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CULTURE CONDITIONS OF SPIRULINA IN FRESHWATER MEDIUM AND ITS EFFECT ON CELL GROWTH

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Abstract

Spirulina is a multicellular and filamentous microalgae that is considered a promising protein source alternative and absolute food and feed supplement for growth and nutrition. Optimizing the culture conditions of Spirulina is detrimental to maximizing biomass yield and minimizing cost by modifying the commercial culture media. This study was conducted to determine the chemical culture conditions *of Spirulina platensis* concerning biomass yield. Chemical conditions of the liquid culture such as the pH level and salt concentration of the cultivated Spirulina were determined. Moreover, the cell length (µm) of Spirulina as affected by pH and salt concentration was measured using Image J. The optimum quality and quantity of DNA as affected by preparation was determined. Results revealed that Spirulina thrived in alkaline conditions and significantly yielded a biomass of 75.43g and 67.47g at pH 8 and 9, respectively. The fresh weight harvest yield was significantly best observed at 1% salt concentration (w/v). In preparing and extracting the DNA of Spirulina using the modified CTAB DNA Extraction protocol, the Non-grinded sample of the DNA yielded the highest nucleic acid concentration of 437.8 ng/µl and had rendered pure DNA quality with an A260/280 ratio of 1.95 nm absorbance as compared with the ground samples. The size in length (µm) of the rod-shaped Spirulina cells cultivated in freshwater media was significantly long (626.75 µg and 494.64 µg) when cultivated at pH 8 and 9, respectively. Similarly, the spiral-shaped Spirulina cells were significantly long at pH 8 (356.67 µm) and pH 9 (348.53 µm). Moreover, the size length of the rod-shaped Spirulina cells is longer when cultivated at 1% (w/v) salt concentration with a length of 568.75 µm while the spiral-shaped cells were significantly long at 5% with a length of 193.68 µm.

Keywords: Spirulina, Culture Media, Production, Microalgae

INTRODUCTION

Cultivation and production of Spirulina have gained so much interest among researchers, industries, and other stakeholders due to its cardinal role in human and animal nutrition, environmental protection, bio-circular economy, food, and feed industry, aquaculture, nutraceutical, and pharmaceutical application (Saranraj, et al, 2014; Soni, R.A. 2017; Lafarga et al., 2021). Its wide array of content of excellent nutritional values like high protein, vitamins, and minerals has made spirulina useful in many nutritional and industrial applications In addition, this photosynthetic, multicellular, and filamentous blue-green algae is easy to grow and harvest.

Much progress has been achieved during the past decade in developing appropriate technology for microalgal mass cultivation. Many studies on optimizing the culture condition such as the chemical culture media, light intensity requirement, ambient temperature, aeration, agitation, etc. have been conducted to obtain maximized algal biomass yield at a considerable input cost. Spirulina, under controlled conditions, grows best using Zarrouk's medium. It is the standard substrate used for growing Spirulina however it incurs higher costs due to the expensive

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components needed in concocting the medium. Hence, studies of finding the alternative cost-effective source of modifying Zarrouk's medium either one or more of its components have been and are continuously being explored. Despite the enormous information on the optimum culture condition of Spirulina that will maximize its biomass yield, little is reported on the effect of the pH level and salinity concentration on the growth (cell length) of the Spirulina. This study has attempted to determine the optimal pH level and salt concentration of the culture medium using freshwater and its effect on the cell growth (length in μm) of Spirulina. Moreover, the DNA extraction method of Spirulina cell that will yield a suitable good quality and quantity of DNA was also explored.

METHODOLOGY

Cultivation Conditions

The strain of Spirulina was maintained in Zarrouks's medium at ambient temperature with 12 h light and 14 h dark photoperiod using a standard light and manually aerated in the flask. All reagents used for preparing the media were technical grade.

Experimental Set up

Five (5) liters of freshwater were placed in ten (10) –liter laboratory open glass containers. An anti-chlorine reagent was added to the water to ensure the removal of the chlorine which is detrimental to the survival of the Spirulina culture. The anti-chlorine reagent was left to be mixed in the water for an hour. Air pumps and accessories such as air hose, hose clamp, and air stone were assembled and installed in the experimental set-up as source of oxygen supply. After some time, carbonates and chloride salts were added to the freshwater to obtain the desired pH levels and salt concentrations. Thorough mixing was employed to ensure proper dissolution of the salts. When careful mixing of the liquid media was done.

Experimental Procedure

Effects of various pH levels and salt concentrations were determined by fresh weight of Spirulina biomass. The optimum quality and quantity of Spirulina through modified CTAB DNA Extraction protocol and the cell length through Image J application.

Inoculum. A concentration of 10 g/l of Spirulina scum were added into the labeled containers representing various pH levels and salt concentrations.

Lighting. Since Spirulina requires lots of sunlight, the experiment was set up outside the laboratory where the right amount of sunlight with minimum direct radiation was ensured. An optimum of 1000 -4500 lux is the best range for growth of spirulina (Sukenik et al., 1991).

Aeration system: Proper aeration is important to meet the CO2 requirement and prevent algae to settle down and form layer at the bottom. Aeration was achieved using manual stirring 3x a day and continuous air pump system. pH and temperature measuring device: PCSTestr 35 multi-parameter instrument and pH strips are used to determine pH value and temperature of media.

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Harvesting. After a month of cultivation, (while maintaining 5L in each of the containers by adding more water), the algal scum was harvested using fine cheese cloth, and the fresh weight was measured for data analysis

DNA Extraction Protocol

Three hundred milligrams (300mg) of pure Spirulina cells were placed in an Eppendorf tube labeled as grinded and non-grinded using a pipette tip. Prior to the grinding of the cells, liquid nitrogen was added to the tube labeled as grinded and none to the non-grinded tube. The tubes were placed in an ice box to ensure the integrity of the cells and the DNA as well. The 600µl of pre-warmed (at 65°C) cationic detergent, Cetyl Trimethyl Ammonium Bromide (CTAB) buffer, then was mixed by inverting tubes several times vigorously. The tubes were then incubated for at least 30 minutes at 65°C using a dry bath. The samples were cooled a bit before adding an equal volume of phenol-chloroform isoamyl alcohol, 24:25:1, and then mix by inverting the tubes several times vigorously.

The samples were incubated for 5 minutes leaving the tubes laid down on the bench, then centrifuged at 13000 xg for 10 minutes at room temperature. The supernatant was transferred to a new 2-ml microfuge tube this time, an equal volume of chloroform isoamyl alcohol, 24:1 was added. The tubes were then mixed by inverting the tube several times vigorously and incubating for 5 minutes leaving the tubes laid down on the bench. After which, the tubes were again centrifuged at a speed of 13000 rpm for 10 minutes at room temperature. The aqueous phase was transferred to a new 1.5 microfuge tube before adding 5µl RNAse a (10mg/ml) and was then incubated for 30 minutes at 37°C. An equal volume of cold 2-Propanol was added, then gently mixed by inversion, and was incubated once more at room temperature for 15 minutes. Tubes were again centrifuged at 13000 xg for 5 minutes. The supernatant, before adding 1ml of 70% ethanol (to wash the DNA pellet) was shaken and tubes were tapped to dislodge the DNA pellet. Once more, the tubes were centrifuged once more at 13000 xg for 2 minutes. Ethanol was decanted and tubes were re-centrifuged for 20-30 seconds to bring down traces of ethanol before removing the remaining liquid using a pipette, taking care not to disturb the pellet. The DNA pellet was allowed to dry for 10-15 minutes (avoid over-drying as the pellet will be hard to dissolve) in the fume hood. The DNA pellet was reconstituted in 70µl TE buffer (1x comprised of Tris-HCl and EDTA, pH 8.0). At this point, the samples were kept on ice. The quality and quantity of the 2µl genomic DNA were checked and recorded using the Nanodrop. The samples were stored in a -20°C freezer for further use.

Measuring the Length of Spirulina Cell

A drop of Spirulina culture, from each replicate per treatment was placed on a glass slide and was covered with a coverslip to keep the specimen flat while under microscopic examination. Using a compound microscope (Olympus) and accessories, the pictures of the Spirulina cells were taken at ROI conditions of Height – 800 µm, Width - 800µm, 20x magnification optics, and 30ms. The images taken were measured using the Image J Application. Before, measuring, a calibrated image was used to standardize the calibration. Ten images per treatment of both the rod-shaped and spiral-shaped were randomly taken and measured.

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Determination of quantity and purity of Spirulina DNA Concentration

DNA concentration was quantified using Nanodrop (ND-100) spectrophotometer. To calibrate the Nanodrop, about 1 µl of molecular biology grade water was pipetted onto the nozzle of the machine while the arm was closed to initiate spectral measurements using the operating software in the computer. When a prompt of "load your sample" on the screen was flashed, 1µL of DNA samples was pipetted on the nozzle while the arm was closed to determine the DNA concentration sample expressed in ng/µL. The purity of the DNA was determined by the A260:A280 (1.8-2.0). The quantity and quality of the DNA were detected by spectrophotometer while the integrity was validated using 1% agarose gel electrophoresis stained with SYBR safe.

DNA Analysis by Agarose Gel Electrophoresis

The genomic DNA was analyzed using 1% agarose gel electrophoresis in 1X TAE buffer (0.04M Tris-HCl pH 8.0, 0.04M acetic acid, 0.001M EDTA pH 8.0) as described by Sambrook et al. (1989). A 10µL sample was loaded with 5 µL loading dye (0.25% bromphenol blue and 30% glycerol) and was loaded in the gel wells. Electrophoresis was performed at a constant 130 V for 45 min. After electrophoresis, the gel was visualized by UV light trans-illumination, with Flour S™ MultiImager (Bio-RAD). The size of the DNA was compared against the 1 Kb ladder, of which 8 µL will be loaded in the gel before the samples.

RESULTS AND DISCUSSION

Determination the pH Levels and Salinity Concentration of Spirulina Culture

Table 1 shows the fresh weight of the Spirulina in grams in different pH levels, 8, 9, and 10, respectively.

Table 1: Fresh weight of Spirulina in different pH Levels

Treatment	Fresh weight (g)
T1 – 8	75.43 *a*
T2 - 9	67.47 *ab*
T3 – 10	59.43 *b*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD.

Spirulina best thrives in alkaline and saline solution (Vonshak, 1997). However, how alkaline and how saline the liquid culture media is posing a concern of interest. Based on the results, pH 8 significantly rendered the highest yield (75.43g) of fresh Spirulina biomass, although it is equally comparable with the biomass harvested in a culture media of pH 9 (67.47). Bicarbonate salts increase the pH level of the freshwater liquid media. Spirulina feeds on the bicarbonates which explains the alkaline requirement in the liquid media. However, increasing the pH level of the media causes apoptosis. This may be attributed to the hypertonic reaction of the Spirulina cells.

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Table 2: Fresh weight of Spirulina in different salinity concentrations (% w/v)

Treatment	Fresh weight (g)
T1 – 1%	59.73 *c*
T2 – 3%	69.97 *b*
T3 – 5%	84.30 *a*
T4 – 8%	0.00 *d*
T5- 11%	0.00 *d*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD

Data in Table 2 reveals that Spirulina cells thrive in saline freshwater conditions. In addition, results show that from among the salt concentrations, the Spirulina biomass is significantly high at 5% salt concentration. The data denotes that the 5% (w/v) salt concentration is the optimum salt requirement for Spirulina cell growth. Obviously, when the salt concentration is increased from 8% and above, cell death of Spirulina is observed. Apoptosis may be attributed to the osmotic reaction of the cell in which in this case, a hypertonic reaction is observed.

Determination of the length of Spirulina based on the Different pH levels

Table 3 shows the lengths (µm) of the rod-shaped and spiral-shaped of Spirulina cell cultivated in different pH levels.

Table 3: Size in length (µm) of t rod-shaped and spiral-shaped of Spirulina cell cultivated in different pH levels

pH Level	Rod-shaped Spirulina Cell (µm)	Spiral-shaped Spirulina Cell (µm)
T1- 8	626.75 *a*	356.67 *a*
T2 - 9	494.64 *a*	348.53 *a*
T3 - 10	240.44 *b*	135.08 *b*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD

Spirulina platensis is morphologically rod-shaped or spiral-shaped. In this study, both forms were observed (Figure 1). Whether both forms were of the same species or not, that information is not included in this study. Although there are images that may provide information that both forms are of the same species; the rod-shaped Spirulina cell during the log/exponential growth phase and becomes spiral when it reaches the mature and reproductive stage (Figures 2 and 3). Interesting findings these may seem. However, until proven with substantial evidence, then this study may be the first to report such a phenomenon.

The length of both forms was measured using the Image J Application. Based on the results, the rod-shaped Spirulina cells are significantly longer in pH 8 and 9. This result may explain the highest yield of Spirulina biomass that was harvested as shown in Table 1. In terms of the Spirulina spiral-shaped cell growth (length), pH 8 and 9 also rendered the optimum growth of Spirulina cells.

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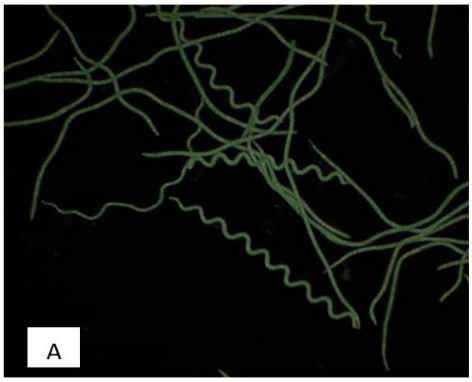

Figure 1A: Rod-shaped and Spiral-shaped Spirulina cells at 10x magnification

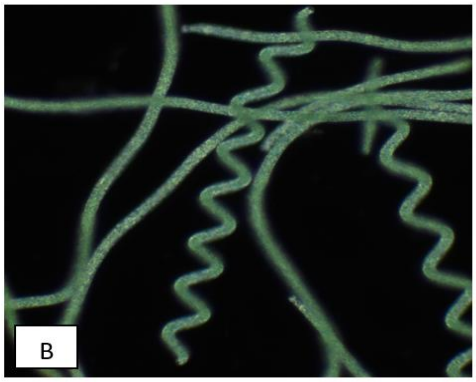

Figure 1B: Rod-shaped and Spiral-shaped Spirulina cells at 20x magnification

Figure 2: Rod-shaped Spirulina cells at 20x magnification

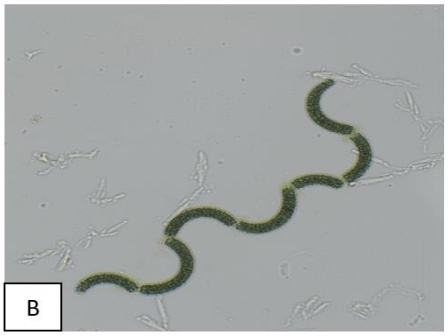

Figure 3: Fission of spiral-shaped Spirulina cells at reproductive stage at 20x magnification

Determination of the size in length of Spirulina based on the different salinity concentration

Table 4 depicts the size in length of Spirulina cells cultivated in different salt concentrations for both the rod-shaped and the spiral-shaped Spirulina cells.

Table 4: Size in length (µm) of t rod-shaped and spiral-shaped of Spirulina cell cultivated in different salinity concentration (% w/v)

Salinity Concentration (%w/v)	Rod-shaped Spirulina Cell (µm)	Spiral-shaped Spirulina Cell (µm)
T1- 1%	568.75 *a*	140.78 *b*
T2 – 3%	239.69 *b*	128.28 *b*
T3 – 5%	224.35 *b*	193.68 *a*
T4 – 8%	0.00 *c*	0.00 *c*
T5 – 11%	0.00 *c*	0.00 *c*

Legend: Means followed by a common letter are not significantly different at 5% level of LSD

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Results in Table 4 reveal that the cell growth of rod-shaped Spirulina in length (µm) is significantly high at 1% salt concentration while 5% with that of the spiral-shaped Spirulina cells. The data may depict that since Spirulina feeds on bicarbonate salts, additional chloride salts may seem to supplement the bicarbonates which is why the cell growth of the rod-shaped Spirulina requires a minimal amount of other sources of salts. In the instance of the spiral-shaped Spirulina cell, this form required a much higher concentration of salts (5%) than that of the rod-shaped. If this study hypothesizes that the spiral-shaped is the reproductive stage, hence it can be interpolated that the additional salts source is much needed in preparation for the cell fission of the Spirulina (Figure 3).

Quantity and Quality Analysis of Genomic DNA of Spirulina

Table 5 shows the quality and quantity of the DNA of Spirulina using spectrophotometer and Agarose Gel Electrophoresis.

Table 5: Quantity and Quality of the Genomic DNA of Spirulina between Grinded and Non-grinded preparation of Spirulina Cell

Spirulina Cell Preparation	Nucleic Acid Concentration (nm/µL)	A260/A280 Absorbance (nm)
T1- Grinded	116. 5	1.95
T2 – Non-Grinded	437.8	1.94

The table shows the purity of the DNA extracted from Spirulina cells. Between the two preparations, the non-grinded yielded a greater amount of nucleic acid concentration as compared with the grinded. The grinding may have ruptured the cell wall as well as the nucleus where the DNA is found which is why the amount of the nucleic acid is lower than that of the non-grinded. In terms of the quality of the DNA, the purity of the DNA from both preparations is almost pure or of good quality as the DNA A260/280 ratio obtained was at the absorbance of 1.95 nm for the grinded and 1.94 nm for the non-grinded preparation. When the A260/280 ratio of DNA is ~ 2.0, the DNA is considered pure. These ratios are commonly used to assess the amount of protein contamination that is left from the nucleic acid isolation process since proteins are absorbed at 280 nm.

CONCLUSIONS

Spirulina platensis, a filamentous blue-green algae that is cultivated in the Tarlac Agricultural University thrived in an alkaline and saline freshwater medium, which is similar to other *Arthrospira sp*. That is found worldwide. Two forms of Spirulina cells, rod-shaped and spiral-shaped cells were observed under a microscope. This finding suggests a hypothesis that these two forms may be of the same species and represent two developmental stages. Based on microscopical studies, the rod-shaped Spirulina cells may be in the log or exponential phase whereas the spiral-shaped cells may be in the reproductive or lag phase. However, until this hypothesis is further substantiated, no conclusive findings may be reported. Paradoxically, if this finding may be proven true, then this study is the first to report this phenomenon based on our knowledge. Despite the differences in forms, both exhibit optimal growth at pH 8 and 9 and 1% and 5% (w/v) salt concentration. The preparation of Spirulina cells for DNA extraction

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plays a crucial role. Mechanical stress like grinding using pipette tips may not seem to yield a high amount of nucleic acid concentration, although it gives a pure DNA A260/280 ratio. The denaturing agent is sufficient to rupture the cell wall of the Spirulina cells.

Conflict of interest

The author declares No conflict of interest to this manuscript.

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