava flour solution. The dry weights of all the replacement levels increased up to the 4th day of incubation followed by a reduction which might be due to declining nutrient concentrations in the growth medium as incubation progressed. The maximum dry weight (1.174 g L^{-1}) was recorded in 50% replacement level implying that cassava has a positive impact on the growth of *Spirulina*.

3.3 Sweet potato flour as an alternative carbon source

As incubation progressed, gradual increase in algal growth was recorded in control (T1), 25% replacement level (T2) and 50% replacement level (T3) compared to the other replacement levels (T4 and T5) which showed negative effect on dry weight starting from the 4th day of incubation. The highest growth was recorded in T2 followed by T3. The significantly higher growth recorded in T2 compared to the other treatments implies that 25% of the carbon in Zarrouk's media could be replaced by the sweet potato flour (Table 4).

Table 4: Effect of different levels of sweet potato flour as an alternative carbon source on the growth of *Spirulina* measured as dry weights (g L^{-1}).

Level	1st day	4th day	7th day	10th day	13th day	16th day
T1 (Control)	0.266a	0.219d	0.283e	0.284d	0.311d	0.541c
T2	0.267a	0.580a	0.596a	0.663a	0.775a	0.862a
T3	0.267a	0.382c	0.568b	0.609b	0.675b	0.827b
T4	0.264a	0.524b	0.430c	0.398c	0.357c	0.318d
T5	0.268a	0.516b	0.393d	0.387c	0.342c	0.293e

Means with same letters are not significantly different (Pr<0.05)

A little information is available on the utilisation of sweet potato flour as a substrate for microalgae cultivation. Ren et al. (2015) studied the effect of different types of wastewater on the energy production of algae using the core culture system. Among the different type of waste waters (sweet potato, potato, cassava and corn starch), sweet potato waste water was reported to be the best substrate with the maximum hydrogen production and total lipids. Even though they have reported some positive impacts of sweet potato on algal growth, the present results declined any such possibility with the use of sweet potato. According to the present results, the higher replacement levels (75 and 100%) of sweet potato flour did not give any support for the algal growth which might be due to the difficulties in accessing the carbon source.

3.4 Taro flour as an alternative carbon source

The growth of algae treated with taro flour is found to be gradually increased in concentration dependent manner implying that taro flour could successfully substitute the carbon source in Zarrouk's medium. Taro flour at 100% replacement level (T5) is found to display the maximum dry weight (1.033 g L^{-1}) while control (T1) showed the lowest (0.303 g L^{-1}) at the end of 16-day incubation period. The significantly (Pr<0.05) higher growth recorded in T5 compared to the other treatments assured that 100% of the carbon in Zarrouk's medium could be replaced with taro flour (Table 5).

Table 5: Effect of different levels of taro flour as an alternative carbon source on the growth of *Spirulina* measured as dry weights (g L^{-1}).

Level	1st day	4th day	7th day	10th day	13th day	16th day
T1 (Control)	0.274a	0.230d	0.219e	0.226d	0.285d	0.303d
T2	0.276a	0.272c	0.299d	0.320c	0.393c	0.424c
T3	0.278a	0.360b	0.313c	0.341c	0.375c	0.420c
T4	0.275a	0.859a	0.751b	0.802b	0.902b	0.960b
T5	0.277a	0.847a	0.842a	0.842a	0.964a	1.033a

Means with same letters are not significantly different (Pr<0.05)

Cultivation of *Spirulina* using taro as a source of carbon has not yet been reported. However, Payne et al. (1983) stated that as taro flour contains 70-80% of starch, it could be used as an alternative source of carbon, which is confirmed by the present findings as well.

3.5 Cost analysis

$NaHCO_3$ and Na_2CO_3 are the inorganic carbon sources in Zarrouk's medium. The higher purchasing cost of these inorganic carbon sources is one of the barriers for large-scale cultivation as it accounts for nearly 15-25% of the total production cost. The present findings proved that $NaHCO_3$ and Na_2CO_3 in the standard media could be completely replaced by taro flour. The production cost of 1 L of Zarrouk's media is calculated as follows.

Price of 1 kg of $NaHCO_3$ = Rs. 2,057.31 (1 g = Rs. 2.05)
Price of 1 kg of Na_2CO_3 = Rs. 2,357.32 (1 g = Rs. 2.35)
Price of 1 kg of taro flour (including processing cost) = Rs. 450.00 (1 g = Rs. 0.45)

$NaHCO_3$
Amount of $NaHCO_3$ in 1 L of Zarrouk's medium =27.22 g
Cost of $NaHCO_3$ to produce 1 L of Zarrouk's medium =27.22 g*Rs. 2.05
=Rs. 55.80

Na_2CO_3
Amount of Na_2CO_3 in 1 L of Zarrouk's medium = 8.06 g
Cost of Na_2CO_3 to produce 1 L of Zarrouk's medium = 8.06 g×Rs. 2.35
=Rs. 18.94
Total cost to produce 1 L of Zarrouk's medium with $NaHCO_3$ and Na_2CO_3 =Rs. 55.00+18.00
=Rs. 73.00
Total cost to produce 10 L of Zarrouk's medium with $NaHCO_3$ and Na_2CO_3 =Rs. 730.00

Amount of taro flour needed to produce 1 L of Zarrouk's medium =1 3.61 g
Cost of taro flour to produce 1 L of Zarrouk's medium = 13.61 g×Rs. 0.45
= Rs. 6.12
Total cost to produce 10 L of Zarrouk's using taro flour = Rs. 61.24

Therefore, cost reduction for 10 L of Zarrouk's medium = Rs. (730.00-61.24)
= Rs. 668.76

4. Conclusion

The carbon sources ($NaHCO_3$ and Na_2CO_3) in the Zarrouk's medium could completely (100%) be replaced by taro flour solution. It reduces the cost of production significantly as well. Therefore taro flour could be considered as a viable low-cost alternative source of carbon. Cassava flour solution could also be used as an effective replacement while mixing with Zarrouk's medium at the ratio 1:1. Table sugar and sweet potato flour are found to be poor sources of carbon to replace the $NaHCO_3$ and Na_2CO_3 in the Zarrouk's medium. The results could be further confirmed by assessing the growth of *Spirulina* under different supplementary levels of taro and cassava flour.

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