COMPARATIVE ANALYSIS OF *Spirulina platensis* GROWTH BY APPLYING UREA AS A VIABLE NITROGEN SUBSTITUTE FOR Na- NITRATE

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This is to certify that the thesis entitled ***"COMPARATIVE ANALYSIS OF Spirulina platensis GROWTH BY APPLYING UREA AS A VIABLE NITROGEN SUBSTITUTE FOR Na- NITRATE"*** *submitted to the Department of Horticulture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE in HORTICULTURE****, embodies the result of a piece of bona fide research work carried out by* ***IFAZ MD ISLAM,*** *Registration No****. 12-05034*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that any help or source of information, received during the course of this investigation has been duly acknowledged.

_____

Dated: ***(Prof. Dr. A.F.M Jamal Uddin)***

Dhaka, Bangladesh ***Supervisor***

DEDICATED TO-

MY BELOVED MOTHER

To whom I owe every fiber of my being

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COMPARATIVE ANALYSIS OF *Spirulina platensis* GROWTH BY APPLYING UREA AS A VIABLE NITROGEN SUBSTITUTE FOR Na- NITRATE

ABSTRACT

An experiment was conducted in the 2a Biotech Laboratory of Horticulture Department of Sher-e-Bangla Agricultural University during the period of 1 March 2018 to 15 March 2018 to observe the growth and yield performance of *Spirulina platensis*. *Spirulina platensis* was allowed to culture in standard Zarrouks Media (T_1) and three different concentrations of Modified Zarrouks Media viz. Zarrouks Media containing 0.01M urea (T_2), Zarrouks Media containing 0.02M urea (T_3) and Zarrouks Media containing 0.04M urea (T_4). This experiment was arranged in a Completely Randomized Design. Fifteen days of production was carried out and to find out a promising Spirulina production technology. The growth rate of *Spirulina platensis* was found to vary in different concentrations of urea solution. The basic concentration of 4mg/L of inoculum gained maximum cell weight (607mg/L) in Zarrouks Medium (ZM) and is significantly ($p>0.05$) higher than the maximum cell weight (454mg/L) found in Modified Zarrouks Medium (MZM) containing 0.01M of urea solution (T_1); 0.02M of urea solution (T_2) and 0.03M of urea solution (T_3) respectively on the 12^{th} day of the culture period. Similar trend was also observed in the case of chlorophyll a content and optical density (OD) measurement of *Spirulina platensis* as well. T_2 showed the best result in the given growth parameters such as specific growth rate (0.086), OD (0.66mg/L) and chlorophyll a content (7.133mg/L) in comparison with treatments utilizing the urea solution viz. T_2, T_3 and T_4. Quality Spirulina production technology can be obtained by using low dosage of urea solution in comparison to other standard type of Spirulina production technology. These findings may be a source of valuable information for a cheap and quality Spirulina production system in Bangladesh by using urea as the readily available nitrogen source.

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LIST OF ACRONYMS

AEZ	= Agro-Ecological Zone
As follows	= viz.
BAU	= Bangladesh Agricultural University
BBS	= Bangladesh Bureau of Statistics
CV%	= Percentage of coefficient of variance
DAE	= Department of Agricultural Extension
DAI	= Days after inoculation
et al.	=And others
FAO	= Food and Agriculture Organization
g	= gram
ha^{-1}	= Per hectare
Journal	= *J.*
kg	= Kilogram
LSD	= Least Significant Difference
Max	= Maximum
Meter	= m
mg	= milligram
mg/L^{-1}	= milligram per litre
Min	= Minimum
MZM	= *Modified Zarrouks Medium*
N	= Nitrogen
No.	= Number
NS	= Not significant
SAU	= Sher-e-Bangla Agricultural University
That is	= *i.e.*
WHO	= World Health Organization
ZM	= *Zarrouks Medium*

CHAPTER I
INTRODUCTION

Spirulina (*Spirulina platensis*) belongs to the family *Arthrospira* is a free floating filamentous microalgae belonging to the class Cyanobacteria (Komárek and Hauer 2009). It has two genera namely *Spirulina* and *Arthrospira* with characteristic photosynthetic ability (Sapp, 2005). Spirulina was primarily classified in plant kingdom but was later placed in the bacteria kingdom based on new understandings on the genetics, biochemical properties and physiology (Vonshak, 1997).

It's native to Central Africa, Mexico, some parts of Asia and America. Spirulina naturally grows on high salt alkaline water reservoirs found across these areas (Vonshak, 1997, Gershwin and Belay, 2007). Spirulina was first known to the modern world by a European scientific mission conducted in Chad. Spirulina or "Dihe" as the Chadians call it has been found growing in the alkaline lagoons scattered all around Chad. Chadians have been taking it as food for several centuries (Abdulqader *et al*., 2000). Later studies show that Mayans, Toltecs, Kanembus during the Aztec civilization had incorporated Spirulina in their diet 400 years ago (Gershwin and Belay, 2007).

During the sixtieth session of the united nations general assembly (second committee, agenda item 52), a revised draft resolution on the "Use of Spirulina to combat hunger and malnutrition and help achieve sustainable development" was submitted by Burundi, Cameroon, Dominican Republic, Nicaragua and Paraguay. As follow up of this resolution, FAO was requested to prepare a draft position paper on Spirulina so as to have a clearer understanding on its use and to convey FAO position on this.

Spirulina has been used as a complementary dietary ingredient of feed for fish, shrimp and poultry and increasingly as a protein and vitamin supplement to aquafeeds. China is using this micro-alga as a partial substitute of imported forage to promote the growth, immunity and viability of shrimp. There has also been comprehensive research on the use of spirulina as aquaculture feed additives in Japan and many other countries. Today, Spirulina is commercially cultivated in several countries, with a total annual production

of a few hundred tons. In addition, some Spirulina products are fortified with extracts of various herbs as well as some vitamins and minerals and sold as a relief for premenstrual syndrome in different countries. Moreover, it offers the possibility of obtaining fine-chemical products such as pigments, lipids, polyunsa- turated fatty acids, polysaccharides, carotenoids, steroids and vitamins (Cohen and Vonshak, 1990; Mahajan and Kamat, 1995; Cohen, 1997; De Philippis and Vincenzini, 1998). For these purposes, research work has been carried out since the early 1950s, especially using the algal genera *Chlorella*, *Scene- desmus* and *Dunaniella* (Vonshak, 1997; Borowitzka, 1999).

Spirulina provides all essential nutrients without excess calories and fats. It is recommended to control obesity and premenstrual stress. Athletes take Spirulina for instant energy. Many herbal cosmetics like face creams biolipstics, hair lotion have been formulated from phycocyanin pigment found in Spirulina. The beta carotene and other carotenoids are having a suggested role in the control of cancer in human and enhancement of pigmentation of eggs, meats and coloration of ornamental fish.

There are a large number of Spirulina species; among them three species of Spirulina namely *Spirulina platensis*, *Spirulina maxima* and *Spirulina fusiformis* are intensively investigated as these are edible and has high nutritional and medicinal properties (Vonshak, 1997, Gershwin and Belay, 2007 Khan *et al*., 2005, Karkos *et al*., 2008).

Spirulina mainly grows in brackish water conditions with the correct chemical balance. It grows well between pH 8-11. Spirulina grows substantially in 20^0C temperature but the growth of Spirulina thrives between 35^0C -37^0C. The growth is seriously hampered if the temperature is above 38^0C. Spirulina growth is also detrimental in low temperature. It requires ample sunlight to thrive as it an autonomous organism but 30% of sunlight is perfect for the growth and development.

The conventional nitrogen source for *S. platensis* is nitrate (Zarrouk, 1966; Paoletti *et al*., 1975; Schlo¨sser, 1982); nevertheless, interesting research work was carried out on using animal wastes (Olguı´n *et al*., 2000) and urban effluents (Bustos Aragon *et al*1, 1992) as low-cost nitrogen sources. *Spirulina sp*. cultivation can then be considered as a promising

alternative for nitrogen and phosphorus removal from wastewater (Chuntapa *et al*., 2003; Olguı´n *et al*., 2003).

The use of urea (U) as source of nitrogen for S. platensis in batch (Stanca and Popovici, 1996) and fed-batch cultures (Danesi *et al*., 2002) increased biomass production and ensured equivalent chlorophyll content (Rangel-Yagui *et al*., 2004). Moreover, it has recently been reported as being a beneficial nutrient for the growth of this cyanobacterium in lagoon water (Costa *et al*., 2004).

Among the possible conventional nitrogen sources—KNO_3, $NaNO_3$, urea, NH_4NO_3, $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$—urea ensured high g-linolenic acid production (Mahajan and Kamat, 1995) and allowed S. platensis to reach concentrations comparable to those obtained with KNO_3 (Danesi *et al*., 2002). The general aim of this work was to check the ability of *S. platensis* to grow in cheaper media where the traditional nitrogen source (nitrate) had been replaced by Urea or ammonium sulphate (A). Considering the capability of most microalgae to hydrolyse urea to ammonia, mainly under alkaline conditions (Torre *et al*, 2003), preliminary batch cultivations were carried out to point out the actual nitrogen requirements of biomass as well as to establish the inhibition threshold of ammonia. Subsequent fedbatch runs, performed according to different feeding protocols, allowed selecting the best conditions for urea supply and demonstrated that the kinetics of growth may be comparable and even better than the ones obtained with the traditional nitrate-based culture media.

The following study was undertaken to observe the culture and growth of *Spirulina platensis* under the influence of Urea as the main nitrogen source.

OBJECTIVES

- To study the effect of Urea on growth & yield of *Spirulina platensis*
- To find out the best possible dosage of Urea application for the optimum growth of *Spirulina platensis*

CHAPTER II
REVIEW OF LITERATURE

Spirulina are unicellular and filamentous blue green algae (BGA) that has gained considerable popularity in the health food industry and increasingly as a protein and vitamin supplement to aquaculture diets. It has long been used as a dietary supplement by people living close to the alkaline lakes where it is naturally found. Spirulina has been used as a complementary dietary ingredient of feed for fish, shrimp and poultry. Among the various species of Spirulina, the blue green alga Spirulina platensis has drawn more attention because it shows a high nutritional content characterized by a 70% protein content and by the presence of minerals, vitamins, amino acids, essential fatty acids etc.

It thrives in tropical and subtropical warm lakes with a high pH ranging from 9.4 to 11.0. *S. platensis* is more widely distributed and found mainly in Africa, Asia and South America. *S. maxima* on the other hand is more confined to areas in Central America.

Many factors are important for the production of *S. platensis* at large scale, of which most important factors are nutrient availability, temperature and light. The earliest attempts to culture algae started about more than a century ago with solutions of a few inorganic salts. A common feature of these media was their high contents of nutrients, particularly nitrogen, phosphorus and potassium (Rodhe, 1978). The commercial production of S. platensis can be made cost effective by reducing the input cost with cheap and readily available materials without sacrificing the production efficiency.

Zarrouks medium (ZM) is the most commonly used medium for *S. platensis* culture (Zarouk, 1996; Phang and Chu, 1999). However, it is expensive and not readily available in Bangladesh. Thus, for mass production of *S. platensis*, particularly in developing countries there is a need to find a way to reduce the cost of culture. In Zarrouks medium (ZM), sodium nitrate is used as a source of nitrogen (N_2), which is very important for *S. platensis* culture. Several studies have demonstrated the feasibility of replacing this conventional nitrogen source with low-cost alternatives such as urea, ammonium sulfate

and ammonium chloride (Bezerra *et al*. 2008; Matsudo *et al*. 2009; Ferreira *et al*. 2010; Avila- Leon *et al*. 2012). Urea is a very common fertilizer that is used as a cheap and rich source of N_2 for plant cultivation (Pathak, 2015). The production cost of *S. platensis* culture will be minimized if urea can be used as cheap source of N_2 in Zarrouks medium. The present study was aimed to replace sodium nitrate-nitrogen ($NaNO_3$-N) of Zarrouks medium by urea-nitrogen for *S. platensis* culture.

Nonetheless, some significant, informative and promising works and research findings related to the current study have been reviewed in this chapter.

Anusuya Devi *et al*., (1981) found that the blue-green algae (*Spirulina platensis*) have been used for hundreds of years as a food source for humans and animals due to the excellent nutritional profile and high carotenoid content. Spirulina is relatively high in protein with values ranging between 55-65% and includes all of the essential amino acids. In addition, it is rich in nutrients such as vitamins (thiamin, riboflavin, pyridoxine, vitamin-B12, vitamin-C), amino acids, gamma linoleic acid, phycocyanins, tocopherols, chlorophyll and β-carotenes, carotenoids and minerals especially iron.

Blum *et al*., (1976) found out that the available energy content of Spirulina is estimated to be 2.50-3.29 kcal/g and its phosphorous availability is 41%

Bosma *et al.* (2007) conducted an experiment where volumetric productivities in the bubble column were predicted and compared with experimental volumetric productivities. The light integration model over-estimated productivity, while the model in which we assumed no light integration under-estimated productivity. Light integration occurred partly (47%) during the period investigated. The average observed biomass yield on light was 0.60 gm/L. The model of partly light integration predicted an average biomass yield on light of $0.57 g.mol^{-1}$ and predicted that productivity could have been increased by 19% if culture temperature would have been maintained at 24^0 C.

It has been reported that Spirulina has health benefits in conditions such as diabetes mellitus and arthritis (Parikh *et al*., 2001; Rasool *et al*., 2006).

Khan *et al*., (2005) found *Spirulina* to have immuno-stimulatory effects and to have antiviral activities.

Reichert *et al*. (2001) studied the specific growth rate and productivity of two Spirulina platensis strains. Spirulina platensis strain used was found that low concentration (0.50g/L) and high renewal rates (50% w/v) resulted in high specific growth rate and productivity. These values are two to four times higher than those obtained in simple batch cultivation and indicate that the semi continuous cultivation of Spirulina platensis is viable.

Manoj *et al*. (1992) reported that the alcohol extract of Spirulina inhibited lipid peroxidation more significantly (65% inhibition) than the chemical antioxidants like α-tocopherol (35%), BHA (45%) and β-carotene (48%). The water extract of Spirulina was also shown to have more antioxidant effect (76%) than gallic acid (54%) and chlorogenic acid (56%). An interesting aspect of their findings is that the water extract had a significant antioxidant effect even after the removal of polyphenols.

Peto *et al*., (1981) reported that Beta-carotene concentration of Spirulina is ten times higher than that of carrot. Food rich in β-carotene can reduce the risk of cancer. It was found that the natural carotene of Spirulina could inhibit, shrink and destroy oral cancer cells.

Zou and Richmond (1999) carried out an experiment that showed the effect of light-path length (i.e. reactor width or thickness) of flat plate glass reactors on outdoor production of eicosapentaenoic acid (EPA) and cell mass of *Nannochloropsis* sp. was tested, using a range of light-paths from 1.3 to 17.0 cm. Volumetric productivity of cell mass and optimal, as well as maximal cell density which represents the highest sustainable cell density under the experimental conditions, decreased with increase in light-path. Daily

areal output rate (g dry weight m-2 day-I) increased with increased light-path, in contrast with results obtained in similar reactors with spirulina cultures, in which areal output rates increased when the light-path was reduced. Maximal areal productivity of *Nannochloropsis sp*. (12.8 and 22.4 g ash-free dry weight per day per m2 of irradiated reactor surfaces, in winter and summer, respectively), reflecting maximal efficiency in light utilization, was obtained with the long light-paths, i.e. 10.4 and 17.0 cm. Increasing the light-path from 1.3 to 17.0 cm resulted in an increase in areal EPA productivity, from 66.7 to 278.2 mg m-2 day 1 in winter and from 232.1 to 515.7 mg m 2 day I in summer. This enhancement in areal productivity of EPA stems from increased productivity of cell mass which was associated with the increase in light-path.

Reichert *et al*. (2001) studied the specific growth rate and productivity of two Spirulina platensis strains. Spirulina platensis strain used was found that low concentration (0.50g/L) and high renewal rates (50% w/v) resulted in high specific growth rate and productivity. These values are two to four times higher than those obtained in simple batch cultivation and indicate that the semi continuous cultivation of Spirulina platensis is viable.

Reichert *et al*. (2006) studied that the cultivation of photosynthetic microorganisms such as the cyanobacterium Spirulina platensis has been studied by researchers in many countries because these organisms can produce products with industrial potential. We studied the specific growth rate (PX, day-I) and productivity (PX, in mg/L/day of Spirulina platensis biomass, dry weight basis) of two S. platensis strains (LEB-52 and Paracas) growing in aerated semi continuous culture in two-liter Erlenmeyer flasks for 90 days (2160 h) at 300C under 2500 lux of illumination in a 12 h photoperiod. Independent of the S. platensis strain used we found that low biomass concentrations (0.50 g/L) and high renewal rates (50% v/v) resulted in a high specific growth rate (PX = 0.111 day-I) and high productivity (PX = 42.3 mg/L/day).

Rijn and Shilo (1986) conducted an experiment on nitrogen limitation in natural populations of cyanobacteria (Spirulina spp. and Oscillatoria spp.) in Israeli fish ponds in

summer. They found that carbohydrates synthesized at the lighted surface partially utilized for protein synthesis at the bottom of these ponds when cells labeled by 14C under simulated pond conditions.

Michele *et al*. (2007) conducted an experiment where they found under controlled conditions the maximum specific growth rate (gmax) was 0.102 day-I , the biomass doubling time (td) was 6.8 d, the maximum dry biomass concentration (X_{max},) was 1.94 g L day-I and the maximum productivity (P_{max}) was 0.059 $gday^{-1}$, while the corresponding values in the greenhouse experiments were = 0.322 day^{-1} , dt = 2.2d, x_{max} - 1 73 g and P_{max} - 0.112$gL^{-1}day^{-1}$. Under controlled conditions the highest values for these parameters occurred when XO = 0.15 g/L, while in the greenhouse XO= 0.4g/L produced the highest values.

Kumar and Singh (2009) studied two strains of Spirulina platensis by enriching of cobalt and iodine, the repeated sub-culturing resulted in increased resistance to these trace metals. The cobalt tolerant strain which grew at 55mg $CoCl_2.6H_2O$ showed maximum uptake of 158.43 m mol Co ion mg^{-1}proteins which was 3.98 times higher than its parent. The iodine tolerant strain which grew at 7.0 g Kl l-1 showed maximum uptake of 0.65 m mol mg-1 of protein which was 1.25 times higher than its parent. Both the strains had the potential in relieving the deficiency of vitamin B12 and iodine in vegetarians.

Abdulrahman *et al.* (2014) observed that dried *S. platensis* found to be of potential effects on growth at an optimum concentration of 5 g/kg for common carp. The mean value level in the group received 5 g/kg is higher in all the tested parameters with significant difference indicates the optimum dietary level of *S. platensis* for C. carpio is 5 g/kg for studying period. Spirulina improved the Feed Conversion Ratio (FCR) and Specific Growth Rate (SGR) to enhance growth performance.

Duncan and Klesius (1996) reported that Spirulina contains high amounts of vitamins and minerals and it is a good source of protein for animal feed. The results of the current study are in accordance with (Ibrahem *et al*., 2013) who found that feed supplemented

with S. platensis powder improved the feed conversion ratio and growth rates in striped jack, *Pseudocaranx dentex* and (*O. niloticus*).

Hirajashi *et al*. (1984) studied 12 adult males were administered an oral hot water extract of Spirulina and the number and activity of their natural killer (NK) cells was measured before and after treatment (NK cells destroy tumor cells by binding to them and delivering lethal chemicals that kill on contact). At the end of the study, there was a significant increase in the production and cancer killing ability to these NK cells. When their NK cells were exposed to a bacterial product after treatment, production of interleukin-12 (IL12), a measure of immune strength, was significantly increased in comparison to IL-12 production in NK cells without pre-exposure to Spirulina.
Gardillo *et al*. studied the effect of increased atmospheric CO2 on photosynthesis and growth of Spirulina. The increase of CO2 did not cause any change in maximum growth rate while it decreased maximum biomass yield as it affected the pigment content of the algae.

Hintak (1985) described *Spirulina fusiformis* from the Kenya with a fusiform trichome construction. He mentioned that a relatively small, inconspicuous, broadly rounded calyptra was occasionally formed with or without an accompanying tapering of the trichome. Spirulina consisted multicellular, filamentous, unbranched and helicoidel trichomes wear formed by a single spirally twisted cell. Motile structure like flagella and heterocysts which are generally present in many blue green algae wear absent. The filaments wear called ‘trichome’. The cells wear cylindrical and the spiral wear loose. The cells exhibited active rotary movements. The helical shape of the trichome was characteristic of the genus but the helical parameters varied with the species even within the same species.

Amotz, (1987) conducted an experiment that showed that the beta-carotene in algae and leafy green vegetables has greater antioxidant effects than synthetic beta-carotene.

Deore (1992) studied the taxanomy of *Spirulina gigantea* var. Schmidle and Spirulina platensis var. tenius. The alga *Spirulina gigantea* var schmidle showed trichome with regular spiral, 4-6 numbers per trichome and deep blue green in colour. Trichome was 3 µm broud, 14.4 µm long and the distance between two spirals was 31 - 33 µm. Trichome was 6 µm broad, 14.4 µm long, regularly coiled. The breadth of the spiral was 26 µm and distance between two spirals was 31µm. Spirulina platensis had pale blue-green and it showed distinct separation.

Pelizer *et al*. (2002) reported that Spirulina platensis is generally produced in open ponds in liquid culture but there is recent production possible also in solid-state cultivation system but the estimation of cell growth is made difficult in separating cells from the cultivated medium in case of solid-state cultivation systems.

Clement *et al*. (1967) observed that *Spirulina maxima* were an algae rich in organic nitrogenous components, used for food in Chad Republic (Africa). Amino acids, vitamins and nutritive value were observed for a strain of the algal growth in an open-air pilot production unit. It contained 62% protein, high digestibility and vitamins like β-carotene, β_1, β_2, β_6, β_{12} and C.

Omstedt *et al*. (1973) observed the nutritive value of processed *Spirulina maxima* by in vitro in skeletal muscle of rats. Rats were fed six consecutive days on a diet containing 20 per cent protein level. Lyophilized *Spirulina platensis* had a nutritional quality between that of wheat gluten and casein supplemented with methionine. Drum dried preparations of *Spirulina platensis* were fairly similar in quality to that of casein sample with methionine.

Wahal *et al*. (1974) reported that water-soluble sugars constituted the major carbohydrates of Spirulina. The low amount of starch was due to the high activity of α and β amylase in Spirulina. The in vitro digestibility of Spirulina had been reported using an amylase enzyme. A sufficient amount of protease activity indicated that the enzyme was mainly involved in protein turn over rather than in storage hydrolysis.

Balasubramanya and Sampath (1994) investigated the PER of Spirulina diet at 10 per cent protein level. Thirty-six albino rats of 21 days old each were used as animal models for the experiment. Concentrated milk, casein and Spirulina diets were prepared at 10 per cent protein level. The PER of Spirulina alga diet was found to be as high as (2.28) than that of casein (2.45) or milk (2.46).

OrioCiferri and Tinoni (1985) studied the biochemistry and industrial potential of Spirulina. The composition of the biomass recorded, high protein content, low nucleic acids content and the presence of cell wall that was more easily digestible than that of yeasts or eukaryotic algae.

Bonotto *et al*. (1988) reported that many species of microalgae and blue green bacteria under suitable *et al*. conditions grew firstly and produced large biomass with high protein content. They constituted a source of single cell protein and the algal biomass supplemented or even replaced animal proteins, thus short circuiting the rather inefficient animal food chain.

Mitchell (1990) demonstrated that Spirulina maxima significantly altered the storage and utilization of vitamins A and E. These were investigated by feeding diets containing 0, 2.7, 10.7, 18.7 and 26.7 per cent protein or by a mixture of them. Growth results indicated that rats did not utilize the diets containing Spirulina maxima as well as the case in control diet. The ingestion of Spirulina maxima caused a significant increase in dry matter and chloroform extractable crude fat in the faeces.

Berg *et al*. (1991) studied the bioavailability of vitamin B12 in rats using Spirulina. Two different seafood products nori and Spirulina were evaluated with synthetic cyanocobalamine as control, in 30 male weaning wistar rats, given a diet deficient in vitamin B12 for six weeks, followed by a four-week repletion period in which the rats were given supplements of equal doses of vitamin B12. After repletion, cobalamine contents of serum and kidney were significantly lower and liver cobalamine content was

higher, for both the nori and Spirulina fed rats than for the cyanocobalamine supplemented controls.

Manjit Kaur and Ahluwalia (1992) carried out the biochemical studies of Spirulina enzymes and sugars. Total water-soluble sugars were more (7.31 percent) than acid soluble sugars (6.19 per cent). The activities of α amylase (8.5 percent), β amylase (5.33 percent) and protease (1.07 percent) were recorded. The activity of acid phosphatase was insignificant.

Manoj *et al*. (1992) reported the application of Spirulina for cancer chemo-prevention. Water extract of Spirulina was found to inhibit the lipid peroxidation to 76 per cent and the alcohol extract to 65 per cent. The chemical antioxidants like α tocopherol, Butylated hydroxyanisole and β-carotene gave 35, 45 and 48 per cent respectively.

Ogawa and Terui (1972) reported that Spirulina did not grow in organic compounds as the sole carbon source. To increase the productions of cyanobacteria, during mass culture, the minimal medium without sodium chloride fertilized with extracts of different organic manures were used. The dry matter production of Spirulina subsalsa and Spirulina platensis was increased by 5 to 30 percent.

Nasima *et al*. (1996) reported that the Rice Husk Ash (RHA) and NaHCO3 l-1 were used as a source of carbon in Spirulina culture. Addition of 2g of NaHCO3 l-1 every other day supported better growth than 1 g RHA l-1 every day. However, from an economic point of view RHA was preferred.

Gardillo *et al*. (1998) studied the effect of increased atmospheric CO_2 on photosynthesis and growth of Spirulina. The increase of CO_2 did not cause any change in maximum growth rate while it decreased maximum biomass yield as it affected the pigment content of the algae.

Vieira Costa *et al*. (2003) reported that, the influence of nutrient for biomass production of *Spirulina platensis*, in open raceway ponds in addition of (carbon as sodium bicarbonate, nitrogen as urea, phosphate, sulfate, ferric iron, magnesium and potassium) on the growth rate of the cyanobacteria *Spirulina platensis*. In unsupplemented lagoon water production of S. platensis was 0.78 ± 0.01 g/l (dry weight basis) while the addition of 2.88 g/l of sodium bicarbonate resulted in 0.82 ± 0.01 g/l after 40 hours of culture.

Tasneem Fatma (1990) reported that synchronous growth of Spirulina platensis was failed to grow both in liquid and solid media at its higher dilution. It was observed that minimum cell population is necessary to initiate and sustain Spirulina cultures. The culture filtrate had an absorbance of 0.96% on ninth day increased 50 per cent growth against control condition (0.63% absorbance).

Dubey (2006) found that, aeration, which could be achieved by propeller rotators, and which provides agitation of growing cells to maintain the cells in suspension, has been described as very necessary in getting good quality and better yields of *Spirulina* species.

Watanable and Hall (1996) investigated the photosynthetic productivity of the filamentous cyanobacaterium *Spirulina platensis* in a conical helical tubular photo bioreactor. It was constructed with 0.255 m^2 basal area and conical shape. The inner surface of the photo stage was illuminated with compact fluorescent cool white lamps and the input radiation energy was 1249 kjd^{-1}. The maximum photosynthetic efficiency obtained was 6.83 per cent which is corresponded to a production rate of 15.9g dry biomass $m^{-2}d^{-1}$.

Dubey (2006) had reported moderate light intensity in the cultivation of Spirulina, suggesting low light intensity at the beginning to avoid photolysis. He also noted that exposing Spirulina to high light intensity photolysis them.

Pandey *et al*. (2010) found that, the influence of light intensity for Spirulina platensis growth at 5 Klux light intensity the dry weight of Spirulina platensis was 0.85g/500ml while protein content and Chlorophyll a were 64.3% and 9.8mg/gm respectively.

Mohammad-Taghi Golmakani *et al*. (2006) studied that, Spirulina platensis is an important source of pharmaceuticals and nutraceuticals such as glinolenic acid (GLnA). GLnA yield of the culture medium (32 mg/L) were obtained at the highest light intensity of 5.0 klx.

Vincent and Silvester (1979) reported that the pH had a direct effect on the physiological properties of algae and the availability of nutrient. pH determined the solubility of carbon source and minerals in the culture directly or indirectly. Spirulina grew well at pH values between 9 and 11. The optimal pH of the Spirulina nutrient medium was shifted from 8.4 to 9.5 during the mass cultivation, due to the consumption of bicarbonate and sodium ions.

Pandey *et al*. (2010) found that, the influence of pH for Spirulina platensis growth, protein and Chlorophyll a content were examined and the dry weight of Spirulina platensis was 0.91g/500ml and protein and Chlorophyll a content were 64.3% and 13.2mg/gm respectively at pH 9.

Dubey (2006) found that, aeration, which could be achieved by rotators, and which provides agitation of growing cells to maintain the cells in suspension, has been described as very necessary in getting good quality and better yields of Spirulina species

Venkatraman and Sindhukanya (1981) reported the insect contamination in mass culture of Spirulina platensis. The mosquito larvae fed on the algal biomass for 2-3 days before entering into pupal stage and the decrease in algal yield was up to 10 per cent. The use of fine wire mesh frame removed all extraneous materials.

Mahadevaswamy and Venkatraman (1987) reported the presence of bacterial contaminants in outdoor cultivation of *Spirulina platensis*. The bacterial forms occurred in cultures were identified as aerobic spore formers. Number of the pathogenic forms affecting the products safety was identified.

Hofner *et al*. (1987) reported that the hexavalent chromium was most toxic and the zinc had little effect on the growth of Chlorella fusce. All elements tested arrest growth of Spirulina maxima at 10-4 M and smaller doses had no effect.

Ahluwalia and Kochar (1992) studied the effect of mercuric chloride, cadmium chloride, nickel sulpahte and zinc chloride on the growth of *Spirulina platensis*. Among the metals, mercury had been most toxic and the algal growth was reduced even at 0.01 ppm. It was followed by cadmium (0.1 ppm). Higher doses resulted in fragmentation, lysis and death of the algae.

Nanda and Padhi (1992) studied the effect of sodium salt to 2,4-Dichlorophenoxyacetic acid (2,4-D), on the growth and pigmentation of Spirulina platensis. It was found to be very sensitive towards 2,4-D and the growth of algae inhibited at 5 mg ml^{-1}.

Ken Sasaki *et al*. (1995) reported that the growth of Spirulina platensis was stimulated by adding 5Aminolevulinic acid (ALA, 500 mg l^{-1}). The photosynthetic activity was enhanced by rapid stimulation and accumulation of phycocyanin and chlorophyll II.

Ahluwalia and Kochar (1992) found that mercuric chloride, cadmium chloride, nickel sulpahte and zinc chloride on the growth of Spirulina platensis. Among the metals, mercury had been most toxic and the algal growth was reduced even at 0.01 ppm. It was followed by cadmium (0.1 ppm). Higher doses resulted in fragmentation, lysis and death of the algae.

Eugenia Olguin *et al*. (2001) evaluated the effect of low light and nitrogen deficiency on growth and chemical composition of Spirulina sp. (straight elements strain, SF) in batch

cultures utilizing a complex medium containing sea-water supplemented with anaerobic digested pig waste, was undertaken. Cultivation was carried out either at a light of 66 (lower) or 144 l mol photon my2 sy1 (higher), utilizing bench raceways. Biomass concentration (as dry weight) after 12 days of cultivation in the complex medium was similar. P < 0:05 to the one observed in a chemically defined medium (Zarrouk's Media), regardless of the light intensity. Protein content of the biomass in the complex medium was significantly lower P < 0.05, compared to the Zarrouk's medium, regardless of the light.

Promya and Traichaiyaporn (2005) studied the mass culture of *Spirulina platensis* in kitchen wastewater and fermented solution of oil extracted soybean. The statistical experimental designed was of Completely Randomize Design (CRD) by having 5 treatments with 3 replications: Zarrouk's medium (ZM), Kitchen wastewater (Kw 90%), (Kw100%) and fermented solution of oilextracted Soybean (Sb5%), (Sb10%) were tested for 30 days. Primary production and water quality from culture of *Spirulina platensi*s were monitored every 5 days. As the results, the highest primary production of *Spirulina platensis* was achieved in Sb5 % effluent (0.83 g/l). All experimental units had decreased removal percentage of NH_3-N, NO_3-N and NO_2-N, where they had met the laws and standards of pollution control.

Phang *et al.* (2001) reported that, the waste water is usually discharged into the rivers, each factory producing about 10- 22 tons waste water per day which contains a very high carbon to nitrogen ratio (105:0.12), but it has been made more suitable for fermentation by anaerobic fermentation in an up-flow packed bed digester. The digested effluent with an average C: N: P ratio of 24:0.14:1 supports growth of *Spirulina platensis*. The highest crude protein, carbohydrate and lipid content of the biomass were 68, 23 and 11%, respectively. The reduction in COD, ammoniacal-nitrogen and phosphate levels of the digested effluent reached levels of 98.0, 99.9 and 99.4%, respectively.

Phang *et al.* (2000) carried out a study that the production of wastewater arising from sago starch has a high carbon to nitrogen ratio, which is improved with anaerobic

fermentation. The effluent supported growth of *Spirulina platensis* with an average specific growth rate (μ) of 0.51 day-1 compared with the average μ of 0.54 day-1 in the inorganic Kosaric Medium in a high rate algal pond. Supplementation with 6 mM urea and 2.1 mM K_2HPO_4 produced gross biomass productivity of 14.4 g m^{-2} day^{-1}. A flow-rate of 24 cm s^{-1} increased the μ and gross biomass productivity (18 g m^{-2} day^{-1}).

According to Rajeev Kaushik *et al*. (2006) anaerobically digested distillery effluent (ADE) of molasses-based distilleries with high BOD and COD cause deterioration of land and ground water quality when discharged in the environment Spirulina platensis (ARM 730) was grown in different dilutions and used for treatment and safe disposal of ADE. It was observed that growth and other parameters like protein, carbohydrate, lipids were significantly higher in 50% ADE over standard Zarrouk's medium and other ADE dilutions. After growing Spirulina for 14 days in all the different dilutions of ADE, a significant decrease was observed in the following: BOD, 5194.6; total C, 27.2-71.8; and total N, 35.4- 58.0 per cent.

Stanca and Popovici (1996) demonstrated that the utilization of urea as a nitrogen source in *Spirulina platensis* cultivation leads to an increase in both the total biomass and the biomass chlorophyll content. Urea is easily assimilated by *Spirulina platensis*, probably due to its spontaneous hydrolysis to ammonia under alkaline cultivation.

Zhang *et al*. (1999) investigated the effects of initial glucose concentration and light intensity on specific growth rate; phycocyanin concentration and cell dry weight concentration in mycotrophic batch cultivation of *Spirulina platensis* using both shake flask and fermenter. Based on experimental results in shake flask culture, a number of mathematical models were constructed, and the optimal initial glucose concentration and the optimal light intensity were calculated. Finally, a time-dependent kinetic model for mixotrophic batch cultivation of *Spirulina platensis* in fermenter was also proposed. This was in good agreement with the experimental results and could be employed to predict the production of biomass and phycocyanin, and the consumption of glucose in fermenter culture.

Tri-Panji *et al*. (2001) attempted to grow *Spirulina platensis* using optimization media from low-cost nutrient sources. Optimum medium composition consisting of mineral salt and organic complex derived from low-cost nutrient sources. *Spirulina platensis* grown on complex media containing latex serum from concentrated latex factory, supplemented with salt minerals might produce high yielding carotenoids. Among eleven media composition containing latex serum examined, best growth on a formulated medium with a ratio of C: N: P: Mg = 1:3:0.3:0.2 yielding 0.350 g biomass/L This amount was slightly lesser than those on synthetic Aiba & Ogawa medium that yields 0.407 g biomass/L, after eight-week incubation.

Pulz *et al*. (1992) conducted an experiment where algal species *S. platensis* was cultured in three concentration of soybean meal medium (SMM) supplemented with 0.2g/L urea and observed positive effect of urea on chlorophyll a content of *S. platensis.*

Elias *et al*. (2002) documented improvement for the mass-scale culture of microalgae with the use of sophisticated closed systems. The proposed photo bioreactor in this study is a combination of helical parts receiving strong light with a main culture vessel mixed and degassed by airlift. A first experimental trial was carried out with the filamentous cyanobacterium *Spirulina platensis* achieving maximal volumetric productivity of 1.2 $gl^{-1}d^{-1}$ comparable with the maximal reported values by other closed culture designs for the same organism. The proposed system is under optimization of its engineering elements in order to comprise a sound modular solution for commercial production of microalgae of economic interest.

Dansei *et al*. (2002) cultivated the microalgae using urea as the nitrogen source by a fed-batch process. The addition of urea was done in four different modes: intermittent addition every 24 or 48 h, continuous addition by exponentially increasing the added mass, and continuous addition by using a constant mass rate. The experiments were carried out at three different temperatures: 27°C; 30°C and 33°C and at a constant light intensity. The results showed a positive influence of urea in the growth of Spirulina but

no effect on the chlorophyll content of the cultures. Best results were obtained by continuous urea addition in exponentially increasing amount, at 30ºC.

Jorge *et al*. (2003) studied the influence of nutrient on the growth rate of the Cyanobacteria *Spirulina platensis* using a 22-factorial design. In unsupplemented lagoon water production of *Spirulina platensis* was 0.78 ± 0.01 g/l (dry weight basis) while the addition of 2.88 g/l of sodium bicarbonate (without added urea, phosphate, sulfate or metal ions) resulted in 0.82 ± 0.01 g/l after 400 hours of culture. The further addition of phosphate and metal ions resulted in growth for up to 750 h and a final *Spirulina platensis* biomass of 1.23 ± 0.04 to 1.34 ± 0.03 g/L.

Hidenore (2004) studied the mass production of Spirulina in the open pond system. They used modified aeration technique produce high mass in ambient temperature and decrease some metals in the medium to obtain more yield, production cost was minimized cost was minimized by improved harvesting and filtration methods.

Reichert *et al*. (2006) studies the specific growth rate and productivity of two *Spirulina platensis* strains namely K-1 and K-2. *Spirulina platensis* strain used was found that low concentration (0.50g/L) and high renewal rates (50% v/v) resulted in high specific growth rate and productivity. These values are two to four times higher than those obtained in simple batch cultivation and indicate that the semi continuous cultivation of *Spirulina platensis* is viable.

Kemka Ogbonda *et al*. (2007) studied the influence of temperature and pH on biomass production and protein biosynthesis in a *Spirulina sp*. isolated from an oil-polluted brackish water environment in the Niger Delta. The isolated organism was identified on the basis of its phenotypic characteristics such as nature and direction of helix, temperature, pH and salt tolerance ranges. Biomass concentration in the culture media was calculated as cell dry weight. The combination of 30°C and pH 9.0 gave the highest values of 4.9mg/ml and 48.2 g/100g for biomass and total crude protein, respectively. The effect of pH was modulated by temperature and vice versa during biomass

production. This native isolate of Spirulina sp. act as a good source of natural protein that could be easily accepted by rural communities as single cell protein in the form of feed, food and health supplement when properly processed.

Ishikawa *et al.* (1989) studied that, GLA has mainly respect to its therapeutic properties such as its ability to decrease blood cholesterol levels. Spirulina has a high amount of polyunsaturated fatty acids, 1.5-2.0 per cent of 5-6 per cent total lipid. Spirulina is rich in linolenic acid, stearidonic acid, eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid.

CHAPTER III

MATERIALS AND METHODS

This chapter demonstrates information regarding methodology that was exploited in accomplishment of the experiment. It encompasses a brief outline of location of experiment, climate condition and material used. for the experiment. It also flourishes the treatments of the experiment data recording procedure and statistical data analysis along with a report general practice adopted during the experiment etc. which are presented as follows:

3.1 Experimental site

The experiment was conducted at the 2a Biotech Laboratory, Sher-e-Bangla Agricultural University, Dhaka during the period from 1 March, 2018 to 15 March 2018 to observe the growth, performance and yield of *Spirulina platensis* by applying urea solution as the nitrogen substitute for Na-nitrate. The location of the experimental site is $23^{o}74^{/}$N latitude and $90^{o}35^{/}$E longitude and at an elevation of 8.2m from sea level (Anon., 1989).

3.2 Growth factors

Spirulina production plants for mass cultivation are to be done in areas with suitable climatic conditions, particularly with the sunshine throughout the year. It is difficult to have an ideal growth due to different environmental factors like solar radiation, rain, wind, temperature fluctuation, etc.

3.3 Liquid media

The liquid used for the production of spirulina is a solution of mineral salts in water. This liquid has to supply the spirulina with all the chemical elements it needs. The pH of the culture media (i.e. its level of alkalinity) should be between 9.0 and l1.

There are various recipes for culture medium for spirulina; namely Zarrouks Media(ZM) and Kosaricks Media (KM) The one shown here is the modified version of if the popular Zarrouks Media(ZM) by replacing urea with the $NaNo_3$; although the ZM is the best for

ensuring an easy culture, even if it is far from being the cheapest (for composition, as seen in Table 2.)

3.4 Climatic Condition for Spirulina Production

3.4.1 Temperature

Temperature in the range of 25-35^{0}C even if the outside temperature as 35^{0}C was most ideally suited for getting maximum yield of S*pirulina*. Below 20^{0}C, growth is practically nil, but spirulina does not die. Temperature above 35^{0}C leads to bleaching of cultures. Partial shading provided a culture of about 30^{0}C even if the outside temperature was 38^{0}C. Rafiqul Islam *et al*. (2003) reported that, the maximum specific growth rate of 0.141 was found at 32^{0}C for Spirulina platensis and that of 0.144 was found at 3l^{0}C for *Spirulina fusiformis*. Maximum biomass production of 2.4 g /land chlorophyll a production of 16.6 mg/l were observed at 32^{0}C for *Spirulina platensis*. Maximum biomass production of 2.3 g n and chlorophyll - a production of 14.2 mg/l were observed at 37^{0}C for *Spirulina fusiformis*. Colla *et al*-(2004) found that, temperature was the most important factor and that the greatest amount of gamma-linolenic acid (GLA) was obtained at 30^o c, the fatty acid profile of the spirulina cultivated showing that (in order of abundance) palmitic, linolenic and linoleic acids were most prevalent.

3.4.2 Light

Spirulina required light intensities during its growth phase. The optimal light intensity was between 20K and 30K lux. Subramanian (1992) reported on the effect of different light quality on growth, protein and pigment synthesis of *Spirulina fusiformis.*

3.5 Water quality

The characteristics of water quality contributed in the algal mass production. It had dual influence, firstly by affecting the solubility of nutrients added in the medium and also selective accumulation of certain heavy metals by algae during the growth phase. That's why its ideal to use de-ionized water as the liquid media in order to suppress the possibility of contamination in the culture media.

3.6 Size of the inoculum

Tasneem Fatma (1990) reported that synchronous growth of Spirulina platensis was failed to grow both in liquid and solid media at its higher dilution. It was observed that minimum cell population is necessary to initiate and sustain spirulina cultures.

3.7 Agitation

Agitation of algal cultures had the advantages of uniform distribution of $C0_2$ and prevention of thermal stratification. Many agitation devices had been reported which range from motor driven pumps, gravity flow, air light systems and manual agitation. Dubey (2006) found that, aeration, which could be achieved by rotators, and which provides agitation of growing cells to maintain the cells in suspension, has been described as very necessary in getting good quality and better yields of spirulina species.

3.8 Research Materials

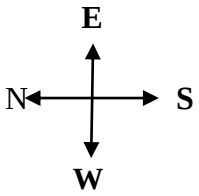
Producing spirulina algae from a mother strain is, for most, the easiest and rapid technique to propagate spirulina. Fresh, healthy and disease-free spirulina were used as experimental materials for the present study.

3.8.1 Sources of Materials

Spirulina strain “K-2” was collected from "Energaia Bangladesh Limited". And urea is collected from the local market.

3.9 Design and Layout of the Experiment

Experiment was propelled in completely randomized design (CRD) with three replications for each treatment as seen in Figure 1,



Number of Replications :3

Design : Completely Randomized Design (CRD)

Number of Treatments : 4

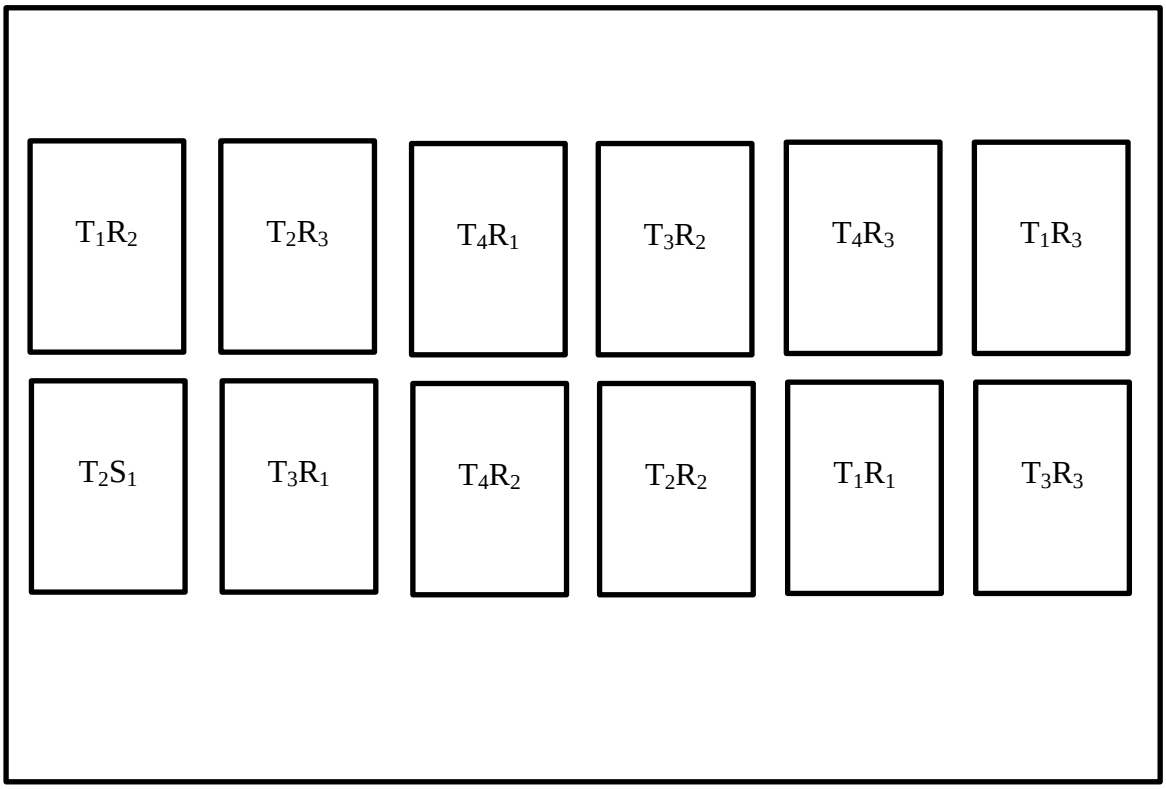


Figure 1 : Layout of the Experiment

Table 01. Various concentrations of urea solution to be used in ZM as MZM (Modified Zarrouks Media)

Treatments	Culture Media	Amount of $NaNO_3$ (g/L)	Amount of Urea (g/L)	Number of Replications
T_1	ZM* with 100% $NaNO^3$	2.25	-	3
T_2	0.01 M of Urea in ZM	-	0.60	
T_3	0.02 M of Urea in ZM	-	1.25	
T_4	0.03 M of Urea in ZM	-	1.80	

Here,

T_1= Standard Zarrouks Media

T_2= Modified Zarrouks Media containing 0.01M (0.60g/L) of urea solution

T_3= Modified Zarrouks Media containing 0.02M (1.25g/L) of urea solution

T_4= Modified Zarrouks Media containing 0.03M (1.80g/L) of urea solution

Number of Replications: 3

3.10 Materials and Chemical Required for *Spirulina* Production

3.10.1 Materials Required for *Spirulina cultivation*:

Materials which was required for *Spirulina* cultivation as follows:

a. Air Pump
b. Autoclave
c. Plastic Tube
d. Drilling machine
e. Connector
f. Erlenmeyer Flasks
g. Syringe
h. Polythene

3.10.2 Materials Required for the analysis of *Spirulina* sample:

a. Electric Balance

b. UV Spectrophotometer

c. Digital pH meter

d. Refrigerator

e. Filter paper

f. Crusher

3.11 Treatments of the experiment

The experiment was conducted to study the comparative analysis of *Spirulina* sp growth by applying urea as a nitrogen substitute for Na-nitrate. The experiment was conducted in completely randomized design with five treatments. Zarrouks medium was used as a control medium for treatment T_1. In treatments T_2 to T_5, sodium nitrate of (NaNO3) of Zarrouks media was replaced with urea as shown in Table 1.

3.12 Protocol for Spirulina Production

A series of steps have carried out for successful spirulina production as follows

3.12.1 Installation of the Containers

12 autoclaved and sterile 500ml Erlenmeyer flasks were autoclaved and placed horizontally in two layers on the 2a Biotech laboratory of Sher-e-Bangla Agricultural University. Food grade silicon tubes branched out from a central plastic pipe which was directly connected with a mini air pump. The silicon tubes were linked to flasks for supplying air. The motor connected with electric line performed two services at a time one was air circulation and another was agitation. The air circulation was continued at one-hour interval automatically by using timer. Cotton balls were used to cover the top of the flasks so that the flasks stay free from any contaminations and foreign objects.

3.12.2 Chlorination of Container

The Chlorination of container was carried out @ 0.02g/L H_2O with bubbling for one day.

3.12.3 De chlorination of container

The dechlorination of container was done Ascorbic acid =0.04g/L with bubbling for one day.

3.12.4 Culture Media

Zarrouk Media will be used as the culture media and urea is added to the Modified Zarrouk Media as the substitute for Na- nitrate. 300ml ZM and MZM was added in the fl

3.12.5 Media Preparation

Zarrouks medium is widely used as the standard medium for *S. platensis* culture. (Zarrouk, 1966) The composition of Zarrouks media is shown in Table 2.

Table 2: Composition of Standard Zarrouks Media (ZM) for *S. platensis* culture

Chemical/Reagents	Concentration in stock solution
$NaHCO_3$	16.8 g/L
$K2HPO_4$	0.5 g/L
$NaNO_3$	2.5 g/L
K_2SO_4	1.0 g/L
NaCl	1.0 g/L
$MgSO_4.\ 7H_2O$	0.20 g/L
$CaCl_2$	0.04 g/L
$FeSO_4.\ 7H_2O$	0.01 g/L
Na_2-EDTA	0.08 g/L
Micronutrient Solution*	1.0ml/L

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

For the preparation of Zarrouk medium, the above-mentioned amount (Table 2) of chemicals from no. 1 to 9 was weighted by the help of electric balance and taken in a 1.0 L conical flask. Then 1 ml micronutrient solution was pipetted in the flask and distilled water was added to make the volume 1.0 L**.**

For the preparation of MZM containing urea solution, 0.01M, 0.02M and 0.03M urea solution was added in the Zarrouks medium as the substitute of Sodium nitrate.

3.12.6 pH adjustment of all media

Prior to culture initiation of *S. platensis* pH of all media were adjusted at 9.0 by incorporating either 0.1 N HCl or 0.1 N NaOH depending on pH condition of the media.

When it was found that the pH value of one media was above 9.0 then 0.1 N HCl was added and when the pH value was found below 9.0, 0.1 N NaOH was added until pH becomes stable at 9.0.

3.13.7 Strain inoculation in Flask

The strain was maintained in 500 mL sterilized Erlenmeyer flasks containing 100 mL Zarrouk's medium(ZM) at 30 ± 2 LC, pH 9 with continuous illumination using cool white fluorescent tubes (2500 Lux). The strain containing a high proportion of coiled filaments is chosen (less than 25 % straight filaments, if possible, none at all), easy to harvest, and containing at least 1 % of gamma-linolenic acid (GLA) based on dry weight. Concentrated spirulina seed culture can be obtained either from the floating layer of an unagitated culture, or by rediluting a freshly filtered biomass (beware of lumps). A concentration of up to 0.03 g spirulina (dry) or 0.85g spirulina (fresh weight) per liter is permissible if storage and transportation last less than a week's time, and provided the seed culture be aerated at least two times a day. If aeration can be continuous, the concentration may be up to 10 g/l (weights of spirulina always refer to contained dry matter). The media was inoculated with 0.75g/l fresh weight basis.

3.13.8 Intercultural operations

Apart from harvesting and feeding, a spirulina culture requires some attention in order to be kept in good condition. Agitation is a requisite. Continuous agitation however is not required. Agitation was carried out by an air pump. One third of full sun will saturate the photosynthetic capacity of spirulina, but shading is not required except to reduce the consumption of water (evaporation) or the temperature (< 38^0C) or the pH (< 11.3). The temperature will practically never be too high, but the pH may soon become too high if insufficient carbon is supplied. The depth of culture must be kept between 10 and 20 cm. Accumulation of "white skins" and foam may float in the afternoon when the temperature of the culture goes above 35^0C. These are not harmful as they will go back to the bottom

again during the night, but their appearance is unpleasant and interferes light transmission during day time. They can be removed using a net. If the concentration of spirulina is too low, the culture may be invaded by chlorella (a unicellular, edible alga). It also interferes light transmission. So, they can be removed using a net. Usual pathogenic bacteria do not survive the high pH (> 9.7) of a spirulina culture in production, however a microbiological assay of the product should be made also at least once a week. Contaminations most generally occur during or after harvesting. The color of the culture should be deep green. If it turns yellowish, this may be due to either a lack of nitrogen or an excess of light (photolysis) or of ammonia (excess of urea). In the latter two cases recovery is generally possible within two weeks while resting the culture under shading.

3.13.9 Harvesting of Spirulina

When the spirulina is in good condition, separating it from the water ("harvesting") is an easy operation (Plate 5g).

The best time for harvesting is early morning for various reasons:

- The cool temperature makes the work easier,
- More sunshine hours will be available to dry the product, - The % proteins in the spirulina are highest in the morning.

There are basically two steps in harvesting:

- filtration to obtain a "biomass" containing about 10 % dry matter (1 liter = 100 g dry) and 50 % residual culture medium,
- removal of the residual culture medium to obtain the "fresh spirulina biomass", ready to be consumed or dried, containing about 20 % dry matter and practically no residual culture medium.

3.13.10 Washing and filtering

Washing was done by tap containing 20% of NaCl that reduced that excess amount of and other chemicals. Filtration was simply accomplished by passing the culture through a fine weave cloth. using gravity as the driving force. Synthetic fiber cloth (especially polyamide or polyester) with a mesh size of about 30 to 50 microns is the preferred filtering medium. Supporting the filtration cloth by a fine net will accelerate somewhat the filtration and protect the cloth against rupturing, but a simple bag made from the cloth works well also. Here I used 80 microns filtering bag. The filtration is accelerated by gently moving or scraping the filter. When most of the water has filtered through, the biomass will often agglomerate into a "ball" under the motion. leaving the cloth clean (this desirable condition happens mostly when the biomass is richer in spiraled forms and the culture medium is clean). Otherwise it may then be necessary to scrape it out from the cloth.

3.13.11 Pressing

The final dewatering was accomplished by pressing the biomass enclosed in a piece of filtration cloth plus a strong cotton cloth, either by hand or in any kind of press.

3.13.12 Drying

The industrial type of spirulina dryer is the spray drier which flash dries fine droplets at very high temperature and yields an extremely fine powder of low apparent density. This type is outside the reach of artisanal producers. Freeze drying is the best way of drying but far too expensive and complicated.

Sun drying is the most popular among small producers, but requires a few precautions. Direct sun drying must be very quick, otherwise the chlorophyll will be destroyed and the dry product will appear bluish. Whatever the source of heat, the biomass to be dried must be thin enough to dry before it starts fermenting. A very simple technique was applied to dry spirulina. Firstly, spirulina pest was poured in a syringe and pressed it. Finally, spirulina came out from the syringe very thin spiral rod-shaped diameter of I to 2 mm. The total duration of the drying should not exceed a few hours, preferably 2 hours.

3.14 Data Collection on Different Parameters

Observations of experimental data were extracted from each pot. Data were recorded respectively on which parameters observation as follows:

3.14.1. Estimation of *S. platensis* cell weight (dry basis) (mg/L)

Sample containing 50 ml *S. platensis* suspension was filtered through a Whitman 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) in order to remove insoluble salts. After that the filter papers were put in a glass Petridis and kept in the oven at 70 ºC for overnight. For cooling, Petridis were put into desiccators for 20 minutes and then filter papers were weighed. Dry weight of *S. platensis* was calculated using following formula.

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

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

3.14.2. Estimation of chlorophyll a (mg/L)

S. platensis sample were collected in order to estimate chlorophyll a content. Collected sample (10 ml) was filtered with an electric filtration unit using filter papers (Whatman GF/C of 0.45 µm mesh size and 47 mm diameter). These filtered samples together with filter paper was taken into a test tube and ground with a glass rod and finally mixed with 10 ml of 100% redistilled acetone. Each of the test tubes was wrapped with foil papers to inhibit the contact of light. The wrapped test tubes were kept into a refrigerator overnight. Then the refrigerated samples were homogenized for 2 minutes followed by

centrifugation at 4000 rpm for 10 minutes. After centrifugation the supernatant was isolated and taken for chlorophyll a determination. Optical densities of the samples were determined at 664 nm, 647 nm and 630 nm by using UV spectrophotometer (DR 5000). A blank with 100% acetone was run simultaneously. Chlorophyll a was calculated using the following formula (Habib, 1998). Chlorophyll a (mg/L) = 11.85 (OD 664) - 1.54 (OD 647) - 0.08 (OD 630).

3.14.3. Specific Growth Rate

Specific growth rate (μ) of *A. platensis* was calculated according the following equation:

$$\mu = (\ln X_m - \ln X_i)\,(T_c)^{-1}$$

where,

X_i = initial biomass concentration (mg L^{-1}), X_m = maximum biomass concentration (mg L^{-1}), and T_c = cultivation time related to the maximum biomass concentration (days)

3.14.4. Measurement of optical density (mg/L)

Optical density was measured during the time of sampling at 640 nm, by using UV spectrophotometer. The sample of *S. platensis* grown in different treatments were taken in puvet and placed in UV spectrophotometer. Then the OD of the samples was recorded.

3.14.5. Measurement of pH level in the Media

The pH value of the culture media was measured from each sub sample by an electric pH meter (Corning pH meter 445).

Bleaching powder

$MgSO_4$

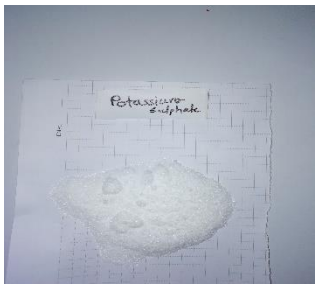
$FeSO_4.7H_2O$

Ascorbic acid

$NaHCO_3$

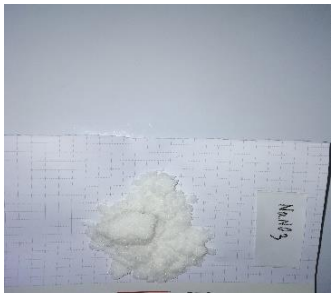
TSP

NaCl



K_2SO_4

Na-EDTA



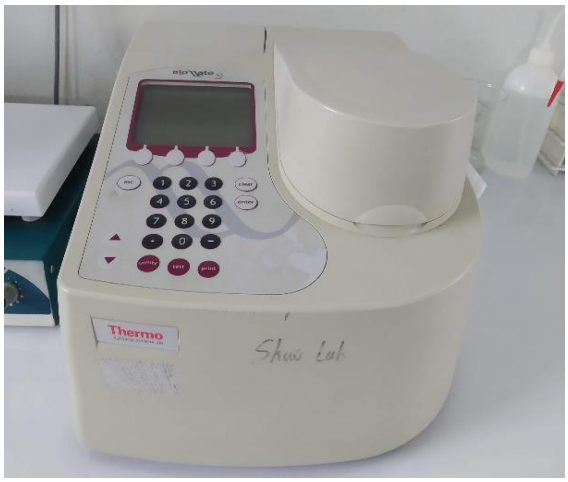
$NaNO_3$

Urea

Plate 01: Different necessary chemicals for *Spirulina* cultivation

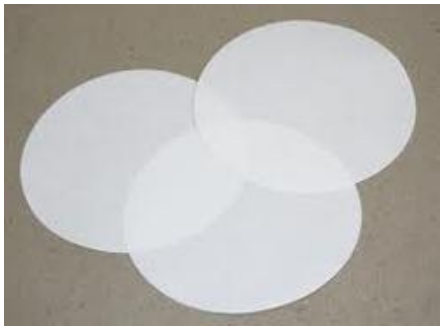
Electrical Balance

UV-spectrophotometer



Optical density meter

P^H meter



Filter Paper

Plate 02: Various equipments used to aid the analysis of *Spirulina* sample

Plate 03: *Spirulina* cultivation in the laboratory

CHAPTER IV

RESULT AND DISCUSSION

The research work on 'Growth and development of Spirulina under various dosage of Urea' was undertaken in the Department of Horticulture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka. Data were collected on various growth and yield indicator and data was statistically analyzed with Statistix10.0 software. Appendices II- VI contains the analysis of variance (ANOVA) of the data on different growth and yield parameters. Figures, graphs and tables were used to discuss the findings of the study as well as their most probable interpretation in this chapter under the following headings.

4.1 Statistical Analysis

4.1.1 Estimation of *S. platensis* cell weight (dry basis) (mg/L)

Cell weight or the cell biomass is one of the most important yield parameters in *Spirulina platensis* which is positively correlated with the growing conditions significantly influenced this trait. Biomass concentration was determined every three days by measuring the optical density at 560 nm to produce a standard curve (Leduy and Therien, 1977).

The difference in varieties for cell biomass in standard ZM and MZM was found significant and has been presented in Figure 2 and Appendix II. Highly significant differences exist among different dosage of Urea solution with regard to cell biomass at 3 days, 6 days, 9 days, 12 days and 15 days after inoculation respectively. The basic concentration of 4mg/L of inoculum gained maximum cell weight of 542.67 mg/L (ZM), 423.33 mg/L in MZM containing 0.01M of urea solution, 423.33 mg/L in MZM containing 0.02M of urea solution and 278.67 mg/L in MZM containing 0.03M of urea solution. Most biomass (dry basis) was found from ZM ($T_{1)}$ (542.67 mg/L) whereas the least amount of biomass was found from MZM containing 0.03M of urea solution (T_4). The highest cellular growth of 607 mg/L was found in ZM (T_1) and from the MZMs, highest growth of 454 mg/L was found from MZM containing 0.01M of urea solution

(T_2) and the difference in the cell biomass at the peak was found very significant. A steady decreasing trend was observed in cell weight was observed in all the culture media from the 12th day of the culture period.

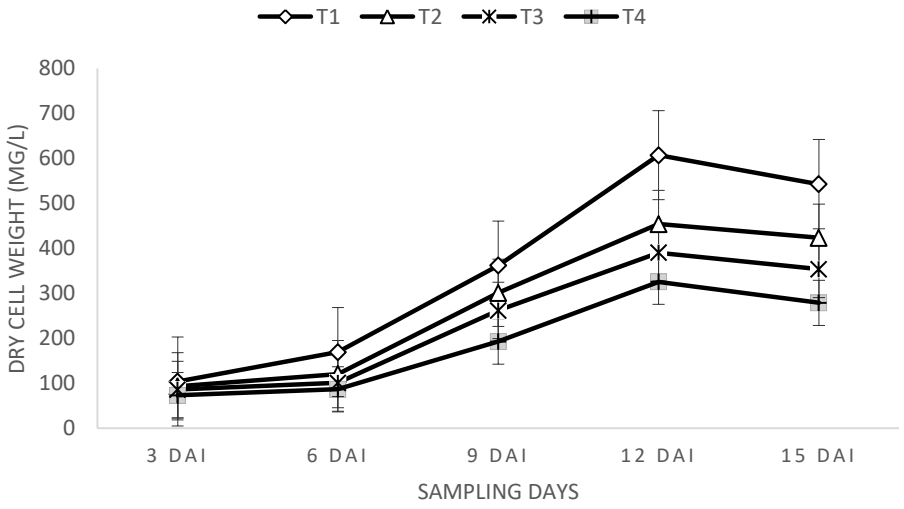


Figure. 2: Mean Cell Weight (mg/L) (dry basis) of *S. platensis* in three different concentrations of MZM and standard ZM (Here,T_1: Standard ZM; T_2 : Modified ZM containing 0.01M urea ; T_3:Modified ZM containing 0.02M urea ; T_4: Modified ZM containing 0.03M urea)

Table 3: Cell weight of *S. platensis* under standard ZM and Modified ZM at different days after inoculation

Cell Weight of *Spirulina platensis* (mg/L) (Dry basis)[Y]

Treatments[x]	3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
T_1	103.670a	169.00a	361.67a	607.00a	542.67a
T_2	93.000b	120.33b	300.67b	454.00b	423.33b
T_3	85.667b	100.67c	261.67b	390.00c	353.33c
T_4	73.000c	86.333d	192.67c	325.33d	278.67d
CV%	5.76	5.57	8.14	4.84	4.76
$LSD_{0.05}$	9.6314	12.489	42.794	40.496	35.791

[X]Here, T_1: Standard ZM; T_2 : Modified ZM 0.01M urea ; T_3:Modified ZM 0.02M urea ; T_4: Modified ZM 0.04M urea

[Y]In a coloumn, means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

4.1.2 Estimation of chlorophyll a (mg/L)

Chlorophyll influences the growth of a plant which is correlated with the yield of *S. platensis*. The preference of Spirulina for nitrate and ammonium salts as a nitrogen source was demonstrated by Richmond (1988) who reported that nitrates and ammonium salts were the main nitrogen sources assimilated by Spirulina as well as urea in lesser con-centration of urea. Chlorophyll a content was measured and analyzed in ZM and MZM showed significant variation among the various medias used for the experiment as shown in Figure 3 and Appendix III. The highest chlorophyll a content (7mg/L) was observed from T_1 (ZM) whereas the lowest chlorophyll a content (4.133 mg/L) was observed from T_4 as shown in Table 4. The highest Chlorophyll a content was obtained from the ZM culture on the 12th day (7.466 mg/L). In Contrast, cultures supplemented with urea showed slower growth rates, when compared to ZM. A steady decline of the chlorophyll a content was observed in the all the growing media of ZM and MZM starting from the 12th day of the inoculation period.

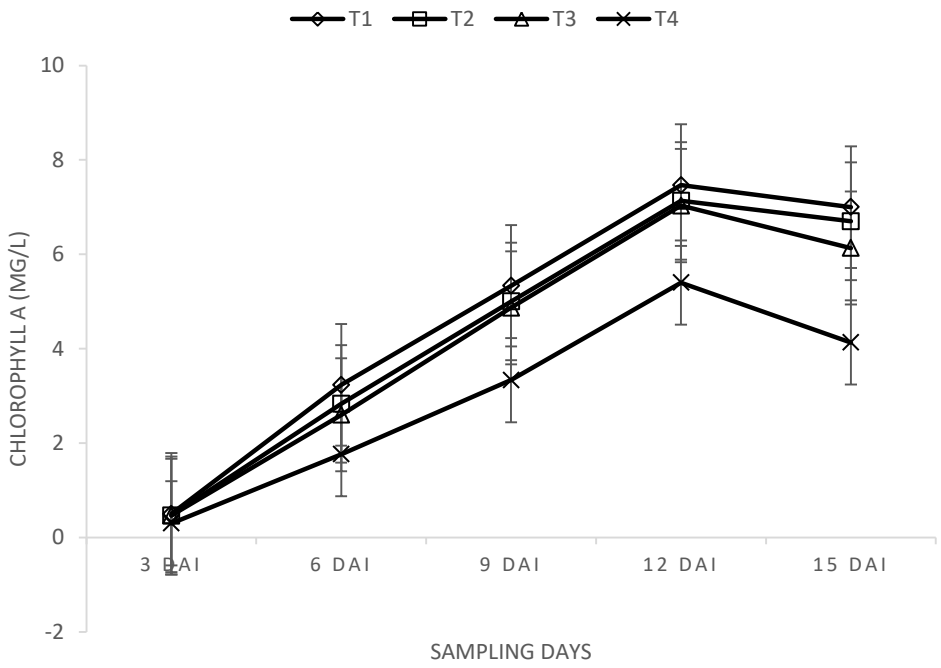


Figure. 3. Chlorophyll a content (mg/L) of *S. platensis* in three different concentrations of MZM and standard ZM (Here,T_1: Standard ZM; T_2 : Modified ZM containing 0.01M urea ; T_3:Modified ZM containing 0.02M urea ; T_4: Modified ZM containing 0.03M urea)

Table 4: Chlorophyll a measurement of *S. platensis* under standard ZM and Modified ZM

Chlorophyll a Measurement of *Spirulina platensis* (mg/L)[Y]

Treatments[x]	3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
T_1	0.500a	3.233a	5.333a	7.466a	7.000a
T_2	0.466ab	2.833ab	5.000ab	7.133b	6.700a
T_3	0.467ab	2.600b	4.866b	7.033b	6.133b
T_4	0.300b	1.766c	3.333c	5.400c	4.133c
CV%	22.09	8.57	4.45	2.22	2.97
$LSD_{0.05}$	0.1803	0.4210	0.3882	0.2824	0.3351

[X]Here, T_1: Standard ZM; T_2 : Modified ZM 0.01M urea ; T_3:Modified ZM 0.02M urea ; T_4: Modified ZM 0.04M urea

[Y]In a coloumn, means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

4.1.3 Specific Growth Rate

Significant difference was found for specific growth rates of the S. platensis cultured in ZM and MZM containing 0.01M, 0.02M and 0.03M of urea solution which is presented in Figure 4 and Appendix IV. Among the culture mediums, *Spirulina* grown in ZM (T_1) showed the maximum specific growth of 0.101 and the MZM containing 0.04M of urea solution as the nitrate substitute showed the least specific growth rate of 0.044 in the 15th day of the culture; which is statistically dissimilar from the other MZMs; (T_2 and T_3); which has the specific growth rate of 0.086 and 0.065 respectively. It is a possibility that the excess nitrogen from the urea solution in the MZM (T_4) actually hampered the whole natural specific growth rate of the *Spirulina platensis* culture. The highest specific growth rate was observed on the 12th day after the inoculum in the MZM containing 0.01M (T_1) urea solution and MZM containing 0.02M urea solution (T_2) which was 0.086 and 0.068

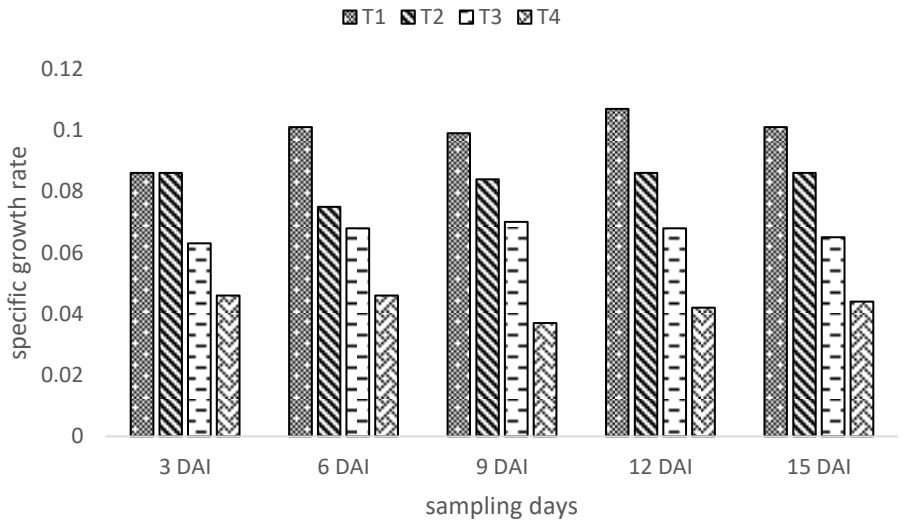


Figure. 4. Specific growth rate of *S. platensis* in three different concentrations of MZM and standard ZM (Here,T_1: Standard ZM; T_2 : Modified ZM containing 0.01M urea ; T_3:Modified ZM containing 0.02M urea ; T_4: Modified ZM containing 0.03M urea)

Table 5: Specific Growth Rate measurement of *S. platensis* under standard ZM and Modified ZM

Specific Growth Rate of *Spirulina platensis* (mg/L)[Y]

Treatments[x]	**3 DAI**	**6 DAI**	**9 DAI**	**12 DAI**	**15 DAI**
T_1	0.086a	0.101a	0.099a	0.107a	0.101a
T_2	0.086a	0.075b	0.084b	0.086b	0.086b
T_3	0.063b	0.068b	0.070c	0.068c	0.065c
T_4	0.046c	0.046c	0.037d	0.042d	0.044d
CV%	4.06	9.26	5.40	5.77	5.48
$LSD_{0.05}$	5.381	0.0127	7.393	8.279	7.629

[X]Here, T_1: Standard ZM; T_2 : Modified ZM 0.01M urea ; T_3:Modified ZM 0.02M urea ; T_4: Modified ZM 0.04M urea

[Y]In a coloumn, means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

respectively. Although the maximum specific growth rate of *Spirulina* sp. cultured in MZM containing 0.03M urea solution was observed on the 3rd and 6th day after inoculation. (0.046).

4.1.4. Measurement of optical density (mg/L)

The mean values of optical density (OD) of *S. platensis* cultured in different Modified Zarrouks Medium and the standard Zarrouks Medium containing urea solution showed significant difference.as presented in Figure 5 and Appendix V. Richmond (1988) reported that The preference of Spirulina for nitrate and ammonium salts as a nitrogen source was demonstrated by nitrates and ammonium salts were the main nitrogen sources assimilated by Spirulina as well as urea in lesser con-centration . The initial OD content was 0.03 mg/L for all of the ZM and MZMs. The initial OD attained a maximum of 0.756 mg/L and 0.630 mg/L when cultured in ZM (T_1) MZM containing 0.01M urea solution (T_2) and the highest optical density content of the *Spirulina* culture was obtained in ZM after the 8th day of the inoculation (0.803 mg/L) and the lowest OD was observed in the MZM containing 0.03M urea solution (0.160 mg/L) as seen in Table 6. A decreasing trend in the OD was observed after the 12th day of inoculation in all of the MZM and ZM.

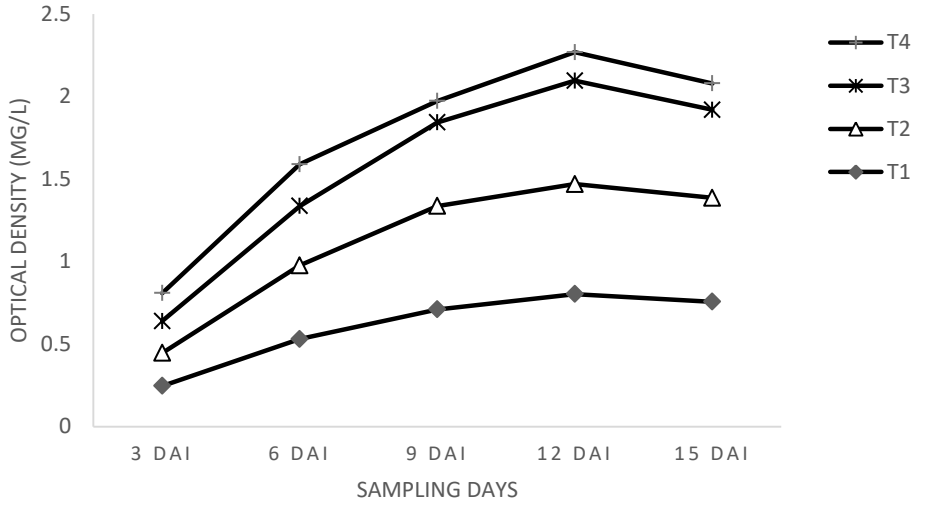


Figure. 5. Optical density (OD) (mg/L) of *S. platensis* in three different concentrations of MZM and standard ZM (Here,T_1: Standard ZM; T_2 : Modified ZM containing 0.01M urea ; T_3:Modified ZM containing 0.02M urea ; T_4: Modified ZM containing 0.03M urea)

Table 6: Optical Density of *S. platensis* under standard ZM and Modified ZM

	Optical Density of *Spirulina platensis* (mg/L)[Y]				
Treatments[x]	**3 DAI**	**6 DAI**	**9 DAI**	**12 DAI**	**15 DAI**
T_1	0.246a	0.5300a	0.710a	0.803a	0.756a
T_2	0.200b	0.446b	0.626b	0.666b	0.630b
T_3	0.193b	0.360c	0.506c	0.626c	0.533c
T_4	0.170c	0.253d	0.130d	0.173d	0.160d
CV%	5.33	3.70	4.38	3.22	4.26
$LSD_{0.05}$	0.0203	0.0277	0.0407	0.0344	0.0417

[X]Here, T_1: Standard ZM; T_2 : Modified ZM 0.01M urea ; T_3:Modified ZM 0.02M urea ; T_4: Modified ZM 0.04M urea

[Y]In a coloumn, means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

4.1.5. Measurement of pH in the media

The mean values of pH in three concentrations of urea solutions present in MZM and ZM containing the *Spirulina platensis* shown no significant variation (Fig. 7) and Appendix VI. The range of pH value was not significant as the pH value ranged from 8.63 to 9.20 During the culture period. The highest pH was observed in ZM in the 9th day after inoculation (9.2). The lowest pH value was the MZM containing 0.03M urea solution (T_4) in the 12th day.

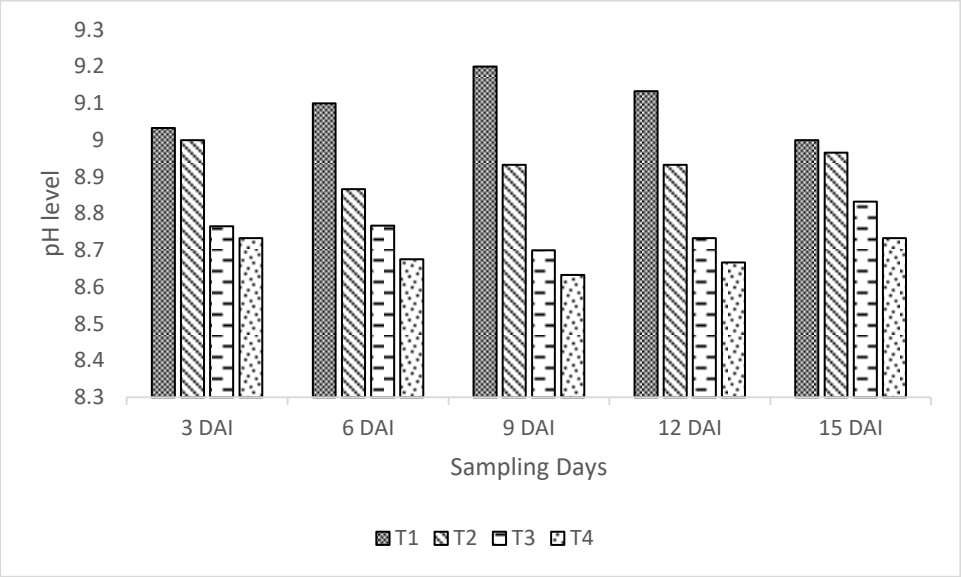


Figure.6. pH level of *S. platensis* in three different concentrations of MZM and standard ZM (Here,T_1: Standard ZM; T_2 : Modified ZM containing 0.01M urea ; T_3:Modified ZM containing 0.02M urea ; T_4: Modified ZM containing 0.03M urea)

Table 7: pH level measurement of *S. platensis* under standard ZM and Modified ZM

	pH level of the growth media[Y]				
Treatments[x]	**3 DAI**	**6 DAI**	**9 DAI**	**12 DAI**	**15 DAI**
T_1	9.033a	9.100a	9.200a	9.133a	9.000a
T_2	9.000a	8.867b	8.933b	8.933b	8.966a
T_3	8.767b	8.767bc	8.700c	8.733c	8.833ab
T_4	8.733b	8.676c	8.633c	8.667c	8.733b
CV%	0.97	1.13	1.08	1.17	1.13
$LSD_{0.05}$	0.1631	0.1883	0.1803	0.1960	0.1883

[X]Here, T_1: Standard ZM; T_2 : Modified ZM 0.01M urea ; T_3:Modified ZM 0.02M urea ; T_4: Modified ZM 0.04M urea

[Y]In a coloumn, means having similar letter (s) are statistically identical and those having dissimilar letter (s) differ significantly as per 0.05 level of probability

CHAPTER V
SUMMARY AND CONCLUSION

5.1 Summary

The experiment was carried out in the 2a biotech Laboratory, Department of Horticulture, Sher-e-Bangla Agricultural University during 1 March 2018 to 15 March 2018 to assess the influence of various dosage of urea solution as a viable substitute for Sodium Nitrate present the Zarrouks Media on the growth and yield of *Spirulina platensis*. It was a single factor experiment and laid out in Completely Randomized Design (CRD) with three replications. Four growth media treatments for *Spirulina platensis* viz. T_1= Standard Zarrouks Media; T_2 = Modified Zarrouks Media with 0.01M urea solution; T_3 = Modified Zarrouks Media with 0.02M solution and T_4 = Modified Zarrouks Media with 0.03M solution were used. Different growth and yield parameters were measured and recorded followed by statistical comparison of data.

Significant variations were observed in respect of in case various urea dosages of all parameters as followed –

The highest cell weight on dry basis or total biomass content (542.67 mg/L) was found in the standard ZM (T_1) after the 15 days of culture period. MZM containing 0.01M urea solution (T_2) also showed very satisfactory result in case of biomass content (423.33 mg/L) which is quite good considering the negative effects urea usually has on the growth of *Spirulina* culture. The lowest biomass content was seen in T_4 (278.67 mg/L). All of the Media peaked at 12 DAI and then showed a slow decrease in biomass production. Among the MZMs, T_2 showed most promise.

The highest chlorophyll a content (7.466 mg/L) was seen in the standard ZM (T_1) at 15 DAI. The easy solubility of the nitrate ion from sodium nitrate is possibly responsible for the increase in Chlorophyll a content. satisfactory result for chlorophyll content was seen in MZM containing 0.01M urea solution (T_2) (6.700mg/L) which is similar to T_3 (6.133mg/L). The lowest chlorophyll a content was seen in T_4 (4.133 mg/L). All of the Media peaked at 12 DAI and then showed a slow decrease in biomass production. The

chlorophyll a content at 12 DAI for T_1, T_2, T_3 and T_4 are 7.466mg/L; 7.133mg/L; 7.033mg/L; 5.400mg/L respectively.

Among the treatments, maximum specific growth rate was found in T_1 (0.107) and the lowest was found in T_4 (0.037). Among the treatments with the urea solution, T_2 showed maximum growth rate (0.086) which is the highest between T_2, T_3 and T_4. Although specific growth rate peaked for T_1, T_2 and T_3 at 12 DAI, T_4 showed a growth rate increase from 12 DAI (0.042) to 15 DAI (0.044).

The highest optical density was seen in T_1 (0.803 mg/L) and T_2 has also shown some promising optical density (0.666 mg/L) when compared to T_1. The lowest optical density was seen in T_4 (0.160 mg/L) which indicates that the dosage of urea has too much for this treatment and it is slowly killing the culture as the OD was significantly low at 15 DAI (0.160 mg/L) when compared to the OD seen in 3 DAI (0.170 mg/L).

Among the treatments, the pH was relatively stable all throughout the experiment. The highest was pH recorded in T_1 (9.02) at the time of 9DAI and the lowest pH was recorded in T_4 (8.67) at the time of 6DAI. The variation of pH among the treatments T_1, T_2, T_3 and T_4 was not significant.

5.2 Conclusion

From the result and discussion above, it can be concluded that the *Spirulina platensis* grown under various dosages of urea solution showed significant variation in the studied characteristics. Although, sodium nitrate present in standard ZM seemed more optimal for *S. platensis* growth, urea can also serve as an alternative nitrogen source, since this cyanobacterium shows utilization of these nitrogen source at low concentrations. As concentration over 0.02M seriously hampers the growth and development of the *Spirulina platensis* strain.

5.3 Suggestions

It is clear from the preceding discussion that fully substituting urea for sodium nitrate is not a ‘silver bullet’ solution to achieve the optimum growth and development of the *Spirulina platensis* culture. But, namely MZM containing 0.01M urea solution (T_2) has shown promising result. As urea is much cheaper and easily available in Bangladesh, it may be used as a supplement of nitrogen rather than a substitute. Nevertheless, this growth media management challenges can be resolved with continued research in this sector.

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APPENDICES

Appendix I. Monthly record of air temperature, relative humidity and rainfall of the experimental site during the period from 1 March 2018 to 15 March 2018

Date	*Air temperature (°C)		*Relative humidity (%)	*Rainfall (mm) (total)
	Maximum	Minimum		
1 March 2018	30.2	28	78	-
2 March 2018	32.4	26.8	74	-
3 March 2018	29.5	27.4	76	-
4 March 2018	30.3	26.7	77	-
5 March 2018	31.4	29.6	74	-
6 March 2018	33.7	27.1	75	-
7 March 2018	34.7	28.5	78	-
8 March 2018	32.8	26.8	82	-
9 March 2018	33,1	30.1	79	-
10 March 2018	32	28.9	77	-
11 March 2018	30.4	28.5	78	-
12 March 2018	29.7	27.1	76	-
13 March 2018	31.2	29.7	79	-
14 March 2018	31.1	29.5	78	-
15 March 2018	32.3	27.2	77	-

*Source: Bangladesh Meteorological Department (Climate & weather division) Agargaon, Dhaka-1207

Appendix II. Analysis of variance on the measurement of Cell weight (dry basis) at different days after inoculation of *Spirulina platensis*

Source of Variation	Degrees of freedom	Mean Square for Cell Weight (Dry Basis)				
		3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
Factor A (Treatments)	3	498.111*	3904.97*	15057*	43666.8*	37796.8*
Error	8	26.167	44	516	462.6	361.3

***: Significant at 0.05 level of probability**

Appendix III. Analysis of variance on the measurement of Chlorophyll a content at different days after inoculation of *Spirulina platensis*

Source of Variation	Degrees of freedom	Mean Square for Chlorophyll a				
		3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
Factor A (Treatments)	3	0.024	1.149*	2.368	2.563*	4.99*
Error	8	0.009	0.05	0.042	0.022	0.031

***: Significant at 0.05 level of probability**

Appendix IV. Analysis of variance on the measurement of pH at different days after inoculation of *Spirulina platensis*

Source of Variation	Degrees of freedom	Mean Square for Specific Growth Ratio				
		3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
Factor A (Treatments)	3	1.143*	1.560*	2.144*	2.258*	1.830*
Error	8	8.16	4.525	1.542	1.933	1.642

***: Significant at 0.05 level of probability**

Appendix V. Analysis of variance on the measurement of Optical Density (OD) at different days after inoculation of *Spirulina platensis*

Source of Variation	Degrees of freedom	Mean Square for Optical Density (OD)				
		3 DAI	6 DAI	9 DAI	12 DAI	15 DAI
Factor A (Treatments)	3	3.097*	0.0422	0.197	0.224	1.198
Error	8	1.167	0.0002	0.0004	0.0003	0.0004

***: Significant at 0.05 level of probability**

Appendix VI. Analysis of variance on the measurement of pH at different days after inoculation of *Spirulina platensis*						
Source of Variation	**Degrees of freedom**	**Mean Square for pH**				
		3 DAI	**6 DAI**	**9 DAI**	**12 DAI**	**15 DAI**
Factor A (Treatments)	3	0.072	0.103	0.19778	0.133	0.045
Error	8	0.007	0.01	0.009	0.0108	0.01
***: Significant at 0.05 level of probability**						