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Development of Low-Cost Growing Media with Mungbean as A Source of Carbon for *Spirulina*

Desarrollo de medios de cultivo de bajo costo con frijol mungo como fuente de carbono para la *Spirulina*

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Abstract

Spirulina is a multicellular, photosynthesis, filamentous blue-green algae which is found naturally in a wide range of fresh, marine, and brackish waters. It is an excellent source of protein, vitamins, minerals, lipids, carbohydrates, and pigments thus considered as the "superfood" of the century. The commercial production of *Spirulina* depends on many factors such as nutrient availability, temperature, and light. Zarrouk's medium is known to be the standard media (SM) which contains all the macro and micro-nutrients required for the growth of *Spirulina*. The cost of nutrients is however found to be the second limiting factor next to labor cost affecting the commercial-scale production of *Spirulina*. The cost of carbon source is higher than that of N and P sources. Therefore, this study aimed at developing a low-cost medium for large-scale production of *Spirulina*. This intention was implemented by substituting carbon sources present in SM with cheaper and locally available carbon sources. Two separate experiments were conducted using a complete randomized design (CRD) with 3 replicates. The conventional carbon source in Zarrouk's medium ($NaHCO_3$) was substituted with low-cost carbon sources i.e., Mungbean flour (before and after germination separately). The carbon content in SM was replaced by 100 %, 75 %, 50 % and 25 % of Mungbean flour. Zarrouk's medium was used as the control. The culture was maintained at 30 ^{0}C ± 2 ^{0}C under 4000 Lux, continuous illumination using a white, fluorescent tube for 16 days. Growth was measured using a spectrometer and optical density (OD) values were recorded at 560 nm with two days interval. Data were analyzed using SAS version 9.4. The best growth of *Spirulina* was recorded at the 50 % replacement of carbon in SM by Mungbean flour (before germination). Mungbean flour (after germination) could also replace 25 % of carbon in Zarrouk's media. Taking the cost factor into consideration, 50 % replacement of carbon in Zarrouk's medium by Mungbean flour (before germination) can be recommended for commercial scale cultivation. The comparative cost reduction of this replacement is estimated to be 50 %.

Keywords: *Spirulina, Mungbean flour, low-cost, carbon sources*

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Resumen

La espirulina es un alga azul verdosa filamentosa, multicelular, fotosintética, que se encuentra naturalmente en una amplia gama de aguas dulces, marinas y salobres. Es una excelente fuente de proteínas, vitaminas, minerales, lípidos, carbohidratos y pigmentos, considerado por ello como el "superalimento" del siglo. La producción comercial de espirulina depende de muchos factores como la disponibilidad de nutrientes, la temperatura y la luz. Se sabe que el medio de Zarrouk es el medio estándar (SM) que contiene todos los macro y micronutrientes necesarios para el crecimiento de la espirulina. Sin embargo, se considera que el costo de los nutrientes es el segundo factor limitante, después del costo de la mano de obra, que afecta la producción a escala comercial de espirulina. El costo de la fuente de carbono es mayor que el de las fuentes de N y P. Por lo tanto, este estudio tuvo como objetivo desarrollar un medio de bajo costo para la producción de espirulina a gran escala. Esta intención se implementó mediante la sustitución de las fuentes de carbono presentes en SM con fuentes de carbono más baratas y disponibles localmente. Se realizaron dos experimentos separados utilizando un diseño completamente aleatorio (DCA) con 3 repeticiones. La fuente de carbono convencional en el medio de Zarrouk ($NaHCO_3$) se sustituyó por fuentes de carbono de bajo costo, es decir, harina de frijol mungo (antes y después de la germinación por separado). El contenido de carbono en SM fue reemplazado por 100 %, 75 %, 50 % y 25 % de harina de frijol mungo. Se utilizó el medio de Zarrouk como control. El cultivo se mantuvo a 30 °C ± 20 °C bajo 4000 Lux, iluminación continua utilizando un tubo fluorescente blanco durante 16 días. El crecimiento se midió usando un espectrómetro y los valores de densidad óptica (OP) se registraron a 560 nm con un intervalo de dos días. Los datos se analizaron utilizando SAS versión 9.4. El mejor crecimiento de la espirulina se registró con el reemplazo del 50 % del carbono en SM por harina de frijol mungo (antes de la germinación). La harina de frijol mungo (después de la germinación) también podría sustituir el 25 % del carbono en los medios de Zarrouk. Teniendo en cuenta el factor costo, se puede recomendar para el cultivo a escala comercial la sustitución del 50 % del carbono en el medio de Zarrouk por harina de frijol mungo (antes de la germinación). La reducción comparativa de costos de este reemplazo se estima en un 50 %.

Palabras clave: *Spirulina, harina de frijol mungo, fuentes de carbono, bajo costo.*

Introduction

Spirulina (*Arthrospira sp.)* spiral–shaped, edible filamentous cyanobacteria is commonly known as blue-green microalgae. (Jiang et al., 2021). *Spirulina*, a microalga that naturally grows in lakes, is known for its nutritional benefits and is widely recognized as a valuable source of nutrients. It is a type of unicellular blue-green microalgae that can be grown in large quantities with a high level of carbonate and bicarbonate in tropical and subtropical water bodies (Shen et al., 2021). The filaments are up to 500 mm long and have a diameter of 3–5 mm (Nosratimovafagh et al., 2023). In commercial cultivations, *Spirulina* can successfully be grown on freshwater, saltwater, and brackish water. It thrives in a pH range of 10-12, which is highly alkaline (Wijayanti et al., 2020).

Although this microorganism grows photosynthetically, an organic substrate can encourage its growth (Jung et al., 2022). *Spirulina* has a photosynthetic conversion rate of 8 % to 10 % (García-Lópeza & Olguína, 2020). The composition of microalgal biomass is highly determined by environmental conditions (Mulokozi et al., 2019). Mexicans have been using *Spirulina* for over 1000 years, dating back to the Aztec civilization. Dangeard, a French phycologist, documented the utilization of Spirulina as a food source by the indigenous people in the Lake Chad region, as reported by Ibrahim et al. (2023). The rediscovery of Spirulina by J. Leonard and Compere in the 1960s paved the way for commercial production in the late 1970s (Ibrahim et al., 2023). *Spirulina* is now commercially cultivated in several countries, with an annual production of a few hundred tons (Manogar et al., 2020). Because of its potential applications in human food additives,

animal feed, and pharmaceuticals, *Spirulina's* commercial production has gained a considerable attention (Torky et al., 2023). *Spirulina* has a huge potential not only for making conventional food but also for obtaining useful chemicals like β-carotene and phycocyanin. The use of Spirulina pigments as feed for tropical fish is popular in aquaculture. It's also used in agriculture and wastewater treatments (Matufi & Choopani, 2020). Vegetable production in most developed countries is insufficient to fulfill the population's dietary needs. *Spirulina* with its high content of β-carotene could be considered as a healthy substitute source of vitamin A (Fernandes et al., 2023).

Spirulina was considered as "the best food for tomorrow" by the United Nations World Food Conference, and it has gained attention as a food supplement in recent years. The WHO has called *Spirulina* "the greatest superfood on earth" (Ramírez-Rodrigues et al., 2021). *Spirulina* has a unique combination of nutrients that no other food can supply. Vitamin B-complex, nutrients, high-quality proteins, gamma-linolenic acid, and the super antioxidants, beta-carotene, vitamin E, and trace elements are all present in *Spirulina* (Suyoso et al., 2022). Every day, 40 000 children succumb to death due to malnutrition and its associated diseases, underscoring the urgent need for global efforts to address and alleviate this devastating public health issue (World Health Organization [WHO], 2013). One of the aims for growing *Spirulina* was to produce a new source of complete food supplement for a starving planet. One tablespoon per day has major health benefits (Al Hinai et al., 2019). The growth in the world's population and forecasts of a protein crisis in the early 1950s sparked a search for new complementary and unconventional protein sources (de Morais et al., 2019).

Spirulina spp. is well recognized for its richness in proteins due to its exceptional value when compared with other microorganisms. *Spirulina's* protein level is high and accounts for between 60 % and 70 % of the dry matter, more than fish (25 %) or soybean (35 %). The amino acids composition of the protein is balanced in *Spirulina* and more than 40 % of amino acids are found such as methionine, threonine, isoleucine, tryptophan, leucine, lysine, and valine. The composition of *Spirulina* is too low in sulfur-containing amino acids (Cysteine & methionine) which are essential for people who are malnourished, particularly children. *Spirulina* must be substituted with other amino acid-rich protein sources, it contains a significant amount of essential amino acids (Shao et al., 2019). Microalgae contain carbohydrates in the form of starch, glucose, sugar, and other polysaccharides (Álvarez & Otero, 2020). Carbohydrates represent approximately 15 % to 25 % of the dry weight of *Spirulina*. Both in *S. platensis* and *S. maxima*, the overall total lipid content is 5.6 % - 7 % of the dry weight. By using a better extraction method can obtain more than 11 % of lipids.

For several years, Zarrouk's medium has successfully utilized as the conventional and standard media for *Spirulina* culture (Fanka et al., 2022). It contains all the nutrients required for the growth of *Spirulina* including C, N, and P. In the standard media, $NaHCO_3$ is used as the source of carbon. The cost of carbon source is higher than phosphorous and nitrogen source. Therefore, high purchasing cost of carbon source is a limiting factor in large scale cultivation. Cost-effective *Spirulina* cultivation is necessary when large-scale cultivation is done for industrial purposes. Therefore, to reduce the cost of growing medium, ($NaHCO_3$) should be replaced with a cheaper and locally available carbon source.

The high cost of the growing medium limits large-scale production around the world, which is highly dependent on Zarrouk's medium. Due to the analytical grade materials used to produce the medium, the medium is expensive (Michael et al., 2019). When considering large-scale production for industrial purposes, it is important to grow *Spirulina* at a lower cost. After the labor costs, the cost of nutrients is the second most significant factor affecting the cost of *Spirulina* biomass production (Rodrigues et al., 2020). The major objective of this research is to identify a possible low-cost alternative source of carbon that could substitute $NaHCO_3$ in Zarrouk's medium. In this study carbon source was substituted with Mungbean flour (before and after germination).

Materials and Method

Experiment location

The research was conducted at the Research laboratory, Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Sri Lanka. The experiment was conducted for a five-month period starting from November 2022 to May 2023. The average annual temperature of the area is 30 ^{o}C, with an average humidity of 79 % and 1813 mm annual precipitation.

Mother stock

To prepare the mother stock the microalgae *Spirulina* was stored in the faculty's culture collection center. The mother stock was prepared in a 5-liter conical flask which was initially sterilized using the autoclave. Distilled water added to the conical flask was boiled and kept at room temperature for 24 hrs. until cooling. All constitutes of Zarrouk's medium were added to the conical flask (according to the calculated weight required for the 5-liter medium) and dissolved using a magnetic stir. After 24 hrs., the media was inoculated with 250 mL of *Spirulina* inoculum which was taken from the culture collection. Mother stock was maintained at 32 ^{o}C ± 2 ^{o}C, pH 8.5, with continuous illumination using 35 W fluorescent tubes which is equivalent to 300 to 500 lux.

Experiment design, experimental layout, data recording and sampling

Experiment 1: Preparation of low-cost media using Mungbean flour (before germination) as a carbon source. Experiment 2: Preparation of low-cost media using Mungbean flour (after germination) as a carbon source and Experiment 3: Preparation of growth enhancement media using coconut water. Fifteen conical flasks with the four modified concentrations and controls, which containing the Zarrouk's medium were placed according to Completely Randomized Design (CRD) with three replicates. Growth of *Spirulina* was measured using a spectrophotometer at 560 nm wavelength within two days intervals and optical density (OD) values were recorded. Samples were withdrawn from the culture flask with a pipette for spectrophotometer readings.

Amount of Mungbean calculation steps

The amount of Mungbean flour (before/after germination) needed to replace the $NaHCO_3$ in Zarrouk's medium was calculated as follows.

$NaHCO_3$

Carbon in $NaHCO_3$ = 14.29 %

Molecular weight of $NaHCO_3$ = 84 $g.mol^{-1}$

Amount of $NaHCO_3$ in Zarrouk's medium = 16.8 $g.L^{-1}$

Mole of $NaHCO_3$ = 16.8 $g.L^{-1}$/84 $g.mol^{-1}$

= 0.2 $mol.L^{-1}$

Amount of g of carbon = 0.2mol*12

= 2.4 g

Carbon in Mungbean = 63 %

The required amount of Mungbean = (100g/63g) *2.4 g

= 3.792 $g.L^{-1}$

Preparation of low-cost media

For the experiment, two liters of Zarrouk's medium (control solution) and Mungbean flour solution (before germination), after germination and coconut water were separately prepared. Distilled water was used for the preparation of all the medium. Experiment 1 - Sundried Mungbean was ground using a grinder and sieved using a 1mm sieve to get a fine powder. A solution was prepared with 7.584 g of Mungbean flour and boiled at 100 ^{o}C using the hotplate to hydrolyze the carbon compound. Four different concentrations of Mungbean (before germination) solution and the control solution are given in Table 1. Experiment 2 Mungbean flour (after germination of Mungbean seeds) were separately prepared. Mungbean seeds were soaked in water and kept until the radical emergence. After the emergence of radical 2-3cm length, seeds were dried at room temperature and ground using a grinder. The powder was sieved using a 1mm sieve and the solution was prepared with 7.584 g of flour. The solution was boiled at 100 ^{o}C using a hotplate to hydrolyze the carbon compound. Four different concentrations of Mungbean flour (after germination) solution and

Table 1. The composition of the Zarrouk's media with Mungbean flour (before germination), Mungbean flour (after germination) and coconut water

Treatment No	Control Solution	Experiment 1 Solution of Mungbean flour (before germination)	Experiment 2 Solution of Mungbean flour (after germination)	Experiment 3 Coconut water
T1 (00 %)	200 mL	000 mL	000 mL	000 mL
T2 (25 %)	150 mL	050 mL	050 mL	050 mL
T3 (50 %)	100 mL	100 mL	100 mL	100 mL
T4 (75 %)	050 mL	150 mL	150 mL	150 mL
T5(100 %)	000 mL	200 mL	200 mL	200 mL

control solution are given in Table 1. Experiment 3 - to prepare the growth media for *Spirulina*, the Zarrouk's medium was replaced by coconut water at the rate of 25 %, 50 %, 75 %, and 100 % on a volume basis. Two-liter conical flasks were used to prepare 100 % of Zarrouk's medium. Two-liter of coconut water was collected for the experiment. The pH of the coconut water was adjusted to 9 (Table 1).

Culture condition

Culture medium (200 mL) was transferred into 250 mL of conical flasks and 50 mL of algal culture (5×10^6 cells in the 50 mL culture) was inoculated separately for each conical flask. Physical conditions such as temperature, light, mixing rate were maintained at standard levels. The experiment was carried out with three replicates for 16 days at room temperature, under 4000 Lux white, fluorescent lamps, with daily handshaking.

Calculation of the Dry Weight (g/L)

The mean values from the statistical analysis were converted into dry weights using the equation, DW = Dry Weight (g/L), OD = Optical Density and (r=0.9927) (Arunakumara et al., 2007).

$$DW\ (g/L) = 0.207+0.960 * OD$$

Pigment analysis

Chlorophyll a and β-carotene content was determined using 90 % acetone method. A sample of 10 ml was kept in a tube with 10 mL of acetone and the tube was immersed in an ice –bath. An Ultrasonic - vibrator was used to break the cells. After the completion of the extraction process, visible two layers can be observed. 5 mL of acetone extract (upper layer) was taken and evaluated.

Chlorophyll a

The optical density was measured using a spectrophotometer at 663 nm and 645 nm wavelength to calculate the chlorophyll content. Optical density values were converted into the chlorophyll a (mg/L) content using the following equation,

$$\text{Chlorophyll a (mg/L)} = \frac{(12.7 * OD\ 663) - (2.59 * OD\ 645)}{\text{Culture volume taken (mL)}} * \text{Acetone volume (mL)}$$

β-carotene

The optical density was measured using a spectrophotometer at 450 nm wavelength to calculate the β-carotene content. Optical density values were converted into the β-carotene (mg/l) content using the following equation,

$$\text{Chlorophyll a (mg/L)} = \frac{(12.7 * OD\ 663) - (2.59 * OD\ 645)}{\text{Culture volume taken (mL)}} * \text{Acetone volume (mL)}$$

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) with the help of statistical analysis system (SAS) version 9.4 computer package, Mean separation was done with Dunnett's Multiple Range Test (DMRT).

Results

Spirulina spp. was cultured at 33 ^{0}C, under 400 Lux illumination for 16 days. On the 1st day of inoculation, no significant growth variations were found among different treatments (Pr = 0.2075). At the end of the 7th day of incubation, a significant reduction in the growth of the algae treated with T4 and T5 was evident. *Spirulina* that was in T4 and T5 died 13th days after incubation. However, as incubation progressed significant differences among other treatments could be observed (T1, T2, and T3). On the 16th day, T2 and T3 recorded the highest growth compared to the control (Table 2). Significantly higher growth was recorded in T2 and T3 compared to the control indicating that 25 %, 50 % of the carbon in Zarrouk's media could be replaced by the Mungbean flour (before germination) as a source of carbon (Table 3).

On the 1st day of incubation, the growth of *Spirulina* was not found to be varied among the different treatments (Pr = 0.1094). However, as incubation progressed significant differences among treatments were observed, implying that supplementation of Mungbean flour (after germination) as a source of carbon could be done. As incubation progressed, optical density value was gradually increased in T1 and T2 due to growth increment. On the 16th day of incubation, significant reductions in the growth of algae were observed in T4 and T5. On the 16th day, T2 recorded significantly higher growth compared to the control (Table 2) Significantly higher growth was recorded in T2 compared to the control, indicating that 25 % of the carbon in Zarrouk's media could be replaced by the Mungbean flour (after germination) as a source of carbon (Table 3).

As incubation progressed, optical density values gradually increased due to growth increment. T4 and T5 treatment showed the lowest optical density values compared to the control. T2 and T3 showed significantly higher optical density values compared to the control. According to the results, incorporating 5 to 10 percent replacement level of coconut water could be deemed a viable option for enhancing the growth of Spirulina. *Spirulina* (Table 2 and 3).

After the 16th day of incubation significantly higher chlorophyll a content was observed in T2 and T3 compared to the control. The lowest chlorophyll a content was recorded at control (T1). According to the results 25 % and 50 % of carbon in Zarrouk's media could be replaced by the Mungbean flour (before germination). After the 16th day of incubation significantly higher β-carotene content was shown in T2 compared to the control. T3 showed the second highest β-carotene content which was however not significantly different from the control. The lowest β-carotene content was shown in T1. According to the results, 25 % of carbon in Zarrouk's media could be replaced by the Mungbean flour (before germination) (Table 4).

After 16th day of incubation, significantly higher chlorophyll a content was recorded in T2 compared to the control. T3 showed the second highest chlorophyll a content. The lowest chlorophyll a content was observed in control (T1). According to the results, 25 % of carbon in Zarrouk's media could be replaced by Mungbean flour (after germination). After the 16th day of incubation, significantly higher β-carotene content was shown in T2 compared to the control. T3 showed the second higher β-carotene content. The lowest β-carotene content was shown in T1. According to the results, 25 % carbon could be replaced by Mungbean flour (after germination) (Table 4). It clearly shows the difference growth of Spirulina on different media is a significantly differ (Figure 1).

Discussion

One of the most important factors attributed to the growth and development of microalgae is the nutrition condition of the growing media. The present investigation was carried out with the objective of developing a low-cost media for culturing *Spirulina*. low-cost medium for large-scale production of *Spirulina* was implemented by substituting Zarrouk's medium with cheaper and locally available organic sources. Ragaza et al. (2020) assessed the ability to utilize glycerol as a source of carbon for *Spirulina* platensis

Table 2. Effect of Mungbean flour (before germination), Mungbean (after germination) and coconut as a low-cost alternative carbon source on growth of *Spirulina*

	1st day			4th day			7th day			10th day			13th day			16th day	
	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After
T1 Control	0.061	0.061	0.060	0.097	0.097	0.064	0.080	0.080	0.087	0.105	0.105	0.108	0.108	0.108	0.124	0.148	0.148
T2-25 %	0.066	0.063	0.063	0.072*	0.075*	0.392**	0.083	0.132**	0.315**	0.136**	0.151**	0.308**	0.164**	0.154**	0.380**	0.161**	0.158**
T3-50 %	0.063	0.062	0.065**	0.145**	0.092	0.395**	0.131**	0.102**	0.296**	0.133**	0.105	0.246**	0.259**	0.221**	0.231**	0.175**	0.114*
T4-75 %	0.062	0.067	0.069**	0.065*	0.070*	0.063	0.061*	0.118**	0.052*	0.042*	0.088	0.051*	0.023*	0.053*	0.050*	0.012*	0.041*
T5-100 %	0.065	0.066	0.068**	0.068*	0.069*	0.067	0.059*	0.108**	0.051*	0.038*	0.070*	0.047*	0.019*	0.051*	0.044*	0.016*	0.038*
Pr > F	0.208	0.109	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV	4.226	4.570	2.245	2.305	3.150	1.116	2.110	2.335	1.651	2.662	8.651	1.419	1.910	2.251	1.110	2.529	2.919

Growth was measured as OD value at 560 nm (Pr < 0.05) (N= 3)
**Means are significantly higher than the control
*Means are significantly lower than the control

Table 3. Dry weight (g/l) of *Spirulina* when Mungbean flour (before germination), Mungbean (after germination) and coconut as a low-cost alternative carbon source

	1st day			4th day			7th day			10th day			13th day			16th day	
	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After	Coconut	Mung Before	Mung After
T1 Control	0.265	0.265	0.265	0.300	0.300	0.268	0.284	0.284	0.290	0.307	0.105	0.310	0.310	0.310	0.326	0.349	0.349
T2-25 %	0.270	0.268	0.267	0.276*	0.279*	0.584**	0.286	0.133**	0.509**	0.338**	0.353**	0.503**	0.365**	0.355**	0.572**	0.362**	0.359**
T3-50 %	0.268	0.266	0.2694**	0.346**	0.295	0.586**	0.333**	0.102**	0.491**	0.335**	0.308	0.443**	0.456**	0.419**	0.429**	0.375**	0.317*
T4-75 %	0.266	0.271	0.2738**	0.270*	0.274*	0.267	0.266*	0.118**	0.257*	0.248*	0.291	0.256*	0.229*	0.258*	0.255*	0.219*	0.246*

Growth was measured as OD value at 560 nm (Pr < 0.05) (N= 3) Depicted values of relevant dry weight for the OD values in each treatment.
**Means are significantly higher than the control
*Means are significantly lower than the control

Table 4. Chlorophyll a content (mg/L) and β-carotene content (mg/L) of *Spirulina* treated with different levels of Mungbean.

Treatment	Chlorophyll a content (mg/L)		β-carotene content (mg/L)	
	Mung before	Mung after	Mung before	Mung after
T1 (0 % Control)	0.306	0.306	0.161	0.161
T2-25 %	0.347**	0.373**	0.208**	0.215**
T3-50 %	0.324**	0.319	0.168	0.172
Pr > F	<0.0011	<0.0004	<0.0007	0.002
CV	2.174	3.061	4.490	5.851

Chlorophyll a content was measured as OD value at 663 nm and 645 nm (Pr < 0.05) (N= 3)
β-carotene content was measured as OD value at 450 nm (Pr < 0.05) (N= 3).
**Means are significantly higher than the control

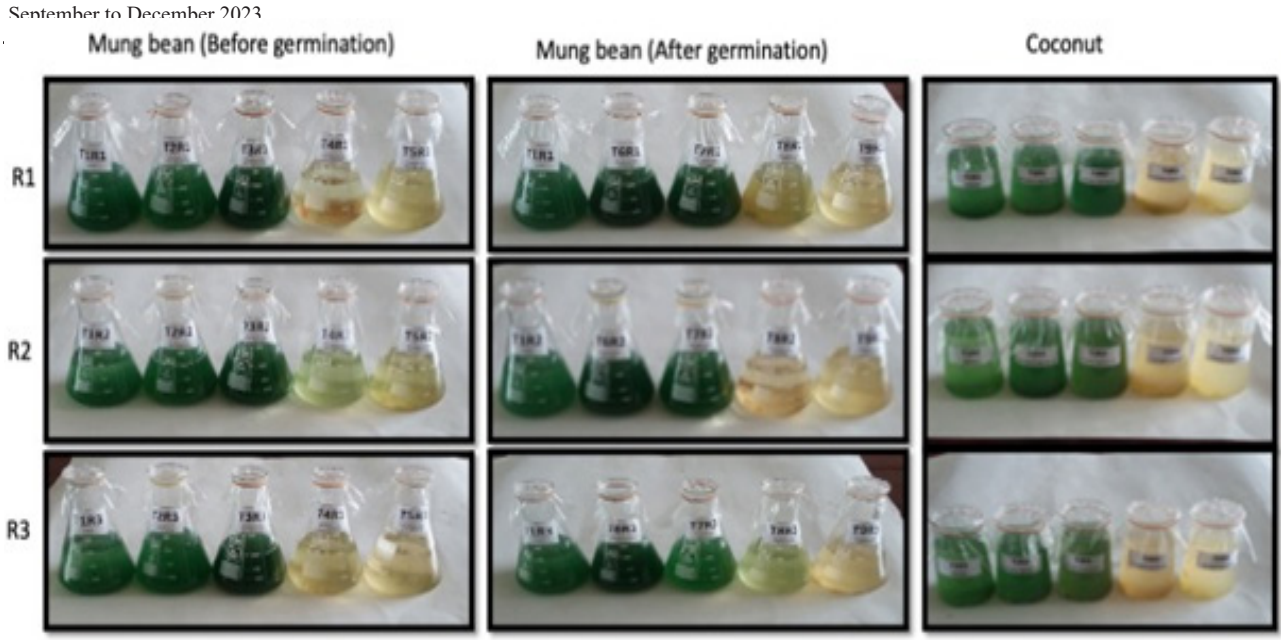

Figure 1. Photographic presentation of the effect of Mungbean (Before germination), Mungbean (After germination), and Coconut water on the growth of *Spirulin*a as a low-cost source of growth enhancement

(Ragaza et al., 2020). Glycerol was added as an alternative carbon source (2.5 mM^{-1} level) in the first experiment and sodium bicarbonate was reduced from 16.8 $g.L^{-1}$ to 2 $g.L^{-1}$. In the second trial instead of bicarbonate, glycerol was used as the sole carbon source with the concentrations ranging from 1.25 mM^{-1} to 12.

5 mM^{-1}. Results showed that *Spirulina* grown on glycerol medium decreased in pigment content of phycocyanin and chlorophyll-a compared to the control. The lipid content of the glycerol-grown groups was like that of the control, though there were differences in the fatty acid profile. The amount of g-linolenic acid (GLA) in the Neutral lipid (NL) fraction was also increased.

Sukanya et al., (2020) conducted a study with five cultures of *Spirulina* platensis using Na_2CO_3, D-glucose, Mannitol, $(NH4)_2CO_3$, $CaCO_3$, urea, and sucrose as a sole carbon source, and sodium bicarbonate containing growth medium served as the control (Sukanya et al., 2020). When $NaHCO_3$ in the standard Zarrouk's medium was substituted with Na_2CO_3 optimum biomass production was observed. Among these various carbon sources, Na_2CO_3 greatly increased biomass production, while $CaCO_3$ produced the least biomass production. The death of culture was detected when $NaHCO_3$ was substituted with some organic carbon source within 10 days of inoculation. According to them, insufficient quantity of carbon for the growth of *Spirulina* provided with an inorganic form of carbon explained the results. Also, they observed the maximum growth when Zarrouk's medium was replaced with glucose (2 $g.L^{-1}$) confirming that glucose can be used as a supplement to enhance growth, but not as a sole carbon source.

Many studies have shown that limitation in nutrients and/or organic carbon supplementation decreases the production of single high-value products from microalgal cultivation. Cultures grown mixotrophically with organic carbon sources typically found in agricultural and industrial waste streams (acetate, formate, glycerol, and oxalate) and under nutrient restriction did not show a clear pattern of growth. Supplementing glycerol and acetate to *Arthrospira platensis* cultured in media devoid of both N and P resulted in a substantial increase in biomass productivity relative to a photoautotrophic reference culture but had a negative impact on both phycocyanin and polyhydroxy butyrate production. According to the findings, a minimum amount of N is needed to maintain phycocyanin level and a higher concentration of the organic C supply is required to achieve a redox balance within the cells that

increase PHB production (Moradi et al., 2021).

Almomani & Bhosale (2021) conducted research to see whether the production of *Chlorella vulgaris* microalgal biomass and biochemical components including photosynthetic pigments, lipids, soluble carbohydrates, and proteins using glycerol and glucose as a complex carbohydrate substrate (Almomani & Bhosale, 2021). The findings suggested that *C. vulgaris* can use glycerol as a single carbon substrate though a lower effect than a combination of glycerol and glucose was reported. The effect of glycerol and glucose on the algal cell growth rate, biomass content, and volumetric productivity could compensate for the lower biomass production when glycerol is used as the sole organic carbon source in a mixotrophic culture medium.

Bandara & Arunakumara (2020) substituted the carbon content in Zarrouk's medium with different concentrations of (25 %, 50 %, 75 %, and 100 %) of alternate carbon sources. They observed the maximum dry weight (0.747 g/ L) from 25 % replacement level at the end of the 16-day incubation, while the lowest (0.419 g/L) dry weight at 100 % replacement level of table sugar. In this study, cassava flour was also used as a source alternative carbon source. The 50 % replacement level has produced the maximum dry weight (1.174 g/L) at the end of the 16-day incubation, followed by 25 % replacement level. Cassava flour which replaced the carbon source in Zarrouk's medium at 75 % and 100 % replacement level significantly decreased in dry weights implying that the carbon source in Zarrouk's medium could not be replaced by cassava flour at high replacement levels (Bandara & Arunakumara, 2020).

In the mixotrophic production of *Arthrospira platensis*, the effects of various organic carbon sources glucose, ethanol, and acetic acid at different concentrations (0.1 g/L, 0.5 g/L, 1.0 g/L for batch and 1.0 g/L, 2.0 g/L, and 3.0 g/L for fed-batch) were investigated by Mohammad et al., (2022). Increasing the concentrations of glucose, ethanol, and acetic acid in the batch media resulted in an improvement in biomass, lipid, and linolenic acid content. Carbon sources in fed-batch media at concentrations greater than 1.0 g/L reported no significant impact on the above parameters. It was also shown that biomass, lipid, and linolenic acid production using ethanol and acetic acid were like those found with conventional glucose-based culture media (Pérez-Juárez et al., 2022).

Al Mahrouqi et al., (2022) investigated the possibility of cultivating microalgae *Spirulina* platensis using molasses as organic substrate under mixotrophic cultivation (Al Mahrouqi et al., 2022). The effects of molasses concentration and light levels on mixotrophic biomass production by *Spirulina* platensis were investigated in this study. Zarrouk's medium supplemented with 0.25 g/L, 0.5 g/L, or 0.75 g/L of molasses. According to the results, increased molasses concentration favored biomass production, though it was unfavorable for the specific growth rate. After 11 days, the light intensity started to influence growth parameters but showed smaller effects than molasses concentration. These results highlighted the potential of molasses as an organic substrate for the mixotrophic cultivation of *Spirulina* platensis and the possibility of using low-cost agricultural by-products for the growth of this microorganism (AlFadhly, et al., 2022). Johny (2022) stated that cassava starch hydrolysate is a good source of carbon to replace carbon source in growing media (Jhony, 2022).

In the present study, higher replacement level of Mungbean flour (before and after germination) showed a significantly negative impact on the growth of *Spirulina* which might be due to the nature of carbon source and level of absorption. Higher replacement levels of Mungbean flour cause to change the pH of the growth media and create an acidic condition. Spirulina needs an alkaline condition for their growth (pH 8-9). Coconut water contains many micro and macro nutrients and hormones. Minerals such as Calcium, Copper, Magnesium, Manganese, Potassium, Sodium, Zinc, and hormones such as auxin, cytokinin's, gibberellins are among them. These elements are a good source for the growth of *Spirulina*. But higher replacement level of coconut water showed a negative impact on the growth of *Spirulina* possibly due to the acidic conditions created in the media.

Cost Analysis

$NaHCO_3$ is the inorganic carbon source present in Zarrouk's medium. The cost of production is higher due to the high purchasing cost of the inorganic carbon source ($NaHCO_3$). Therefore, replacing $NaHCO_3$ in standard medium with Mungbean is suggested. The production cost of 1L Zarrouk's medium with $NaHCO_3$ could be calculated as follows,

Price of 1Kg of $NaHCO_3$ = Rs. 2057.31 (1 g= Rs 2.05)

Price of 1Kg of Mungbean including processing cost = Rs. 300.00 (1 g= Rs 0.3)

Amount of $NaHCO_3$ in 1L of Zarrouk's medium = 16.8 g

Cost of $NaHCO_3$ to produce 1L of Zarrouk's medium = 16.8 g * Rs. 2.05
= Rs.34.44

Total cost to produce 10L of Zarrouk's medium with $NaHCO_3$ = Rs.344.4

To get the maximum economic benefit
Amount of Mungbean in 1L 0f Zarrouk's medium with 50% of replacement level of Carbon source = 1.89g

Cost of Mungbean with 50% replacement level = 1.89 * Rs. 0.3
= Rs.0.56

Amount of $NaHCO_3$ need to replace remain 50% of carbon amount = 8.4 g

Cost of $NaHCO_3$ to replace 50% carbon in Zarrouk's medium = 8.4 g * Rs.2.05
= Rs.17.22

Total cost to produce 1L Zarrouk's with 50% carbon replace = Rs.17.22+ Rs.0.56

Total cost to produce 10L of Zarrouk's medium with $NaHCO_3$ and Mungbean = Rs.177.8

Therefore, profit for 10L = Rs.344.4 - Rs.177.8
= Rs.166.6
= $ 0.5 ($ 1 = Rs 330)
= Percentage wise 50 %

Conclusion

Alternative carbon sources prepared with Mungbean seeds (before germination) can be used in replacing 50 % carbon in Zarrouk's medium. Alternative carbon sources prepared with Mungbean seeds (after germination) can be used in replacing 25 % carbon in Zarrouk's medium. Comparatively high chlorophyll-a and β-carotene contents can be obtained from 25 % replacement level of carbon with Mungbean flour (before and after germination). Taking the economic benefits into account, 50 % replacement of carbon in Zarrouk's medium by Mungbean flour (before germination) can be recommended for the commercial cultivation of Spirulina. *Spirulina*.

Recommendation

Growth of *Spirulina* under alternative carbon sources should be further evaluated with their different concentrations.

Conflicts of Interests

The authors assert that there are no conflicts of interests. Additionally, all authors have approved the final version of the manuscript.

Author contribution statement

AOTT, KKIUA and FMMTM: Conceived and designed the experiments. AOTT and KKIUA: Performed the experiments, KKIUA and FMMT: Finalized the manuscript.

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