Laboratory scale cultivation, Nutritional Evaluation and Development of Fortified Food from Spirulina sp. (*Arthrospira fusiformis)*

Isolated from Lake Chitu, Oromia, Ethiopia

Demile Idao Mechi

A Thesis Submitted to the Department of Applied Biology

School of Applied Natural Science

Presented in Partial Fulfillment of the Requirement for the Degree of Master's in Biology Specialization in Applied Microbiology

Office of Graduate Studies

Adama Science and Technology University

July, 2024

Adama, Ethiopia

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Seid Mohammed (PhD)

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DECLARATION

I declare that this thesis entitled **"Laboratory scale cultivation, Nutritional Evaluation and Development of Fortified Food from Spirulina sp. (*Arthospira fusiformis*) Isolated from Lake Chitu, Oromia, Ethiopia"** is my own work and has not been submitted to any university for similar purpose. The references used in this thesis are duly recognized by proper citations.

Name of student	Signature	Date

RECOMMENDATION

We, the major advisor/supervisor of this thesis hereby certify that we have closely advised/supervised and read the revised version of the thesis entitled "**Laboratory scale cultivation, Nutritional Evaluation and Development of Fortified Food from Spirulina sp. (Arthospira fusiformis) Isolated from Lake Chitu, Oromia, Ethiopia**" prepared under our guidance by **Demile Idao** submitted in partial fulfillment of the requirements for the degree of Masters of Science in Applied Biology. Therefore, I recommend the submission of revised version of the thesis to the department following the applicable procedures. .

Major Advisor	Signature	Date
Co-advisor	Signature	Date

APPROVAL PAGE

I/we, the advisors of the thesis entitled "**Laboratory scale cultivation, Nutritional Evaluation and Development of Fortified Food from *Spirulina sp.* (Arthospira fusiformis) Isolated from Lake Chitu, Oromia, Ethiopia"** and developed by **Demile Idao**, hereby certify that the recommendation and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by **Demile Idao** have read and evaluated the thesis entitled "**Laboratory scale cultivation, Nutritional Evaluation and Development of Fortified Food from *Spirulina sp.* (Arthospira fusiformis) Isolated from Lake Chitu, Oromia, Ethiopia**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfilment of the requirement of the degree of Master of Science in Applied Microbiology.

Chairperson	Signature	Date
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Final approval and acceptance of the thesis is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the Department Graduate Council (DGC) and School Graduate Committee (SGC).

Department Head	Signature	Date

School Dean	Signature	Date
Office of Postgraduate Studies, Dean	Signature	Date

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ANOVA	Analysis of variance
AOAC	Association of official Analytical chemists
CSA	Central statistics agency
EF	Essential fatty acids
FAs	Fatty acids
FDA	Food Drug Administration
GRAS	Generally Recognized as Safe
HDE	Helix diameter at end
HDM	Helix diameter at middle
HP	Helix pitch
MD	Mean diffence
MUFAs	Monounsaturated
NASA	National Aeronautics and space Administration
NCT	Number of trichome per trichome
OD	Optical density
PUFAs	Polyunsaturated fatty acids
SCP	Single-cell protein
SE	*Standards Error*
SFAs	Saturated fatty acids
SM	Spirulina media
TD	Trichome diameter

TL	Trichome diameter
UNESCO	United Nations Educational, Scientific and cultural organization
UV-Vis	UV Ultraviolet-visible spectroscopy
ZSD	Sechi disk depth

ABSTRACT

Microalgae offer great promise in the production of food supplements for humans. Spirulina is reach in various nutritional contents. Thus, this research aimed at evaluating Spirulina's nutritional contents and formulate food fortified with Spirulina sp. obtained from Lake Chitu. For this study, appropriate sampling stations were carefully selected in Lake Chitu and Spirulina sp. containing samples were collected from the selected sampling stations. The growth media was prepared for algal culture in the laboratory. Trichome of Spirulina was isolated from water sample using the serial dilution technique. Then, Spirulina sp. was separated and cultured using Zarrouk medium in a small flask. The pure culture was introduced into 500 ml sized Erlenmeyer flask. After enough pure culture of cell density was achieved in the Erlenmeyer flask, the inoculums were transferred to 1000 ml sized Erlenmeyer flask and then serially transferred to larger Erlenmeyer flasks and the 40-l aquarium. The trichome was observed using a light microscope for their morphological profile. The morphotypes and morphological parameters were recorded for Spirulina isolates. Growth measurement was estimated for Spirulina isolate. Nutritional profile analysis was conducted to determine the composition of protein, carbohydrates, lipids, and minerals. Food fortifications were performed using Spirulina powder. Analysis of the nutritional profile of Arthrospira fusiformis and the selected food was carried out. All data was performed via the t-test or analysis of variance (one-way ANOVA), followed by Turkey's Post Hoc test at a significance level of 95 % ($p < 0.05$), using the IBM SPSS (version 24).In this study, tightly coiled (H-type), spiral or loosely coiled (S-type), and intermediately coiled (C-type) morphotypes were obtained against the samples Spirulina isolates. Morphological parameters such as Trichome length (TL), Helix diameter at end (HDE), middle (HDM), Trichome diameter (TD), Hilex pitch (HP), and No of coil per trichome (NCT) were found. The highest number of morphological parameters was recorded against TL (302.88±133.81) for S-type morphotypes whereas the lowest was recorded against HDE (7.06±1.46) for the same morphotypes. The lowest (12.3±5.87) and highest (23.85±11.08) NCT were obtained from C-type and H-type, respectively. No HP was recorded for H-type morphotypes. The $OD_{680\ nm}$ was recorded over 0.0152±0.0002 to 1.795±0.004 against 0 to 21 day of incubation period for Arthrospira fusiformis. The Spirulina powder contained higher quantities of all the studied macroelements than the microelements. Furthermore, total proteins were found in the supplemented food than in controlled food (18.2% and 7.7%, respectively). The research showed significant differences in the content of protein and essential minerals such as Na, Fe, Zn,and K between supplemented food and controlled food. Thus, the results of this study indicated that the Spirulina is a promising source of many mineral as well as serve as a rich

source of protein confirming the importance and role that Spirulina can play to improve the health and nutrition particularly in malnourished populations.

***Keywords**: Algal cultivation, Food fortification, Microalgae, Nutritional value, Spirulina*

1. Introduction

1.1. Background of study

Algae are veritably protean in their modes of nutrition. Most algae are photoautotrophs that is they use energy from light, CO_2, and water to produce organic compound through oxygenic photosynthesis (Beardall & Raven, 2016). Algae are categorized into two groups based on their biological structure: macroalgae (red, green, and brown seaweeds) and microalgae (blue-green algae; normally a unicellular organism) (Jalilian et al., 2020). Microalgae are unicellular photosynthetic organisms, forming the base of the aquatic food web. This is a group of heterogeneous microorganisms having a great biodiversity of colors, shapes, and cell characteristics with a size of approximately 3 – 10μm (De Morais et al.,2015). The term microalga includes prokaryotic and eukaryotic organisms, which can grow in fresh or marine water. With their extraordinary biodiversity, more than 50,000 species of microalgae represent an important resource with a wide range of active biomolecules. Only some species of microalgae are authorized for human consumption and have exceptional nutritive properties such as *Spirulina* or *Arthrospira species*, *Chlorella vulgaris, Dunaliella* and *Nostoc* (Mata et al., 2010). These micro-organisms are announced as major elements in the challenge of global nutrition, in recent year, *Spirulina* has attracted the attention of numerous scientists. It is a non-poisonous prokaryotic cyanobacterium with the advantage of containing high value-added compounds such as proteins (50 – 70%) of its dry weight (Vernes et al.,2019).

The cyanobacteria are prokaryotic Gram-negative oxygenic photosynthetic autotrophic organisms and as a group are veritably successful, being set up in most found in most habitats on earth, from aquatic to terrestrial, as well as extreme habitats such as hot springs, salt lakes, deserts, and the polar regions (Khalifa et al., 2021). The multicellular filamentous, alkaliphilic cyanobacterium *Spirulina sp.* extensively culture around the world as both a source of food and as a source of blue color cyanophycin which is used as cosmetics and food (Richmond & Hu, 2013).

Spirulina sp. have been employed for human consumption since 16th century (Lafarga et al., 2020). *Spirulina species* are a group of photosynthetic microorganisms that originated 3.5 billion years ago. *Spirulina* has been reported as a spiral shaped type of Cyanobacteria that can

exist within fresh-water bodies, lakes, in sea water and brackish water bodies (Saad & Atia, 2014)

The name of *Spirulina* has been obtained from its spiral structures of filaments. The *Spirulina* has been found to be flourished in the alkaline region which is pH10-12 and belonging to the members of *Cyanophyta, Cyanophycean,* and *Oscillatoria* families (Trottaetal., 2022). *Spirulina* was employed as a food source for the Mayas, Kanembu and Toltec's societies in Mexico during the Aztec civilization. It is a well-known and valuable food supplements that rich in amino acids, fatty acids, minerals, vitamins, proteins and It is also used for animal nutrition (Asghari et al., 2016). For instance, it has been confirmed that *S. platens* has many uses in a healthy diet because it is high in phenols, polysaccharides, polyunsaturated fatty acids, protein and vitamins (Abu-Taweel, Antonisamy, et al., 2019; da Silva et al., 2019). *Spirulina* spp. have recently served as a significant biomedical source (Abu-Taweel, et al., 2019). It is also extensively employed as a nutraceutical food supplement almost throughout the world owing to its high levels of functional compounds including such as polysaccharides, phycocyanins, and phenols (Trotta et al., 2022). It is rich in various nutrients such as carotenoids, microelements, and phycocyanin with a blue pigment protein strong free radical scavenging activity. It has been approved as a food source by the US Food and Drug Administration (FDA) (McCarty, 2007; Nuhu, 2013).

These *Spirulina* species have been reported for various economic importance. For instance, having biological value, these microbial cells are employed for body weight gaining, and facilitate the digestive system with better protein efficiency ratio (Srivastava et al., 2015). It has been stated that *Spirulina* spp found to be employed for mitigation of certain toxic heavy metal concentration from natural ecosystems (Bhattacharya, 2020). *Spirulina* has also found to be shown to perform a regulatory role for the carbohydrate and lipid metabolisms (AlFadhly et al., 2022). These *Spirulina* spp have also found to be important as a protective role in order to alleviate the toxicity of sodium dodecyl sulphate effects on *Clarias gariepinus* (Sayed & Authman, 2018). Certain of *Spirulina* spp have been used as therapeutic food to control glycemia and lipidemia disease in a type 2 diabetes with a considerable high concentration (Koyande et al., 2019).

Diversity of *Spirulina* sp. are existed in the various water body. But, the most dominant *Spirulina* species are belonging to *Spirulina maxima and Spirulina platensis* with various morphology and biological structures (Gentschewva et al., 2023). It was reported that *S. platensis* is phycoremediation and /or phyco-valorisation of brewery effluents with integration of the phycobiliprotein and phycocianin biosynthesis which is a promising mixotrophic microalgae (Querques et al., 2015). S. *platensis* was also found to contain exopolysaccharide. It is a photosynthetic type of microalgal species. S. *platensis* has been found to be associated with NADH *biosynthesis* in the central metabolic pathways (Santo et al., 2019). Its protein content has also increased with its biomass that increased with increasing phosphorus concentrations. However, the under phosphorus limitation condition, the protein content of *S. platensis* has been reduced due to accumulation considerable amount of carbohydrates (da Silva et al., 2018). Like others microalga cells, *S. maxima* found to employed as a potential candidate for SCP production (Rajkumari, 2019).These *Spirulina* species are one of the most important biological organisms employed for food security having blossomed national value, and health benefit for all type people. It can be also used to generate incomes for these low incomes which is thus employed for creating job opportunity. Therefore, the current study aimed at evaluating of nutritional contents for *Spirulina sp.* and development of fortified food for it products.

1.2. Statement of problem

Despite the natural abundance of *Spirulina* in Ethiopia, its nutritional and functional values have not been well studied so far. However, the issue of malnutrition, particularly among women and children continues to pose a monumental challenge to national and regional development efforts.The prevalence of stunting, underweight and westing have high, with 39.3 percent of children under 5 years stunted, 28.6 percent underweighted and 16 percent wasted (Lamessa et al., 2023).This study will evaluate nutritional content of *Spirulina* and development of fortified foods *Spirulina* in diets in order fighting of the mal nutrition problems. Particularly with children, to promote extensive production, consumer acceptance, industry utilization and demand of the microalgae in food systems in Ethiopia.

1.3. Objectives of the study

1.3.1. General objective

- To evaluate nutritional contents and development of fortified food from *Spirulina sp* obtained from Lake Chitu, Oromia, Ethiopia

1.3.2. Specific objectives

- To assess the physico-chemical quality of the water in Lake Chitu.
- To isolate and characterize Trichome of *Spirulina* morphology from sampled water collected from Lake Chitu.
- To conduct qualitative and quantitative assessment of the nutritional value of spirulina sp.
- To cultivate and biomass production of Spirulina SP. in laboratory scale.
- To develop fortified food from *Spirulina sp.*

1.4. Significance of the study

Ethiopia is in the phase of a fast development process, in which agriculture has a profound role to play. Food security is one of the major factors essential for the development of any nation, without which a healthy population cannot be expected to survive. Malnutrition as well as adulterated and unsafe food causes health problems among the population. Microalgae offer great promise in the production of food supplements for humans. *Spirulina* is a microscopic blue-green algae and it is considered one of the richest sources of organic nutrients, making it a good nutritional supplement for humans worldwide. This study will be useful in improving health and nutrition, particularly in malnourished populations in Ethiopia. The scientific information that resulted from the study will be used as base line information for further research and food industries.

2. Literature review

2.1. History of *Spirulina SP.*

Spirulina sp. standout among the most cultivated microalgae that has been utilized as complete nourishment and additive since 1970s because of its rich nutrient supplements such as proteins, carbohydrates, minerals, and vitamins (De Bhowmick et al.,2014) It has gained significant acceptance in the food industry in the various countries of the world. *Spirulina* has become a major source of protein, human health food, and vitamin supplement to aquafeeds (Soni et al., 2021). For centuries native peoples have harvested *S. platensis* from chad lake in Africa and Texico in Mexico for use as source of food a fact which means that *Spirulina* deserves special attention both as a source of (single cell protein (SCP). and because of nutraceutical properties (Colla et al.,2007).

Spirulina is a microalga that has a unique set of natural characteristics which are very useful for a broad range of operations. It can be used as a nutritional supplement or pharmaceutical cumulative with no pitfalls to health. *Spirulina* is useful for human nutrition, because of the high quality and quantity (60- 70 of its dry weight) of protein (Prasad et al., 2014). *Spirulina platensis* is a blue-green microalga, which has been retailed and consumed as a human food and has been approved as a food for human consumption by numerous governments one of them is Egyptian health agencies and associations. It's one of the naturally being sources of protein, which, reaching to five times that of meat and can give the majority of essential and non-essential amino acids (El- Moataaz et al., 2019). *Spirulina* (phylum Cyanobacteria) is an ideal nutritive supplement and can be the answer to malnutrition problems in developing countries. *Spirulina* contains a remarkable concentration of nutrients, thereby making it a whole food alternative to isolated vitamin and mineral (Bhattacharya & Shivaprakash, 2005).

2.2. Cultivation of Spirulina

Among commercially exploited microalgal species, Spirulina (commercial name for Arthrospira) has been cultivated on a large scale for over 40 years as a food source and animal feed, owing to its high nutrient content and easy digestibility (de Jesus et al., 2018).The large-scale commercial cultivation of Spirulina began in the 1970s in Mexico whereas currently, Arthrospira sp. is commercially produced in various parts of the world including China,

Thailand, India, Australia, and the U.S.A. particularly the production of Spirulina is fast-growing in the Asia-Pacific region (Ma et al., 2019). According to the International Energy Agency (IEA), the worldwide annual production of Spirulina is approximately 10000 tons (dry biomass), half of which is produced by China (Costa et al ., 2019). Typically, open raceway pond systems are used for the commercial cultivation of Spirulina (Thevarajah et al., 2022).

2.3. Physicochemical property of water with spirulina

Numerous chemical constituents and physical characteristics, including temperature and turbidity, are known to be present in water. The composition, distribution, and abundance of aquatic ecosystems are significantly influenced by the interaction of water's physical and chemical properties (Ferencz & Dawidek, 2021).Soda Lakes are well-known for their distinctive diversity, advantages for science, economic importance, and scenic attraction. Currently, these in threat due to both natural and human-caused occurrences (Nkambo et al.,2015).The seasonal variations in the wet and dry seasons naturally cause variations in the physicochemical parameters of saline lakes. The biota that inhabits saline lakes is directly impacted by these changes (Melese & Debella, 2023).

Lake Chitu is one of the a soda lake in Ethiopia, having extremely high primary productivity and algal biomass associated with the super abundance of Arthrospira and supporting the huge flocks of the Lesser Flamingos (Ogato et al., 2015). Basically,sirulina develops in water that contains different minerals and is free of contaminants that prevent nutrient intake, unlike terrestrial crops that are produced on soil. Hence, water with proper nutrient supplementation enhances nutrient uptake, promoting microalgal growth and intracellular substance accumulation. Spirulina is capable of growing in high alkalinity with the presence of carbonate, bicarbonates and inorganic nitrogen (Yiglet & Damitew, 2023).Lake Chitu is a creator lake situated in the central refit valley, highly salty, surrounded by hot springs flow into the lake with high $CaCO_3$ containing types of soil. The high salinity and alkalinity with temperature makes Chitu Lake the preference habitats of Spirulina SP.(Ogato et al., 2015).

Table 1. Geographical and limnological features of Lake Chitu (data source: Elizabeth Kebede *et al*., 1994; Otago et al.,2014; Melese & Debella, 2023).

Variables	Values
Geographic position	7 º 24' N 38º 25' E
Altitude (m)	1600
Maximum depth (m)	21
Surface area (km^2)	0.8
pH	10.46
Conductivity (K25, µS cm^{-1})	49100
Salinity (g L^{-1})	37.45 g/L
Na^+ (mgL^{-1})	20,500
K^+ (mgL^{-1})	1,200
Ca^{2+}(meq L^{-1})	0.16
Mg^{2+}(meq L^{-1})	0.05
HCO_3^- + CO_3^{2-} (meq L^{-1})	758
Cl^- (mgL^{-1})	12,336.6
SO_4^{2-} (meq L^{-1})	21.1
SRP ((mg L^{-1})	1.75 ± 0.34
NO_3^- +NO_2 (µg L^{-1})	ND
NH_4^+ (µg L^{-1})	ND
SiO_2 (mg L^{-1})	222

ND nondetectable

2.4. Microalgal Biomass Production Systems

Microalgae are microscopic organisms that have been utilized in a wide range of industries and their biomass are used with in production of nourishment and supplements, feed, cosmeceuticals, nutraceuticals, and pharmaceuticals (Mu et al., 2019). Microalgae are grown in an aquatic environment, which must be kept under appropriate conditions of light, temperature, pH, and CO_2 concentration (Bear et al.,2016). Biomass production of microalgae connects positively with nutrient concentration, particularly nitrogen and phosphorus which

must be sufficient for microalgae to achieve maxima biomass productivity (Yaakob et al., 2021). Under natural conditions, microalgae utilize sunlight, whereas under laboratory or industrial conditions, artificial lighting is most frequently utilized (Carvalho et al., 2011). Generation of huge amount of biomass requires optimization of cultivation conditions. There are numerous components that influence microalgae growth. Other than nutrients accessibility, basic environmental parameters for cultivation include light, temperature, and pH. have a positive impact on *Spirulina* sp. and *D. Salina* providing optimal growth conditions enhanced biomass yield and productivity (Hawrot & Sąsiadek, 2023).

Microalgae biomass production is usually accomplished utilizing an open or closed system**.** The open system utilized in microalgae biomass production is a pond-like type of system**,** in spite of the fact that, the open systems are utilized in large scale microalgae cultivation due to low power demand**,** simple cleaning handle, low construction cost and appropriate scale-up (Costa & de Morais, 2014). The closed system of microalgae biomass generation includes the utilize of a closed photobioreactor for the development of microalgae biomass. This system has high microalgae biomass productivity and a high reduction within the risks of contamination and favors the capacity of microalgae to fix and convert CO_2 into microalgae biomass. The mechanism included within the generation of biomass in both open and closed systems by microalgae depends on the mode of nutrition of the them (Randrianarison & Ashraf, 2017). Microalgae are most often grown in an autotrophic method in open pond systems or in photobioreactors. Usually since the development prerequisites of autotrophic microalgae tend to contrast from those of heterotrophic and mixotrophic microalgae. Additionally, the growth conditions of mixotrophic microalgae tend to contrast from those of heterotrophic microalgae (Ugya & Meguellati, 2022).

The mechanism of biomass generation by autotrophic microalgae includes the utilization of CO_2 and water within the presence of sunlight and the utilization of nitrate, phosphate, and other basic elements display in open and closed systems (Mohammad Mirzaei et al., 2016). The autotrophic culture method is considered the most economical and most viable method since a high biomass yield is achieved. The mechanism includes within the production of biomass by heterotrophic microalgae by the utilization of organic carbon substrate as source of carbon and energy includes glycerol, glucose and acetate (Ugya & Meguellati,2022). The

process occurs in dark, since it is free from light. Mixotrophic method of microalgae biomass production includes the use of light and organic source of energy for the production of microalgae biomass. The method moreover utilizes both inorganic and organic carbon, leading to higher biomass production. The method produces the microalgae biomass that riches more lipids, carbohydrate, proteins and other biomolecules since the method overcomes the limitation of both heterotrophic and autotrophic method of microalgae biomass production because both standards are combined in the mixotrophic method (Zhan et al., 2017). *Spirulina* sp. can grow either photoautotrophically (source of energy: light, source of carbon: CO_2), heterotrophically Under darkness, can use organic compounds as carbon and as energy sources. (source of energy and carbon: glucose) and mixotrophically (source of energy: light and source of carbon: glucose and CO_2) (Chojnacka, & Zielińska,2012).

2.5.Growth condition and factor affecting cultivation of *Spirulina* sp.

Like other autotrophic organisms, *Spirulina* grows best when certain conditions are fulfilled. Microalgae growth and compositions depends on the nutrient accessibility in the medium, the distribution of the radiant energy inside the culture, and environmental stresses (Vonshak & Tomaselli, 2000). Light, temperature, agitation, culture medium, and water source are very important factors for growing *Spirulina. Spirulina* can be one of several algal species, under the normal water conditions. The more alkaline water becomes, the further negative it's to other life forms, there by permitting *Spirulina* to thrive as a single species. *Spirulina* thrives in alkaline lakes where other microorganisms are difficult or impossible to survive (Soni et al., 2021)

2.5.1. Light

Light is one of the most important parameters to consider. Light distribution inside the reactor doesn't only affect the morphology of the filaments but also has a strong influence on the growth rate and the composition of microalgae. There are studies about the effect of light intensity on the growth rate, lipid, carbohydrate, and protein content as well as pigment concentration (Zapat at al.,2021). Via process known as photosynthesis, all phototrophic organisms, including of higher plants, photosynthetic microorganism, and cyanobacteria, are capable of convert the energy from light into chemical energy. High light intensity affects

maximum growth the rate, while as a low intensity leads in the synthesis of biomass rich in pigments and proteins, increase the concentration of cultured cells, increases self-shading, and leads to a decrease growth rate of *Spirulina* (AlFadhly et al.,2022). Both the quality and intensity of light are essential factors in the manufacturing of algae. The majority of photosynthetic plant cells, along with those of *Spirulina*, derive maximum of their required energy from light. They may be capable of capturing light energy from the sun or from artificial light sources, and they convert inorganic carbon to organic carbon-based compounds such as lipids, proteins, carbohydrates, pigments, and phenols via photosynthesis. Their growth is slowed when the amount available to them is lowered. *S. platensis* and *S. maxima* are well-known species that growth at an excessive optical density that eventually reaches saturation. Their light intensities variety 150 to 200 μmoL photons/m /s and from 420 to 504 μmoL photons/m /s, respectively (Mogale, 2016).

2.5.2.Temperature

The temperature is one of the most critical environmental factors that can influence on the rate at *Spirulina* grows. *Spirulina* does not grow below 17 °C, but it does not perish above 38 °C. The top-quality temperature for growth is 35 °C, however temperatures which are higher than 38 °C both prevent or disrupt its growth (Batista et al.,2019). It became validated that temperatures of 35 °C decreased the amount of biomass *Spirulina*, while temperatures of 30 °C promoted its growth. That is because *Spirulina* is capable of eat carbon dioxide (CO_2) quickly (ten to fifty times quicker than terrestrial flora because of rapid growth rate) and convert it into biomass via the process of photosynthesis (Mogale, 2016). At 25 °C in the morning and evening, the photosynthetic activity of the culture shifted from being directed towards the synthesis of carbohydrates from it towards the synthesis of protein (Ahsan et al.,2008).

2.5.3.pH

For *Spirulina* to flourish, the environment where in it's grown must be pretty alkaline, and it must have access to bicarbonate ions. The upward thrust in carbon dioxide ranges within the surroundings has an influence on photosynthesis as well as the development of *Spirulina*; nevertheless, it did not produce any change in the maximum growth rate, a loss in biomass

productivity, or any impact in any way on the number of pigments in algae (Celekli et al.,2009). *Spirulina* flourishes in environments with a pH starting from 9 to 11, and its pH rises from 8.5 to 9.5 in the course of the cultivation technique due to the consumption of bicarbonate and sodium ions. The potential of *S. Platensis* to conform to high pH values is pretty unique, because it permits the species to flourish in extremely alkaline situations. From 9 up to 10.5, it varies (Michael et al.,2018).

2.5.4.Agitation and Aeration

It's miles essential to offer sufficient aeration and to move the culture on the way to save the formation of thermal stratification, homogenize and maintain the suspension of the *Spirulina* filaments, distribute nutrients evenly, and prevent the filaments from becoming clumped together. Then *Spirulina* can be cultivated efficaciously. The culture will benefit from the elevated biomass of better exceptional because of this (Dubey, 2006). Similarly, to this, it assists in the uniform distribution of carbon dioxide and gets rid of inhibitory chemical substances such as oxygen. When ventilation is insufficient, there can be a reduction in both power performance and the generation of biomass. Due to the presence of air filled with holes, the floating cells which can be discovered at the surface are situation to photoinhibition, which results in a discount in both increase and biomass production (Delrue et al.,2017).

The agitation has the capacity to have adverse effect on the increase of cultures; for instance, little agitation may cause self-shading, while immoderate agitation can reason culture stress (Michael et al.,2018). Regular mixing, along with the bubble mixing system, more than doubles the efficiency of photosynthesis by way of movement the *Spirulina* cultures to homogenize the scattering of light and shield the filaments from photolysis that happens at some stage in extended exposure to light. However, stirring at high speeds can harm the *Spirulina* filaments and reduces the rate of photosynthesis (Ahsan et al.,2008). The paddle wheel is the most common place stirring tool in commercial *Spirulina*. Special devices can be used for mixing and stirring, consisting of pumps and pneumatic mixing system, as well as gravity and manual agitation and mechanical mixing can be done by way of vibration, stirring, or oscillation (Lee et al., 2013). Proper aeration of *Spirulina* is required satisfy its CO_2 requirement and also aeration does now not allow algae to settle down and form a layer at the

bottom. Aeration may be executed via both mechanical stirring or air pump system (Soni et al.,2019).

2.6. Morphology of *Spirulina*

Spirulina are oxygenic, multicellular, gram negative, they thrive in environments with a pH ranging from 9 to 11, and its pH rises from 8.4 to 9.5 throughout the cultivation process as a result of the consumption of bicarbonate and sodium ions. Photoautotroph, filamentous and non-heterocystous cyanobacterium characterized by coiled spherical fiber or trichomes with a length of 200- 500 μm, width range between 3 and 12 μm and diameter varies from 30 to 70 μm *Spirulina* species are reported to have three different forms as spiral, straight and wavy (Ciferri, 1983). The typical morphology of *Spirulina* is characterized by its regularly coiled trichomes, and the morphological features, such as the degree of spiralization and the arrangement of the spirals, are the main taxonomic criteria for *Spirulina* (Wang & Zhao, 2005). Although the helical shape of the trichome is considered to be a stable and constant property maintained in culture, there may be considerable variation in the degree of helicity between different strains of the same species and within the same strain (Vo et al.,2015). The spiral shape of the filaments (or trichomes) is characteristic of the genus and is maintained only in a liquid environment or culture medium. The presence of gas- filled vacuoles in the cells, together with the spiral shape of the filaments, affect in floating mats. The trichomes have a length of 50 to 500 μm and a width of 3 to 4 μm (Ahsan et al., 2008). The trichome width of *Arthrospira* populations sampled from nature ranges from about 2.5 to 16 μ m, while the helix pitch typically ranges from 0 to 80 μm and its diameter from 15 to 60 μm. The dimensions and other morphological features of *A . fusiformis* not only vary markedly between populations, but also within one population. Both under laboratory and mass cultivation conditions, the helix architecture (pitch and diameter) is highly dependent on growth and environmental conditions. (Abdulqader et al.,2000)

The morphology of *Spirulina* affected by environmental factors like light intensity, nutrient concentration, growth temperature and salinity of medium. It became reported that light intensity and nutrient concentration initiated three morphological versions of *Spirulina. Fusiformis* trichomes forming loose helices, trichomes having tight coiled and trichome extended coiling to very tight variant. High light intensity and high nutrient concentration

which caused the transformation from loose helices to the tighter coil. While the high light intensity and low nutrient concentration lead to forming of very tight trichome (Thammathorn, 2001). Another factor is growth temperature, the increasing of temperature gave rise to more tightly coiled trichome of *Spirulina* (Vonshak, . 2017). Light intensity and nutrient awareness have shown the effect on the morphology of *Spirulina fusiformis.* The culture, which became grown in nutrient deficiency condition, increased in degree of coiling causing tight trichome. At the same time as the cells in high nutrient have loose trichome. Under high light intensity and high nutrient awareness condition the morphological conversion from loose helices to tight coil is induced whereas the excessive light intensity and low nutrient concentration lead to the formation of very tight trichome (Jeeji Bai & Seshadri,1980). *Spirulina* strains have been observed to occur in a varied range of saline habitats which shows its ability to adapt to freshwater, alkaline conditions as well as saline–alkaline and even hypersaline environments (Kaggwa et al.,2013).

2.7.Nutritional value and biochemical composition of *Spirulina*

Spirulina is an incredible natural source of nutrition which has been used since ancient times. *Spirulina* is a major alga which is used as food supplement. The different nutritional compositions of microalgae have been analyzed since 1970. They were shown to be excellent sources of protein, amino acid, minerals etc (Hoseini et al.,2013). The *Spirulina* is known a rich foods source of micro and macronutrients such as protein, vitamins, gamma-linolenic acid, phycocyanin and sulfated polysaccharides. *Spirulina* has a cell wall consisting of proteins, carbohydrates, and fats that are easily digested so that it has more nutritional content than other vegetable food sources. Therefore, *Spirulina* is potentially used as a functional food and supplements that are safe to consume in the right amount. The term functional refers to foods that have been shown to help certain body functions, resulting in promoting health and /or reducing the risk of disease beyond nutritional function. The nutritional composition of *Spirulina* varies greatly dependent on the culture conditions and analysis methods (Liestianty et al.,2019).

2.7.1. Proteins

Spirulina is one of the best sources of proteins. Plant protein is prevalent in *Spirulina* and accounts for 60% to 70% of its dry weight. *Spirulina* offers complete proteins because it contains all nine essential amino acids, which account for 47% of the total protein weight in comparison to beef, which has a protein content of 22%, *Spirulina* has a protein content that ranges from 60% to 70% of its dry weight (Ravindran et al., 2016). For a plant source, this kind of algae has an incredibly high protein content; in fact, it has twice as much protein as the best source of protein found in vegetables. Compared to the percentages present in animal meat and fish (15–25%), soybeans (35%), powdered milk (35%), peanuts (25%), eggs (12%), cereals (8–14%), and whole milk (3%), this amount is significantly larger (Falquet and Hurni, 2007). Depending on the time of harvest, the amount of protein in these algae varies by 10% to 15%, morning harvesting *Spirulina* yield has contained highest levels of protein. *Spirulina's* high protein content and quality make it a valuable addition to human nutrition. A protein's biological value, digestibility coefficient, and amino acid quality all influence how it is nutritious. The three amino acids with the highest concentrations in *Spirulina* are leucine (10.9% of the total amino acids), valine (7.5%), and isoleucine (6.8%) (AlFadhly et al., 2022).

2.7.2. Lipid

Lipids, involving their fatty acids (FAs), are all important human nutrients that can be classified as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), according to the absence or presence of unsaturated bonds. Humans are suitable to synthesize SFAs and MUFAs, but unfortunately, PUFAs with the first double bond on the third or sixth carbon atom (essential fatty acids-EFAs) are essential because they cannot be synthesized by humans. Lipids represent one of the main sources of energy for human metabolic processes. *Spirulina* lipid contains polyunsaturated fatty acids (PUFAs), mainly c-linoleic acid with 30 to 35% of total lipid content, which are functional and structural components of cell membranes, while the main lipid membrane constituents in *Spirulina* are glycolipids and the essential fatty acids from the ω3 and ω6 families (Seghiri et al., 2019).

The total lipid content of *Spirulina* spp. cyanobacterium ranged from 6.4% to 14.3% by dry weight. Despite a low lipid content, seaweeds are rich in ω-3 and ω-6FAs (Ambrozova et al.,2014). The blue-green alga, *Spirulina maxima*, examined in the form of a spray-dried powder, contains 11% of lipid, which has been analyzed in detail with a view to establishing both the classes of lipid present and their fatty acid profiles. The dominant lipids are mono-glyceride-,di-glyceride and probably higher galactosyl diglycerides and phosphatidyl glycerol. The lipid composition varies in different strains of microalgae including *Spirulina* and may also vary according to culture conditions. The lipid accumulation in algae usually increases during environmental stress, including growth under nutrient deficient conditions. The increased concentration of salt affects the rate of respiration, distribution of minerals, ion toxicity, photosynthetic rate and permeability of the cell membranes. It also affects the lipid content both quantitatively and qualitatively (Bhakar et al., 2013).

2.7.3. Carbohydrates

Microalga carbohydrates (e.g., starch, glucose, sugars, and other polysaccharides) normally generate energy and cellular structure. In edible seaweeds the soluble carbohydrates constitute a major part of the total carbohydrate content, whereas in higher plants, insoluble carbohydrates are a major constituent of total carbohydrates (Seghiri et al.,2019). *Spirulina's* total carbohydrate content varied between 15% and 25% dry weight. The carbohydrates in *Spirulina* algae are not composed of cellulose, but rather of a range of sugars, including glycogen, glucose, mannose, galactose, and xylose. As a result, the *Spirulina*'s carbohydrates are easy to digest and packed with nutrients, making them suitable for older people and people with intestinal malabsorption (Jung et al., 2019).

2.7.4. Minerals

Spirulina is rich in minerals such as calcium, potassium, iron, nickel, chromium, magnesium, manganese, copper, sodium, zinc, selenium and lead (koli et al.,2022). The mineral makeup of *Spirulina* is attractive as the iron level is 12 times higher than other food. *Spirulina* is also rich in magnesium, potassium, and calcium and is good for blood rejuvenation and the healthy function of bones and teeth *Spirulina* has a unique quality to detoxify (neutralize) or to chelate toxic minerals, a characteristic that is not yet confirmed in any other microalgae

Spirulina can be used to detoxify arsenic from water and food. It also may be used to chelate or detoxify the poisonous effect of heavy metals (minerals) from water, food and environment (Fayaz et al., 2005).

2.8.Use of *Spirulina* as a Human Food

Spirulina has been used as an additive in a variety of human foods and animal feeds. (Vo et al., 2015*). Spirulina* known as *Arthrospira*, has a long history of consumption. The chemical composition of *Spirulina*, which consists of proteins, carbohydrates, essential amino acids, mineral (Especially iron), essential fatty acids, vitamins, and pigments, has recently sparked interest in its potential health good and nutritional benefits. *Spirulina* is one of the most nutritious nutrients. It contains between 65 and 71 percent complete protein in its natural state. This is more than nearly all other unprocessed foods. It's necessary to consume a sufficient of complete proteins (Colla et al., 2007*). Spirulina* is a nutrient-packed super-food that promotes exceptional health. In addition to their typical nutritive value, super-foods are differenced by their fresh health benefits and sickness-preventing properties. It's the utmost nutrient-packed natural dietary source in its entirety. *Spirulina*, a type of blue-green algae, can be consumed as a dietary supplement. Due to its high nutritive value and various health benefits, *Spirulina* is an excellent nutritive supplement for vegetarians and vegans due to its high protein and vitamin content (Kanojia, 2022).

Spirulina is one of the top trends in the food industries. It's a superfood component, which may be used for the medication of functional foods. Numerous food products containing *Spirulina* are produced daily, similar as bread, biscuits, and pasta (Hussein, et al., 2021). Because of its alimentary value, *Spirulina* has been consumed from a long past time in many parts of the world as a food supplement by humans as well as animals in various forms like a health drink, tablets and powder, etc. In Europe, Japan and North America cyanobacteria tablet have been sold. *Spirulina* known to be the richest source of protein and vitamin and can be used to treat children suffering from malnutrition. *Spirulina* is a rich source of nutrients with., essential amino acids, essential fatty acids, carotenoids, minerals and vitamins, it has been considered as "Food of the future" and ideal food for astronauts by National Aeronautics and space Administration (NASA) (Mohamed et al.,2020**).** As the use of synthetic antioxidants is reduced due to their presumed activity as carcinogenesis as well as the general

refusal of the addition of synthetic food by the consumer (Emami & Olfati,2017).Most of the *Spirulina* available as dietary supplements is sold in the form of dried powder, capsules and tablets. Present forms of food aid by various agencies such as Algama, Phycon and few others, focus on fighting hunger as well as treating malnutrition especially in context of the needs of young children who are at maximum risk (koli et al., 2022)

3. Materials and methods

3.1. Description of the study area

Lake Chitu is located at the south point of Abijata-Shalla National Park (7° 23′ N 38° 24′ E), 286 Kilometers South of Addis Ababa, Ethiopia as shown in figure (1). Some morphometric and physicochemical features of Lake Chitu are given in Table 1.

The lake is a soda-lake with a water salinity of 37.45 g/L, pH of 10.0 and HCO_3^-& CO_3^{2-} alkalinity 758 $meq_{/L}$ (Kebede et al. 1994; Kebede, 1997; Ogato et al., 2015; Assaye et al., 2017). And also they showed the water surface temperature of the lake ranges from 21-24^oC. These highly alkaline-saline conditions of the lake could be ideal for production of *S. platensis* and the species are proven to be found dominating this habitat in immense amount almost as a unialgal

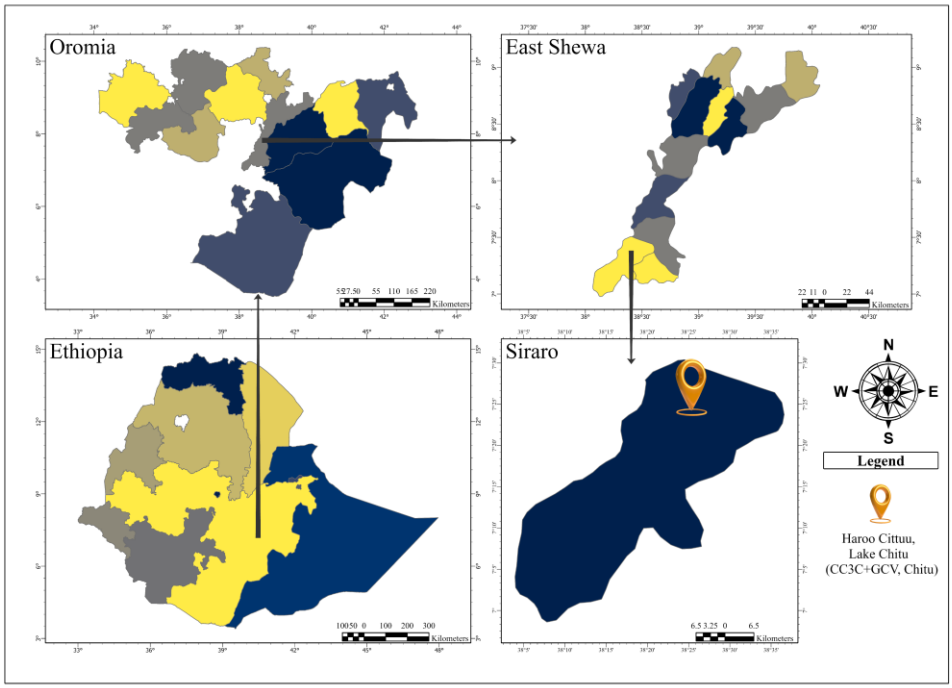


Figure 1: Geographical map of the study area (drawn using ArcGIS Pro 3.0.0)

3.2 Sampling Protocol

The samples were collected in March 16/2023 from Lake Chitu from selected three sites, one central and the other two near shores using plankton nets in sterilized polythene bottles, and *in situ* measurements of some physicochemical parameters were done at these stations. The

samples were taken from the lake's surface, 0.5m and 1m depths from each location using a Van Dorn bottle sampler (horizontal model) and mixed in equal proportions to produce composite samples. The composite sample was used for the determination of total alkalinity and inorganic nutrients. Surface water samples were collected using a phytoplankton net (mesh size 20 µm) in polythene bottles of one-liter capacity and were used to isolate the pure *Spirulina* species and cultivated in the laboratory using Zarrouk medium. The collected samples were transported to the Institute Pharmaceuticals Science laboratory at Adama Sciences and Technology University for *Spirulina* isolation, cultivation, and characterization.

3.2. Measurement of physicochemical parameters of the Lake Chitu

The transparency of the water was estimated using a 20 cm diameter white Secchi disc. Secchi depth (ZSD) was measured using a standard black and white Secchi disc of 20 cm diameter. The depth of the euphotic zone (Zeu) was calculated in meter from ZSD reading using mathematical relationship: Zeu≈ 2.7*ZSD (Salmaso & Padisák ,2007). Some chemical parameters of the lake waters were determined *in situ* and in the laboratory before the experimental culture. pH was measured *in situ* using a digital pH meter (model Jenway 370) and Alkalinity of the unaltered water sample was determined by titration with 1 N HCl while carbonate-bicarbonate alkalinities were calculated from total alkalinity and pH (Clesceri et al., 1999). The composite water samples collected from each site with sterilized plastic containers were used for the analysis of some chemical features in the laboratory. NO_3^- + NO_2-N, NH_3^+ NH_4^+-N and PO_4-P were analyzed using samples filtered through Whatman GF/F (Clesceri et al., 1999). Na-, Cl^-, Ca^+, K^+, Mn, Na^+ SiO2, Bo, Zn, Fe,and Co were tested using spectrometer Arcos_Sop- ICP-OES (model FSH-12-2010).

3.4 Isolation, Characterization and In vitro Cultivation of *Spirulina* sp. and scaling up its culture

3.4.1 Isolation of *Spirulina* sp.

The water samples collected from Lake Chitu were initially cultured in Zarrouk media and trichomes of *Arthrospira fusiformis* were isolated from mixed culture using serial dilution method (Andersen & Kawachi, 2005). Homogenized samples were inoculated growth media. In which,1ml, inoculated in to 10ml growth media, 1.5ml inoculated in to 50ml growth

media and 2ml inoculated in to 100ml. The composition of Zarrouk's culture media is shown in Table 2. The inoculated flasks were incubated in appropriate growth place under fluorescent lamps at a photon flux density of about 20 µmol quanta m^{-2} s^{-1} (produced by two fluorescent lamps, 36 W each), and maintained at 25 degree Celsius and pH 10-11 under 12 hours of light and 12 hours dark variation. The cultures were continuously aerated using air pumps with air stones to improve CO_2 availability. Cultivation was carried out in triplicate and daily microscope investigation was carried out to check the purity and overall cell maintenance. The pH of the medium is continuously controlled over time by pH equipment. During the process of their growth, fresh sterilized Zarrouk media was added to the cultures every four days to amend nutrient accessibility for their growth and scale up the culture. After attaining enough cell density, the pure culture was transferred into the 3 sterilized flasks capacity 500 ml and gradually scaled up into a large volume of 1000 ml sized three Erlenmeyer flasks, 2000ml Erlenmeyer flasks and finally subsequently into two large glass 100 l aquariums under indoor conditions to cultivate enormous biomasses for six months.

Table 1: Standard Spirulina Medium (SM), Zarrouk medium modified by Aiba& Ogawa (1977).

Compoents	**Stock Solution**	**Quantity added**	**Molar Concentration in the Final Medium**
Solution I	500ml	--	--
$NaHCO_3$	--	13.61 g	1.62×10^{-4} M
Na_2CO_3	--	4.03 g	3.80×10^{-5} M
K_2HPO_4	--	0.50 g	2.87×10^{-6} M
Solution II	500ml	--	--
$NaNO_3$	--	2.5 g	2.94×10^{-5} M
K_2SO_4	--	1.0g	5.74×10^{-6} M
NaCl	--	1.0g	1.71×10^{-5} M
$MgSO_4$ $7H_2O$	--	0.2g	8.11×10^{-7} M

$CaCl_2$ $2H_2O$	--	0.04g	2.72 x 10^{-7} M
$FeSO_4 7H_2O$	--	0.01g	3.60 x 10^{-8} M
Na_2EDTA $2H_2O$	--	0.08g	2.15 x 10^{-7} M
Trace metals solution	--	1 ml	--
Na_2EDTA $2H_2O$	--	0.8g	2.15 x 10^{-6} M
$FeSO_4$ $7H_2O$	--	0.7g	2.52 x 10^{-6} M
$ZnSO_4.7H_2O$	0.1g L^{-1} dH_2O	1ml	3.48 x 10^{-9} M
$MnSO_4$ $7H_2O$	0.1 g L^{-1} dH_2O	2ml	8.97 x 10^{-9} M
H_3BO_3	.0.2 g L^{-1} dH_2O	5ml	1.62 x 10^{-7} M
$Co(NO_3)_2$ $6H_2O$	0.02g L^{-1} dH_2O	5ml	3.44 x 10^{-9} M
Na_2MoO_4 $2H_2O$	0.02 g L^{-1} dH_2O	5ml	4.13 x 10^{-9} M
$CuSO_4$ $5H_2O$	0.005 g L^{-1} dH_2O	1ml	10^{-11} M

3.4.2. Morphological identification for *Spirulina* spp

Trichomes were measured carefully in triplicate sub-samples using a light microscope (40X) with an ocular micrometer photo graphed with dual-lens camera (KC8 Spark 4). These morphological parameters included the number of coils (turns) per trichome, helix diameter, helix pitch (distance between coils), diameter, length, ends, color, and abundance of trichomes. The average values of quantitative parameters were determined by measuring of at least 30 trichomes of each morphotype, including large, medium, and small ones.

3.4.3. Determination of growth rate and growth curve of *Spirulina i*solates

The growth of *Arthrospira fusiformis* was determined during the cultivation of batch culture by measuring optical density (OD) using a direct reading spectrophotometer. The absorbance of *Spirulina* isolates was measured at 680nm at intervals of three days for 21 days. Each

measurement of optical density was recorded to make a curve against *Spirulina* biomass concentration versus the growth period (Costa et al., 2004) protocols. Specific growth rate (μ) and doubling time (DT) were calculated using the following equations used for batch culture of microalgae in the exponential growth phase (Vonshak, 1997). following the formula:

$$\mu = \frac{\ln N2 - \ln N1}{t2 - t1}$$

Where: μ= specific growth rate in mg/day; N_1= biomass at time t_1; N_2= biomass at time t_2; t1= initial time; t_2= final time.

Doubling (dt) or generation time of the microalga was calculated from its specific growth rate as follows: $dt = \frac{ln2}{\mu} = \frac{0.693}{\mu}$

3.4.3.2. Biomass Harvest and Drying

After attaining enough cell density, *Spirulina* culture was harvested using silk clothes (filteration method) and washed with distilled water to remove salts. Then, the wet biomasses of *Spirulina* were oven-dried at 40 °C for 24 hours stored in refrigerator at 4°C till their further use.

3.5.Determination of Biochemical composition

3.5.1.Crude protein determination

Nitrogen was determined and crude protein was calculated using the Kjeldahl method according to AOAC standard methods (AOAC,2003). In the procedure, the basic steps include digestion, distillation, titration and calculation.

Digestion: Half a gram (0.5g) of *Spirulina* powder, was heated with 10 ml of concentrated H_2SO_4 in the presence of a one-gram catalyst (K_2SO_4 and Cu). The processes were carried out in a heat-resistant tube at a temperature of ~ 230 °C for 2hrs. This step was a wet oxidation of the sample, and the organic ingredients were originally carbonized, as substantiated by the appearance of a dirty black colour. The mixture was continuously heated, resulting in

complete oxidation to CO_2, and the Digestion became clear. The sample N was converted into a soluble nonvolatile form $(NH_4)_2SO_4$ after the digestion step was carried out.

Distillation: The clear digest was cooled, suitably diluted, and made alkaline by adding 50 ml NaOH (40%, w/v), which releases the N in a quantifiable form as free NH_3. The neutralized digest was also subordinated to steam distillation, which separates the volatile NH_3 from the other ingredient, and the condensed NH_3 was trapped in dilute boric acid (AOAC,2003).

Titration: The final step was quantifying the trapped NH_3 by titration with a 0.1 N standard HCl acid. The quantum of acid used in titration was related to the N content of the sample. The nitrogen concentration was determined according to the following formula: N % =Volume of acid(ml)×normality of acid($moll^{-1}$) ×14 ($gmol^{-1}$) ×100/ Weight of sample(g)×100.

The protein contents were determined against an algal sample. The N content was multiplied by a factor reflecting the N percentage in the protein sample. Proteins contain an average of 16% N, giving a conversion factor of 6.25 = (100). Thus, the protein concentration was determined according to the following formula: Protein = N%×6.25 (AOAC,2003).

3.5.2.Lipid extraction

Total Lipid extraction was carried out using the method suggested by (Pohndorf et al., 2016). Cold extraction. The extraction was conducted to obtain total lipids (neutral and polar). Two grams (2 g) of *Spirulina* powder samples were placed in Erlenmeyer flasks of 250 mL, added with 20 ml chloroform: methanol (2:1 v/v) and stirred for 1 h in a shaker within an ice box. After the extraction period, the samples were filtered to separate the solids. In a separator funnel, 10 mL of aqueous KCl (0.74%. w/v) was added to the sample for extract purification and to aid in phase separation. The lower fraction with chloroform was separated, and the samples were filtered again through anhydrous sodium sulfate Na_2SO_4, to remove water. Finally, the solvent was evaporated in a rotary evaporator. The lipid contents were quantified using the following method:

$$Quantification\ of\ lipid\ (L) = \frac{ml \times 100}{mb}$$

where; ml is the mass of extracted lipids (g) and mb is the mass of dried Spirulina (g)

3.5.3. Carbohydrate extraction and quantification

The total sugar was determined using the standard phenol-sulphuric acid method (DuBois *et al.*, 1956). Ten mg of dry *Spirulina* was suspended in 100 ml of water and the supernatant was collected via centrifugation for 10 min. Then, 0.2 ml supernatant was taken and made up 1 ml of water. One ml of 5% phenol and 5 ml of concentration sulphuric acid were added to the supernatant, and the mixture was allowed to stand for 25 min at room temperature. The concentration of the carbohydrate was measured using Uv-Vis spectrophotometer at 490 nm. The calibration curve was obtained using a range of D-glucose concentrations from (0.01-0.06 mg/ml) to determine the carbohydrate content. Using the formula that $E = y_2\text{-}y1/ x_2\text{-}x_1$ and C=A/E*l. where y_1 and y_2 the absorption of standard concentration and x_1 and x_2 mg/ml the concentration of standards. Concentration C=A/E*l, where C=concentration A= the absorption of carbohydrates extracted, E=epsilon and l= length of cuvet. Finally, the percentage of total carbohydrate content in sample was determined as follows: Absorbance corresponds to 0.1 ml of test sample = xmg of glucose. 100 ml of sample solution contain = X*100 mg glucose/0.1 = % of total Carbohydrate present.

3..5.4. Determination of Moisture content, Dry matter percentage, and total ash

The moisture content, dry matter percentage and total ash were determined by following AOAC

procedure. Moisture content was determined by drying a representative (3.08g) sample at 105°C

for 4 hrs. (AOAC 2000; 925.09). The moisture content and dry matter percentage was calculated

as:

$$Moisture\ \% = \frac{W1 - W2}{W1} \times 100\%$$

where, W1 = weight (g) of sample before drying.

W2 = weight (g) of sample after drying

$$Dry\ Matter\ \% = \frac{mass\ of\ oven\ dried\ sample}{Total\ mass\ of\ sample\ before\ oven} \times 100$$

Total ash was determined by complete burning of the oven dried sample in a muffle

furnace at 500°C overnight and total ash percentage was calculated as:

$$Total\ ash\% = \frac{weight\ of\ ash}{weight\ of\ sample} \times 100\%$$

3.6. Analysis of inorganic elements from *Spirulina*

Macronutrients (Ca, K, Mg, Na, P and S), micronutrients (Cu, Fe, Mn, Ni, Co,B and Zn), and toxic substances such as As, Cd, Cr, Hg and Pb were determined using spectrometer Arcos_Sop- ICP-OES (model FSH-12-2010) (Akbarnezhad *et al.*, 2016).

3.7. Preparation of fortified biscuit with dried *Spirulina*

The biscuits were prepared in the laboratory following commercial formulation which was recommended by experts from food complex companies The study incorporated 5% spirulina into the biscuits. The recipe included 95g of wheat flour, 5g of spirulina powder, 35g of ground sugar, 0.5g of milk powder, 0.7g of salt, 0.5g of sodium bicarbonate, 0.25g of vanilla flavor, and 40ml of olean oil. The mixture was baked in an oven at 180°C for 15 minutes and then packed in plastic bags for further analysis. The proximate composition analysis of both the fortified and controlled biscuits involved determining moisture and ash content using AOAC (2003) methods. Macronutrients and micronutrients were assessed using an Arcos_Sop ICP-OES spectrometer (model FSH-12-2010). Crude protein was quantified using the Kjeldahl method, carbohydrate content using the phenol-sulfuric acid method, and total lipids using the method recommended by Pohndorf et al. (2016).

Table 2: Sample Preparation of fortified biscuit with dried Spirulina

N.S	Sample ingredient	Ratio of ingredients
1	Flour with *Spirulina* powder	95%+5%
2	Flour without *Spirulina* powder	100%

3.8. Statistical analysis

Statistical analysis of the experimental data was performed through the t-test or analysis of variance (one-way ANOVA), followed by Turkey's Post Hoc test at a significance level of 95 % ($p < 0.05$), using the IBM SPSS (version 24). Certain data were computed and analyzed using Origin Pro (2019b version). All results were presented as mean ± standard deviation. The interdependency of variables was analyzed using regression and correlation.

4. Results

4.1. Measurements of some physicochemical parameters of the Lake Chitu

Some physico-chemical parameters of Lake Chitu recorded in this study which are believed to have a bearing effect on the growth of *Spiruluna* are presented in Table 4. During the sampling period, the Secchi depth (Z_{SD}), an estimator of lake water transparency, of Lake Chitu was 0.3m and the euphotic depth (Zeu), surface productive layer, was 0.9m. The pH and surface water temperature for Lake Chitu were also predicted to be 10.6 and 23 ^{o}C, respectively.

Table 4: Some physico-chemical features of Lake Chitu

S. N.	Parameters	Amount
1	Ammonia NH3-mg/L	119.06mg/mL
2	Ammonium NH4-	0.16mg/L
3	Phosphate (PO_4-P)	9.48±1.27mg/L
4	Nitrate (NO_3^- N)	119.04mg/L
5	Sodium (Na^+)	17896.43±233.43mg/L
6	Potassium(K^+)	2262.05±25.03mg/L
7	Calcium(Ca^+)	3.78±0.22mg/L
8	Manganese (Mn)	0.02mg/L

9	Chloride (Cl-)	8,695.55mg/L
10	Silica (SiO2)	128.23±.53mg/L mg/L
	Copper (Cu)	0.02mg/L
	Zink (Zn)	0.02mg/L
	Boron(B)	17.25±0.52mg/L
	Iron (Fe)	0.04mg/L
	Phosphors(P)	67.50 mg/L
	Temp. (T)	23
	pH	10.6
	Carbonate (CO_3^{-2}	21400 mg/L
	Bicarbonate(HCO_3	33750 mg/L
	Alkalinity ($CaCO_3$)	55150 mg/L

4.2. Isolation of *Spirulina* sp.

The isolation and cultivation of *Spirulina* sp. in the laboratory level was mainly achieved considering the pH of the culture between 10 & 11. This included daily monetary availability, nutrient availability, and other growth conditions maintained throughout the experimental times to succeed in cultivating *Spirulina* sp. and obtain enough biomass productivity.

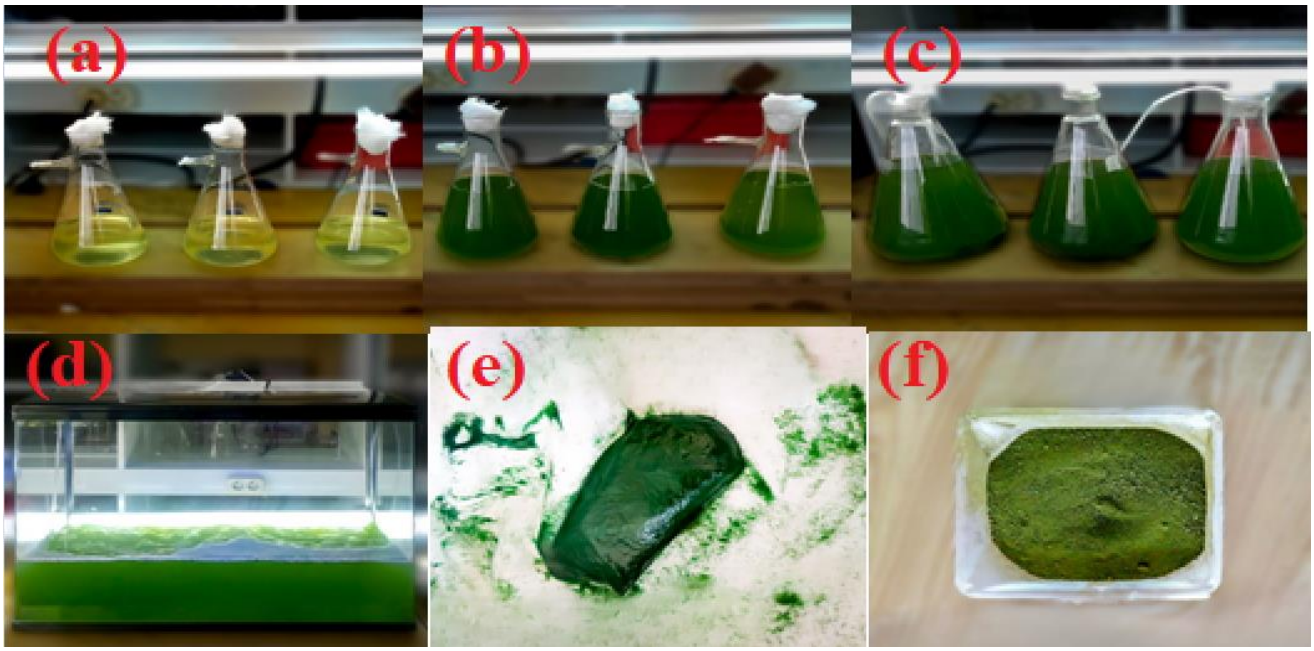


Figure 3. Diagram of Arthrospira fusiformis starts from inoculum up to end products; (a) Inoculate of *Arthrospira fusiformis* (b) Broth culture of *Arthrospira fusiformis* (c) indicated scaled up of *Arthrospira fusiformis* broth culture (2000ml) (d) scaled up of *Arthrospira fusiformis* culture in aquarim (e) *Arthrospira fusiformis* harvested wet biomass and (f) Powder form of *Arthrospira fusiformis* biomass.

4.2.1. Morphological identification of *Arthrospira fusiformis*

Morphotypes of *Arthrospira fusiformis* were identified, including the degree of coiling of trichomes. This study characterized *Arthrospira fusiformis* morphology according to morphological parameters using a light microscope at 40x magnification power with an ocular micrometer. The *Spirulina* contained a water sample collected from Lake Chitu when observed under the microscope during the study, it was noticed that their morphotypes were tightly coiled form, and few were loosely coiled. However, the cultivated *Spirulina* in the laboratory contained three different morphotypes: H type, tightly coiled; S type, loosely coiled; and C type, intermediately coiled. In the current study, the morphotypes of *Arthrospira fusiformis* collected from natural habitats were H types and a few S type. The study demonstrated that the spiral shape was constant. However, the degree of helicity wasn't stable since it was affected by growth conditions like light intensity and nutrients.

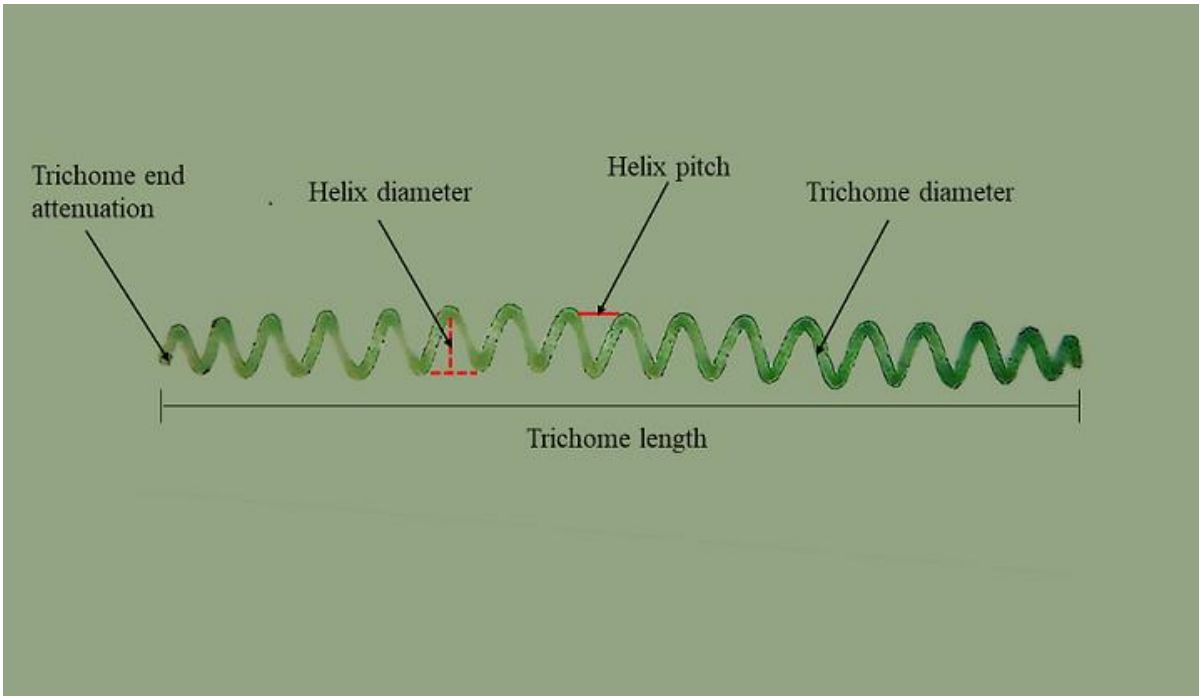


Figure 4: Parts of *Arthrospira fusiformis* filament

After the morphotypes of *Arthrospira fusiformis* had been performed, morphological parameters such as degree of spiralization, trichome end attenuation, helix pitch, trichome length, trichome diameter, helix diameter and number of coiling were obtained. Based on microscopic observation, *Spirulina* isolates were categorized into three morphotypes. These morphotypes were identified as C-type (Figure 5a), H-type (Figure 5b) and S-Type (Figure 5 c & d). In the meantime, it was observed that these morphotype were not stable. It was also noticed that these morphotypes are determined by growth conditions like light and nutrients. H-type forms when high light intensity and low nutrients are in the medium. It can be converted to S-types when there are low nutrients and intensity and S-type changed to C-type when there is high nutrient accessibility and high light intensity in medium.

These various morphotype were belonged to the same species. Lake Chitu is known for its monoalgal population of *Arthrospira fusiformis*.The various morphotype of *Arthrospira* observed in this study belong to a single species,*Arthospira fusiformis.*

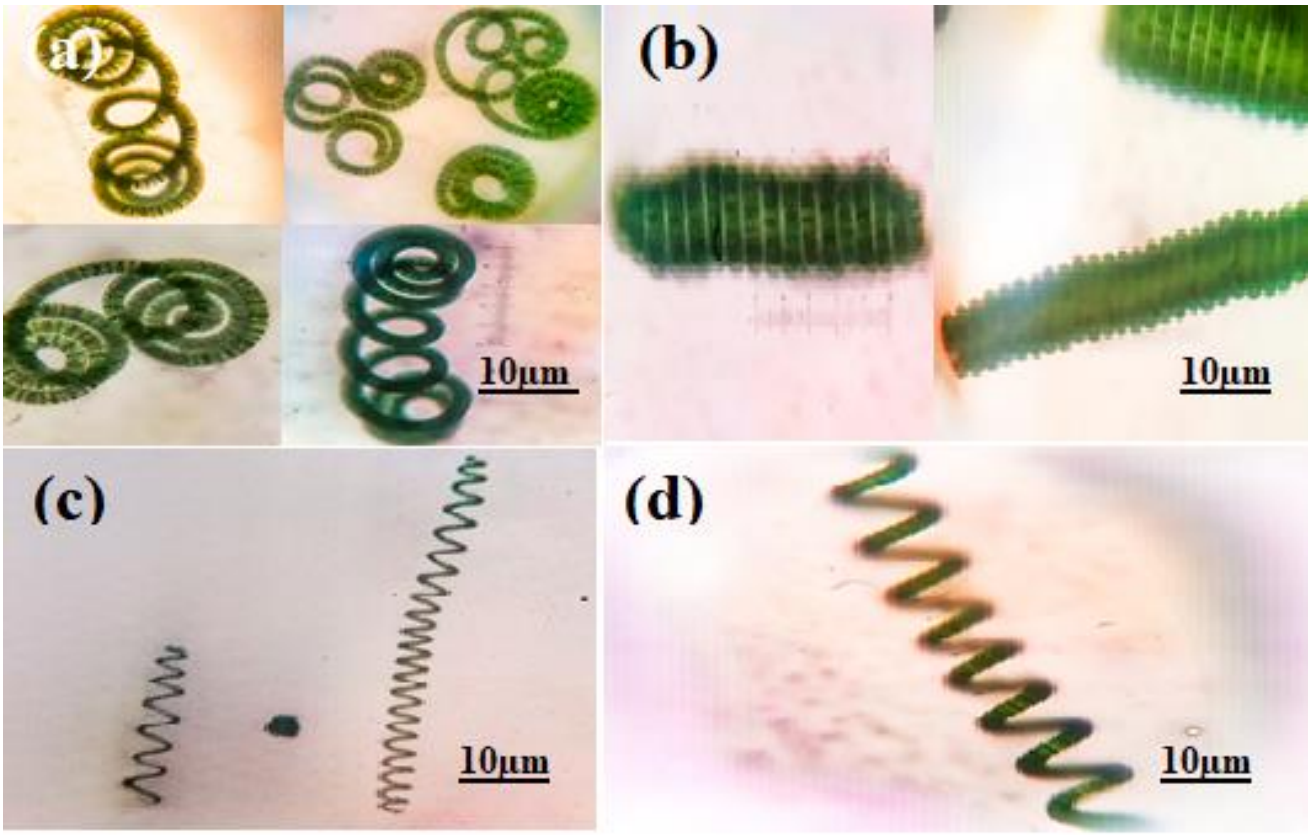


Figure 5: Morphotypes of *Arthrospira fusiformis* sp. That obtained from Lake Chitu water sample. (a) C-type, (b) H-type and (c and d) S- morphotype. Microscope observation from current study.

These above morphotypes were mainly explained by the difference in degree of coiling as highly coiled (H-type), loosely coiled (S-type) and intermediately coiled (C-type). Similarly differences in other parameters have been observed, measured and compared.

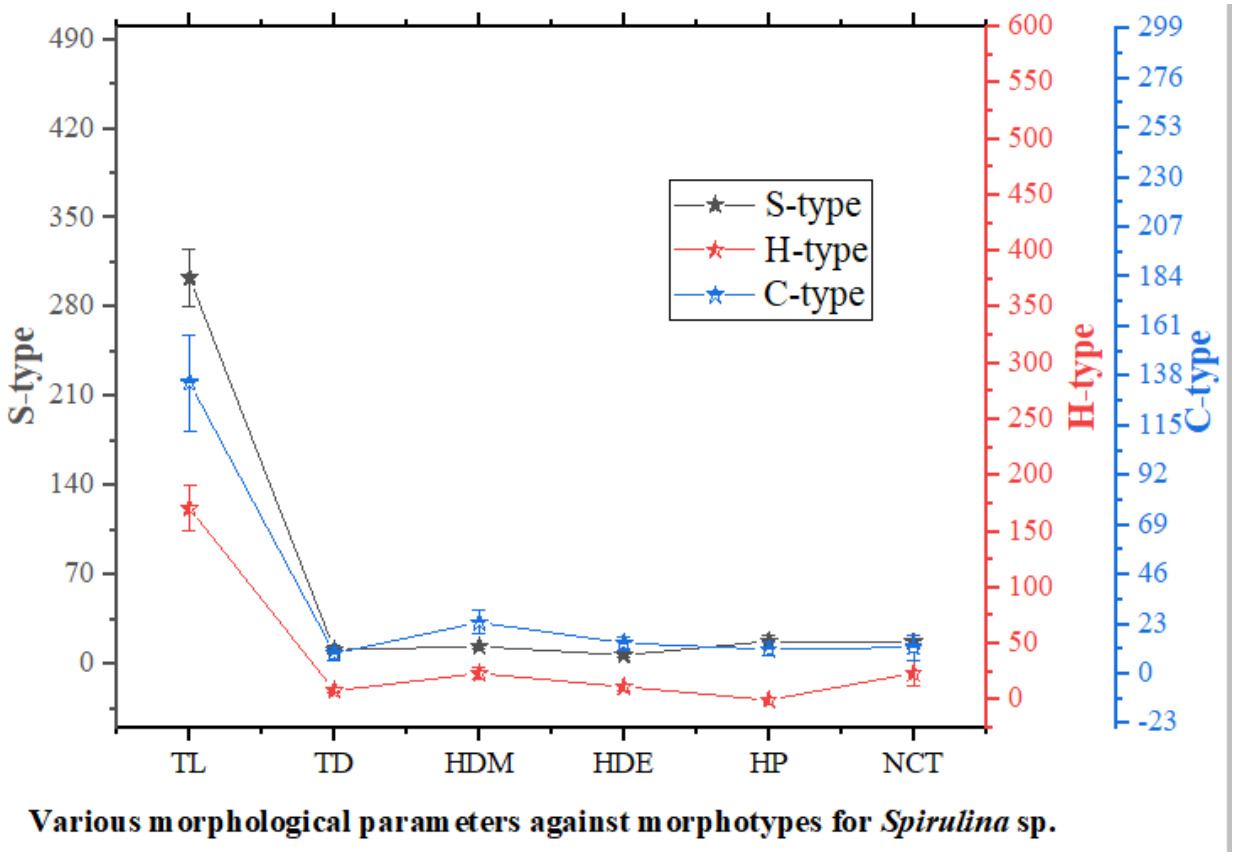


Figure 6: Mean value comparison of morphological parameters of the morphotypes of *Arthrospira fusiformis* of Lake Chitu. These morphological parameters Trichome length (TL), Helix diameter at end and middle (HDE),Trichome dimeter (TD), Hilex pitch (HP), and No of coil per tericome (NCT). Data represented mean ± SD of three replicates (n = 3) for pure culture of Spirulina isolate against various morphological parameters

The identified morphotypes in current study were three different morphotypes. They have a few variety of color and end attention of their trichomes. Among identified morphotypes, the first ones C-type (Figure 5a) intermediate coiled, light blue green and highly attenuated at ends of trichome than others. The Second one H-type (Figure 5b) was tightly coiled more deep blue-green color than S-type and more attenuated at the ends of trichome. The third one's S-type(Figure 5 c & d) loosely coiled. It had less deep blue-green color compared to the H-type. and slightly attenuated at the ends of trichome compared to other morphotypes.

One-way ANOVA and multiple comparisons analysis showed that all morphotypes identified in current study were significantly different at 95% confidence interval using LSD test except few parameters. Interims of trichome length there a significant variation between (*p =0.000*) S-type & H-type with 302.8775±133. 81 and 170.695±88.02 mean value, respectively(Additional file: Table A_2). However, study exhibited that no significance difference (*p=0.272*) were observed between C-type and H-type morphotypes against Trichome length (TL) with 35.695±32.15 mean differences (Additional file: Table A_2). The lowest (13.875±2.38) and highest (23.918±5.61) HDM, a morphological parameter was detected against S-type and C-type, respectively (Additional file: Table A_3**)**. It was noticed that no variation (*p=0.841*) detected between C-type and H-type morphotypes for HDM morphological parameter for the *Spirulina* isolates (Additional file: Table A_4) with 0.298±1.48 mean difference. As indicated in Additional file: Table A_5**,** the lowest (8.12± 2.12) and highest (11.09± 3.45) mean values were recorded agaist H-type and S-type for Trichome diameter (TD), respectively. No variations were obtained among C-type & H-type (p= .133) and C-type & S-type (p=0.091) morphotypes for the TD morphological parameter (Additional file: Table A_7). As illustrated I Additional file: Table A_7 , over 7.0625 ±1.46268 to 14.3075± 3.06797 mean values were recorded against C-type, H-type, and S-type morphotypes for Helix diameter at the end (HDE) morphological parameter. Variations (*p=0.000*) were detected among C-type, H-type, and S-type morphotypes (Additional file: Table A_8). Mean values (9.765± 8.149 to 17.94± 4.748) of C-type, H-type, and S-type morphotypes documented against Hilex pitch (HP) morphological parameter with very significant variation (p=0.000) (Additional file: Table $A_{9\&10}$). The number of coil per tricome (NCT) exhibited variation among C-type & H-type (*p=0.000*) and C-type & S-type (*p=0.033*). whereas no variation observed between S-type & H-type (*P=0.016*) (Additional file: Table A_{12}) for NCT. Additional file: Table A_{11} displayed that mean value were recorded between 12.3± 5.87 and 17.7± 5.06 against C-type, H-type, and S-type morphotypes for NCT morphological parameter.

4.2.2. Variety of *Spirulina* morphotypes

In the current study among observed three distinct morphotypes against *Arthrospira fusiformis*. H-type and S- type morphotype have constant coil form. H-type was happened

during low nutrient and high light intensity and also have constant coil arrangement. H-type always tightly coiled could be due to difference length. Similarly S-type was happened during low nutrient however, with low light intensity. S-type with loosely coiled might be due to difference in length. In case of C-type it happened with high light intensity and high nutrient accessibility. These C-type is called intermediate coiled and it coil haven't constant property Some time seen at many coils and different forms. Therfore C-type couldn't find in only one of the morphotype types.

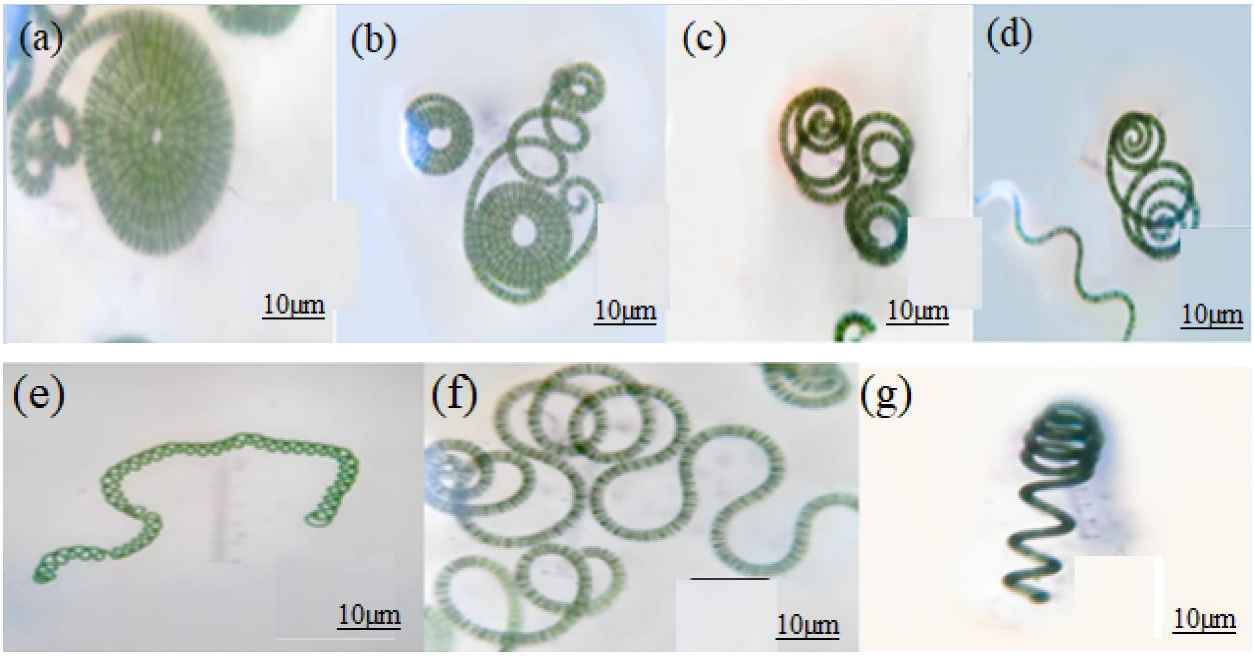


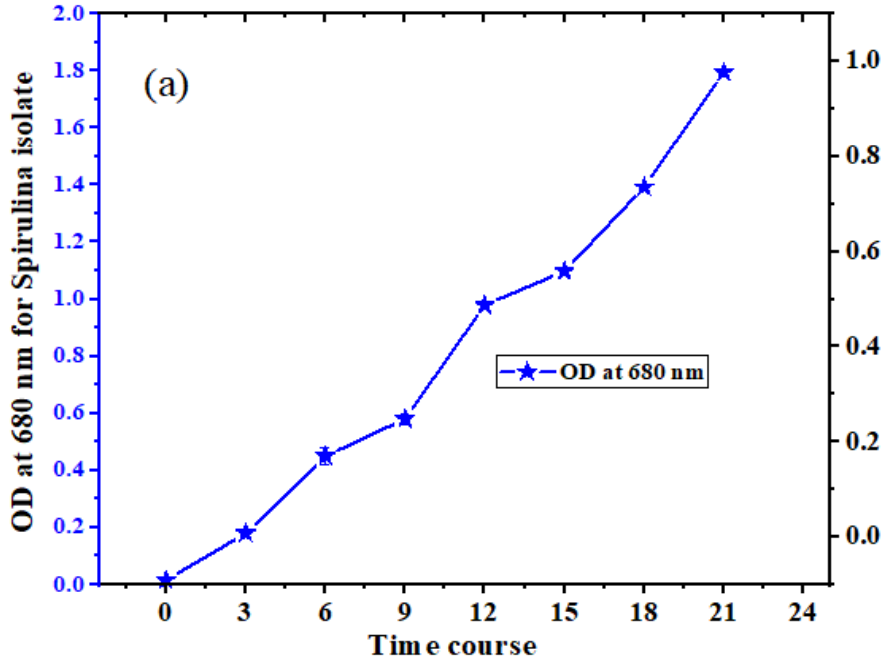
Figure 7: Different coil forms of C-morphotype against Arthrospira fusiformis sp.obtained from Chitu Lake. (a-f) C- type with difference coil forms and (g) showed Situation of converting from H-type to S-type.

4.3. Determination of growth rate and growth curve of *Spirulina* isolates

4.3.1.Growth measurement (OD_{680}) of *Spirulina*

The OD_{680nm} for the growth of a pure culture of *Spirulina* isolate was increased over the time course, as illustrated in Figure 8. *Spirulina* growth was obtained in three days using a spectrophotometer. The development of the *Spirulina* was observed when the culture gradually changed its colour from yellowish green to blue-green. From day zero up to day six, OD_{680nm} was found to be 0.015±0.0002 to 0.45±0.03 (Figure 8). During this

time, *Spirulina* isolate showed the adaptation phase (lag phase) (Figure 8). The lag phase is the stage for *Spirulina* isolate to adapt to its new environment, so the cell increase is still low (Figure 8). When the culture of *Spirulina* isolate entered one week, the colour of the culture also changed to light green. It was noticed that the highest OD_{680nm} (1.795±0.004) was recorded on the 21-date incubation period for the pure culture of *Spirulina* isolate. Followed by the 18 date of the incubation period in which 1.392±0.0035 OD_{680nm} (Figure 8). Specific growth rate of *A.fusiformis* in current study was found to be $0.22/d^{-1}$ with a doubling time of 3. 15days.



The growth of pure culture of *Spirulina* isolate in terms of OD_{680nm} against various date of incubation period

Figure 8. Growth curve graph in terms of OD680nm at various dates of the incubation period for *Arthrospira fusiformis* isolate, which was obtained from Lake Chitu, Ethiopia. Data represented mean ± SD of three replicates (n = 3) for pure culture of *Arthrospira fusiformis* against OD at 680 nm for growth pattern.

4.4. Determination of nutritional profile

The nutritional contents of dried Spirulina powder for their moisture content, total ash, dry matter crude protein, Lipid, and total carbohydrates are shown in Figure 9. The crude protein content was found to be 61.25% based on the nitrogen contents present within a sample, which was found to be 9.8% multiplied with conversion factors (6.25) (Protein = 9.8%×6.25 = 61.25%). The crude lipid was obtained based on the protocols, lipid content was found to be 10.5%. The carbohydrate concentration and absorption of the sample was found to be 0.0139 and 0.117 estimated from the standard curve. The total carbohydrate content present within a sample was 13.9%, in which the concentration of sample was divided by 0.1 and multiplied by 100. In which 0.0139/0.1* 100) =13.9%. The percentages of dry matter, moisture content, and total ash were obtained, 92.85, 7.14 and 7.6% where the sample used was , air dried biomass of *A. fusiformis* before oven = (3.08g) and after oven-dried =(2.86g).

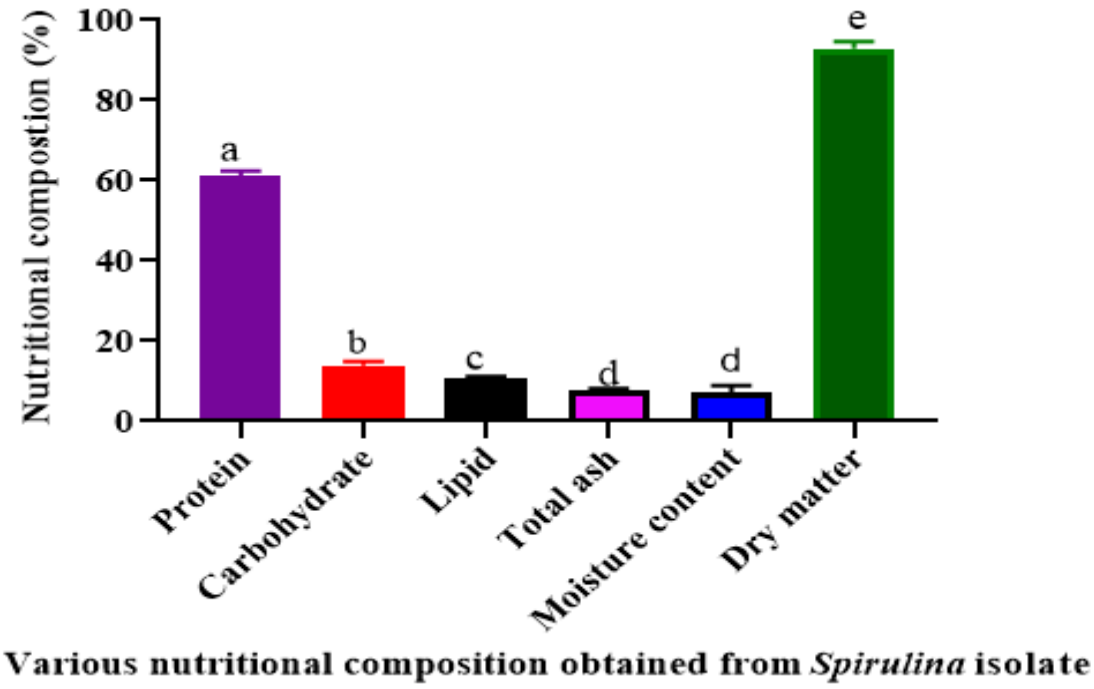


Figure 9: Biochemical composition (nutritional contents) of Spirulina isolates in terms of percentage for isolates which obtained from water sample of Lake Chitu. Data represented mean ± SD of three replicate (n = 3) against nutritional composition of *Spirulina* isolates. The figure is plotted using Graph Pad Prism.

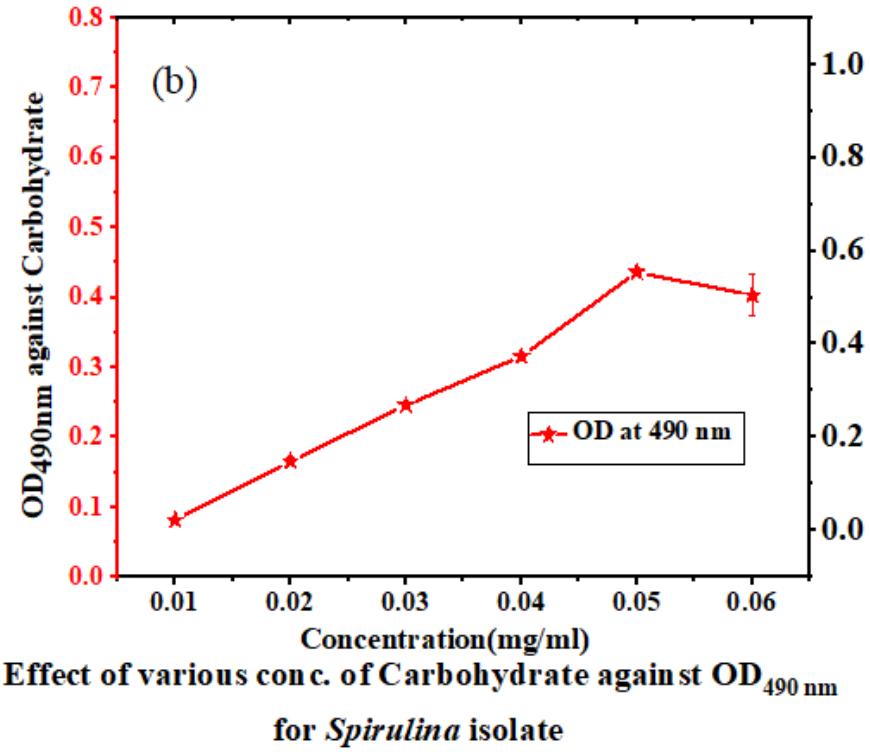


Figure 10: Standard curves for D-glucose (Carbohydrate) concentration against OD490nm.

In the present study, various OD490nm were recorded for different concentrations of carbohydrates against D-glucose (Figure 10). The lowest (0.082±0.002, at 0.01 $_{OD}$) and the highest (0.4032±0.299, at 0.06 $_{OD}$) mean values of OD490nm were obtained as indicated in (Figure 10) for standard *against* concentration of carbohydrate. A considerable amount of OD490nm was also obtained against *A. fusiformis* 0.117 $_{OD}$ and the concentration of the carbohydrate against *A. fusiformis* 0.0139 mg/ml) which was estimated from standard curve at between 0.01 and 0.02 mg/ml concentration of carbohydrate (Figure 10).

Table 5: Absorbance at 490nm (OD_{490nm}) with various concentration against standard D-glucose solution.

D-glucose solution (ml)	Distilled water (ml)	5% of phenols (m1)	96% of Sulfuric acid (ml)	Concentration(mg/ml)	Absorbance at490nm
0.1	0.9	1	5	0.01	0.082
0.2	0.8	1	5	0.02	0.166
0.3	0.7	1	5	0.03	0.246
0.4	0.6	1	5	0.04	0.316
0.5	0.5	1	5	0.05	0.436
0.6	0.4	1	5	0.06	0.576
Blank 0	1	1	5	0	0

In this table the preparation of standard D-glucose and with it's results in OD value. Which was used in to plot standard curves and estimated for concentration of carbohydrates in sample.

Table 6: Concentration and absorbance of total carbohydrate content observed in sample.

Sample solution (ml)	Distilled water (ml)	5% of phenols (1)	96% of Sulfuric acid (ml	Concentration (mg/ml)	Absorbance at490nm	Total carbohydrate content
0.2	0.8	1	5	0.0139	0.117	13.9%

In this table the results of concentration of carbohydrates 0.0139 in sample estimated from standard D-glucose curves and the absorbance 0.117 was direct from spectrophotometer. Finally the total carbohydrates content in sample 13.9% were obtained.

4.6. Minerals and heavy metals from *Spirulina*

The concentration of macronutrients, micronutrients and heavy metals are presented in Table 7. High concentrations were obtained for essential minerals. And deficient levels were found for these toxic substances.

In this table the macro nutrients, micro nutrients and heavy metals obtained for spirulina isolates was indicate that Spirulina is rich in essential minerals, and very low levels were found for the toxic substances.

Table 7: Minerals and heavy metals from Spirulina

S.N.	Elements	contents (mg/kg^{-1})	**S.N.**	Elements	contents (mg/kg^{-1})	**S.N.**	Elements	contents (mg/kg^{-1})
A	Macro nutrients		B	Micro nutrients		C	Heavy metals	
1	Calcium (Ca)	973.91±6.73	1	Iron (Fe)	139.23±3.84	1	Lead (Pb)	< 0.03
2	Potassium (k)	10,104.68±144.51	2	Copper (Cu)	0.69±0.09	2	Mercury (Hg)	< 0.08
3	Sodium (Na)	27,557.07±147.73	3	Zinc (Zn)	2.97±0.07	3	Chromium (Cr)	2.43
4	Magnesium(Mg)	559.46±12.07	4	Manganese(Mn)	1.76±0.06	4	Cadmium (Cd)	< 0.003
5	Phosphorus(P)	3,943.64±71.67	5	Nickel (Ni)	< 0.006	5	Arsenic As	< 0.0004
6	Sulfur(S)	4,363.44±32.84	6	Cobalt (Co)	< 0.013			
			7	Boron (B)	3.58±0.25			

4.7. Fortified biscuit with *Spirulina*

The dry biomass of *Spirulina* incorporated in biscuits enhanced the products' nutritional profile. The results indicated that the proximate composition of fortified product with *Arthrospira fusiformis* showed higher minerals, crude protein(18.2%, moisture (5.5%) and total ash content (4.2%) than that of controlled biscuits, as illustrated in Table 8. As indicated in Figure 9b, the fortified biscuit sample was obtained from *Arthrospira fusiformis* species. As shown in Figure 9a, it was different from the controlled biscuit sample**.** The fortified biscuit sample was found to be green (Figure 9b) in color.

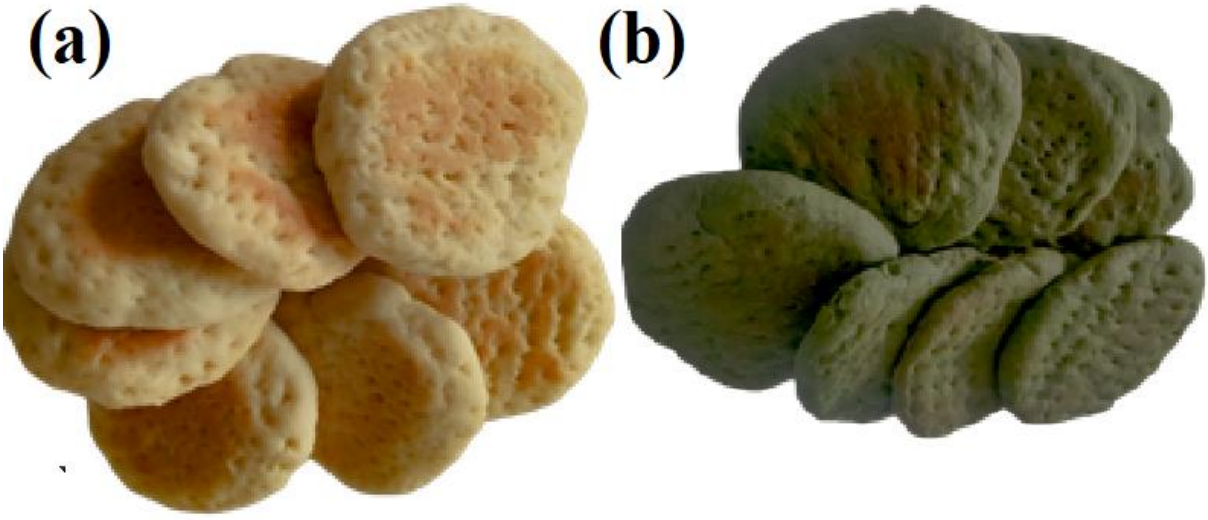


Figure 11: Prepared biscuit sample. (a) indicated the controlled biscuit sample. (b) Indicated the fortified biscuit obtained from *Spirulina* species.

Table 8: Chemical composition of fortified and control biscuits

NS (%)	Fortified biscuit (95%flour5% *Spirulina*) (%)	Controlled biscuit (100% flour) (%)
Protein	18.2	7.7
Carbohydrate	48	67
Lipid	16	24.3
Moisture	5.5	5.05
Ash	4.4	3.1
Ca	652.60+17.23	786.77+16.99
Mg	191.99+ 2.3	171.54+4.32
Na	2,586.86+47.86	1,832.83+42.65
K	1,302.53+27.87	1,050.65+39.44
P	1,103.25+12.52	4,067.80+5,272
S	645+14.35	506.31+7.86
B	3.71+0.22	0.64+0.03
Zn	5.47+0.62	2.42+0.05
Cu	0.007	0.007
Fe	69.30+1.74	62.46+1.07
Mn	0.008	0.008

5. Discussion

The specific composition and dynamics of algal populations are influenced by countless chemical factors such as the amount of bio-available nutrients, alkalinity and pH and physical factors such as light and temperature (Soni et al., 2019). The surface water temperature of Lake Chitu during the sampling period was 23^0C which is closer to room temperature and comparable with the mean surface water temperature recorded by Ogato et al. (2015) (22.8±1.0 ºC) for the same lake. One of the most important factors that influence aquatic production is the pH. pH variation is an essential parameter in water bodies (Melaku & Endale, 2019). In this study, the pH value of Lake Chitu was high, 10.6 which is comparable with the mean pH values recorded by Ogato et al.(2015) (10.45±0.16). Results of this study also showed high carbonate and bicarbonate alkalinity which is used as carbon sources for *Spirulina* and high total alkalinity and inorganic nutrients that favor the production of *Spirulina* and help to dominate this lake. The alkalinity of Lake Chitu was 55150 mg/L which close to is a previous study by Yiglet & Damitew (2023) (56100.0mg/L). Several studies have revealed that some of the East African soda lakes are characterized by high pH and salinity and a large reserve of inorganic carbon sources and Soluble reactive phosphorus are which favor dense populations of microalgae (Ogato *et al.*, 2015).

Studies showed that there are morphological variations among *Spirulina* sp. This could be based on their natural habitats and culture systems. Similarly, it was reported that environmental conditions can affect the morphology of *Spirulina* sp. (Ogato & Kifle, 2014). The culture medium and conditions also affect *Spirulina* sp.'s growth, with the maximum biomass of *Spirulina* sp. recorded against fresh culture medium (Yuan et al., 2019). Previous studies confirmed that levels of nutrients and light intensity affect the morphology of *Spirulina* sp. For instance, low levels of light and nutrients favored trichomes with loose helix (S-type). In contrast, high levels of light and low nutrients enhanced its conversion to a very tight coil (H-type) with the intermediately coiled type (C-type) (Jeeji Bai & Seshadri, 1980).

A previous study indicated that Arthrospira sp. (Spirulina) and other related phytoplankton communities were reported from Lake Chitu (Karssa et al., 2018). In line with this study, it was reported that *Spirulina* sp. displayed three morphotypes for water samples collected from Lake Chitu in Ethiopia (Karssa et al., 2018; Ogato et al., 2014) . The same authors reported H-

type, S-type and C-type morphotypes against *Arthrospira* sp. from Lake Chitu. Other study by Ogato & Kifle (2014) confirmed *A. fusiformis's* as tightly coiled (H-type), spiral or loosely coiled (S-type), and intermediately coiled (C-type) from the same Similarly This study also obtained the same morphotype of *Spirulina* sp. from Lake Chitu (Figure 4). Another study reported temporal morphological changes of *Arthrospira* in Kenyan soda lakes that were determined by elevated levels of soluble reactive phosphorus, wind speed, temperature and conductivity with medium-sized cells, large, and widely coiled filaments of morphotype in Kenyan soda lakes, Nakuru and Bogoria (Kaggwa et al., 2013). Karssa et al., (2018) reported *Arthrospira fusiformis* as the dominant species in Lake Chitu. In the current study, the Morphotypes of *Spirulina* collected from natural habitats were H types and a few S type and helical shape was constant. However, the degree of helicity wasn't stable because it was affected by growth conditions especially light intensity, and nutrients. Although the helical shape of the trichome is considered to be a stable and constant property maintained in culture, there may be considerable variation in the degree of helicity between different strains of the same species and within the same strain (Vo et al.,2015).

Among morphological parameters, reported by Ciferri, (1983) trichomes length ranging from 200- 500 μm, width range between 3 and 12 μm and diameter varies from 30 to 70 μm for *Arthrospira* isolates. In the current study trichome length of S-type was found to be whith in the range reported by Ciferri, (1983). Ogato & Kifle, (2014) also measured trichome length 155– 209 μm for H-type, 319–566 μm for S-type and 32–119 μm for C-type . In the present study, the trichome length of H-type was found to be within the reported range, while that of the C-type was high and S-type was close to with ranged (Additional file: Table A_1). Trichome diameter 9.6–11 μm for H-type, 9.1–11.5 μm for S type and 8.6–9.6 μm for C-type was reported by Ogato & Kifle, (2014) which in line with this study. Number of coil 10–18 μm for H-type, 7–13 μm for S-type and 3–5 μm for C-type was reported by same authors, number of coil of H-type was also within the range. while the number of coil of S-type was larger. Another previous study reported by Abdulqader et al.,(2000) also report the hilex diameter 15 to 60 μm and hilex pitch 0 to 80 μm respectively. In this study the helix diameter of the H- and C-types and hilex pitch of the three morphotype were also within the range of reported on *Arthrospira*.

The established laboratory cultivation system in this study was able to generate a harvest of 1.795±0.004 of dry *Arthrospira fusiformis* per liter of media with a doubling time of growth of 3.15 days which was found to be closer to previous reports of 3.85 days (Costa *et al.*, 2002). The results in Figure 8 demonstrated the variation of OD of the culture suspension. As growth indicators, the growth curve of the optical density (absorbance) was plotted against the number of days. At the beginning of the growth period *Arthrospira fusiformis* grew slowly, but it gradually increased through time until the time, *Arthrospira fusiformis*. showed the adaptation phase (lag phase). According to Suyoso, et al., (2022) the lag phase is the stage of *Arthrospira fusiformis* to adapt to its new environment, so the increase in cells is low. The logarithmic phase occurred on days 9^{th}-21^{th} indicated by a significant increase in the growth of *Arthrospira fusiformis* in this phase, the microalgal cells uptake the excessive nutrients in the medium to support their growth to obtain energy, so the number of cells was increased logarithmically. Similar observation was reported for Spirulina sp. growth optical densities against time for biomass concentration per day increased on 10^{th} -20^{th} days using modified Zarrouk media (Gabal et al .,2018 ; Tiruha, et al., 2018)

In the present study, the protein was found to be the major constituent (61.25% of its dry weigh) in the Spirulina powder grown at laboratory conditions. This value was higher than the protein content reported by Assaye et al. (2018) (ranged from 47.9 to 55.7%) and Getachew et al. (2019) (31.30 ± 0.05 %) for Lake Chitu's Spirulina, Emami, & Olfati (2017) (46.5%) of Egypt. However it was found to be comparable to those reported by Khan et al. (2022) (62.1%) in Pakistan and Liestianty et al.2019 (64.24%) in Indonesia. A higher protein value was reported by Seghiri et al.,(2019) (76.65%). This variation may suggest that, the chemical composition of Spirulina varies greatly dependent on the culture conditions and analysis methods (Liestianty et al.,2019). Particularly protein content in *Spirulina* could vary with harvesting time. For instance morning harvesting *Spirulina* yield has contained highest levels of protein (AlFadhly et al., 2022). Protein is essential for normal function, growth and maintenance of body tissues. The high protein level in spirulina powder in the present study indicates their high nutritive values. Moreover, the Spirulina also contained lipid which reached 10.5% and carbohydrate which reached 13.9% . Seghiri et al. (2019) found that the carbohydrates value of *Spirulina* 6.46% and lipid value 2.45% which is lower than this study. The ash content observed during this study was 7.6% while, the

moisture content of Spirulina powder was 7.14% which indicates that the Spirulina powder contains some nutritionally important minerals.

Furthermore the results obtained in this study indicate that Spirulina is rich in essential minerals, and very low levels were found for the toxic substances (Table 7). The study reported by Galinytė et al., (2023) showed heavy metal content in food must be maintained as low. The most abundant mineral in Spirulina powder was Sodium (Na) followed by potassium (K) and Sulfur and the least content was copper. Sodium is an important source of electrolytes within the body and it regulates acid-base balance in the cell. Potassium reduces blood pressure and it works with sodium to maintain the water balance in the body and lowering the blood pressure. Spirulina powder also contains a moderate amount of calcium. Calcium plays a key role in bone health, for nerve impulses and muscle contractions and it is the major components of the teeth and bones.

The finding of this study showed that the Spirulina powder contained higher quantities of all the studied macroelements than the micronutreints which is similar to the report by Tolpeznikaite et al. (2021) who worked on nutritional characterization of macro-and microalgae extracts bioactive compounds. The values of heavy metals (Table 7) during this study is closer to those reported by Al-Homaidan, (2006) (As,0.002; Cd, 0.031; and Pb,0.109) although, the value of Hg is higher than the his results. The author also reported that the higher value of micronutrients expressed mg/kg^{-1} dry weight follows Cu,8.51; Mn,27.88; and Zn, 21.81 which are higher than the current results. The maximum levels of lead, cadmium and mercury in food supplements is set at 3 mg/kg, 1 mg/kg, and 0.10 mg/kg, respectively and also the maximum levels of arsenic are set only for infant or baby products and, depending on the product, should not exceed 0.010–0.020 mg/kg. The concentrations of the heavy metals and micronutrients in this study was found to be lower than the permissible limit recommended by WHO (2008). Another study reported on heavy metals in algae products evaluated the content of these heavy metals such as Ni 0.4mg/daily, Zn13mg/daily, Hg 0.01mg/daily, Mg 400mg/daily, and Mn 4mg/daily, in *Spirulina* food products for human consumption (Al-Dhabi, 2013). The recent study reported by Janda-Milczarek et al. (2023) showed that the variation in mineral contents of *Arthrospira fusiformis* could be affected by

different growing conditions (under natural conditions or in media under controlled conditions), and cultivation method. Also the method used to determine the mineral content, including the equipment used and the accuracy of the measurement can affect the results.

In this work, supplementation of 5% (w/w) *Spirulina* positively influenced the amount of certain minerals, protein, moisture and total ash contents for supplemented biscuits. The finding of this study revealed that the protein content of fortified biscuit and most essential minerals was much higher than the controlled products and this confirms the importance and role that Spirulina can play to improve the health and nutrition particularly in malnourished populations. Similar results also reported by Şahin (2019) adding dry *Spirulina* to biscuits and Chocolate and Morsy et al., (2014) adding dry *Spirulina* to snack food blends. Commercially produced biscuits have a smaller amount of protein than the labels.. Furthermore, the result showed that the mineral value of supplemented biscuits was higher than that of controlled biscuits except for Ca and P, which aligns with the study reported by Srivastava (2017). The most abundant minerals in *Spirulina*-supplemented biscuits were Na, K and P, and the least abundant were B, Cu, and Mn.

6. Conclusion and Recommendation

6.1. Conclusion

Analysis of some physicochemical parameters of Lake Chitu revealed a high carbonate and bicarbonate alkalinity which is used as a carbon source for *Spirulina* and high pH, high total alkalinity, and inorganic nutrients that favor the production of *Spirulina* and is one of the reasons for the dominance of *Arthrospira fusiformis as* monoalgal culture in this lake. *Arthrospira fusiformis* was grown in a lab scale and succeeded using the Zarrouk medium. Its morphotype variations and their morphological parameters are associated with some environmental variables for this reason, *Arthrospira fusiformis* undergo morphological changes in response to environmental stresses in their natural habitats or artificially cultivated places.

In the present work, the results of the nutritional analysis of Spirulina sample at laboratory conditions indicate the presence of a high amount of protein, low level of carbohydrates, moderate content of lipids, and a considerable amount of important minerals (Ca, K, Mg, P, Fe and Zn) that is suitable to combat malnutrition. These make it a potential algal source of food that is suitable for fortification of foods. The concentrations of the heavy metals in this study was found to be lower than the permissible limit recommended by WHO. Moreover, the addition of powdered Spirulina in biscuits showed that green biscuits prepared with supplementation of *Spirulina* biomass submitted significantly higher in chemical composition such as protein, minerals and ash over the control biscuit. This is especially significant for consumers with a limited protein intake, especially in developing countries like Ethiopia. Overall, these findings demonstrate that Spirulina is an excellent microalgal candidate to be used to improve the health and nutrition of the communities.

6.2. Recommendation

Based on the results obtained during the study period, the following recommendation are made.

- Optimization should be done on *Spirulina* growth. It might be used to generate more biomass productivity with necessary value added compounds.
- Determination of amino acid and fatty acid in the Spirulina should be done, to obtain more nutritional data especially for essential elements.
- Large scale microalgal cultivation should be carried out to evaluate large-scale biomass production for protein, lipids, carbohydrates, and minerals production.
- To get complete nutrition from food, fortification food with *Spirulina* and producing very important and palatable economic products such as Spirulina powder and Spirulina juice in future is needed.
- Further research is required to evaluate the strain type of *Spirulina* and to make a clear distinction among the observed morphological variants using DNA molecular gene sequence analysis.

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8.Appendices

Table A1: Mean value, SD, SE, LB, UP, MIN. and MAX value against Trichome length for S, H, and C-type

Descriptive								
Trichome length								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
S-type	20	302.877500	133.8069215	29.920	240.253933	365.501067	79.6500	507.400
H-type	20	170.695000	88.0181050	19.681	129.501259	211.888741	53.1000	324.500
C-type	20	135.000000	73.1991731	16.367	100.741732	169.258268	23.6000	265.500
Total	60	202.857500	123.6481959	15.96	170.915784	234.799216	23.6000	507.400

Table A2: Post Hoc tests against TL at 0.05 confidence interval

Multiple Comparisons						
Dependent Variable: Trichome length LSD						
(I) CODING	(J) CODING	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S-type	H-type	132.1825000*	32.1504785	.000	67.802277	196.562723
	C-type	167.8775000*	32.1504785	.000	103.497277	232.257723
H-type	S-type	-132.1825000*	32.1504785	.000	-196.562723	-67.802277
	C-type	35.6950000	32.1504785	.272	-28.685223	100.075223
C-type	S-type	-167.8775000*	32.1504785	.000	-232.257723	-103.497277
	H-type	-35.6950000	32.1504785	.272	-100.075223	28.685223
*. The mean difference is significant at the 0.05 level.						

Table A_3: Mean value, SD, SE, LB, UP, MIN. and MAX value against Hilex diameter at middle for S, H, and C-type

Descriptive								
Hilex diameter at middle								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
S-type for HDM	20	13.87500	2.380983	.5324	12.76067	14.98933	11.800	17.750
H-type for HDM	20	23.62000	5.349924	1.196	21.11616	26.12384	11.800	29.500
C-type for HDM	20	23.91750	5.610711	1.254	21.29161	26.54339	14.750	29.000
Total	60	20.47083	6.581537	.8496	18.77064	22.17102	11.800	29.500

Table A_4: Post Hoc tests against HDAM at 0.05 confidence interval

Multiple Comparisons						
Dependent Variable: Hilex diameter at middle LSD						
(I) Coding HDM	(J) Coding HDAM	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S-type for HDM	H-type for HDAM	-9.745000*	1.480662	.000	-12.70998	-6.78002
	C-type for HDAM	-10.042500*	1.480662	.000	-13.00748	-7.07752
H-type for HDM	S-type for HDAM	9.745000*	1.480662	.000	6.78002	12.70998
	C-type for HDAM	-.297500	1.480662	.841	-3.26248	2.66748
C-type for HDM	S-type for HDAM	10.042500*	1.480662	.000	7.07752	13.00748
	H-type for HDAM	.297500	1.480662	.841	-2.66748	3.26248
*. The mean difference is significant at the 0.05 level.						

Table A5: Mean value, SD, SE, LB, UP, MIN. and MAX value against TD for S, H, and C-type

Descriptive								
Trichome diameter								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
S-type for TD	20	11.0900	3.45408	.77236	9.4734	12.7066	5.90	17.75
H-type for TD	20	8.1150	2.11791	.47358	7.1238	9.1062	5.90	11.85
C-type for TD	20	9.5125	2.97728	.66574	8.1191	10.9059	1.85	14.75
Total	60	9.5725	3.10533	.40090	8.7703	10.3747	1.85	17.75

Table A6: Post Hoc tests against TD at 0.05 confidence interval

Multiple Comparisons						
Dependent Variable: Trichome diameter LSD						
(I) Coding TD	(J) Coding TD	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S-type for TD	H-type for TD	2.97500*	.91798	.002	1.1368	4.8132
	C-type for TD	1.57750	.91798	.091	-.2607	3.4157
H-type for TD	S-type for TD	-2.97500*	.91798	.002	-4.8132	-1.1368
	C-type for TD	-1.39750	.91798	.133	-3.2357	.4407
C-type for TD	S-type for TD	-1.57750	.91798	.091	-3.4157	.2607
	H-type for TD	1.39750	.91798	.133	-.4407	3.2357
*. The mean difference is significant at the 0.05 level.						

Table A7: Mean value, SD, SE, LB, UP, MIN. and MAX value against Helix diameter at the end for S, H, and C-type

Descriptive								
Helix diameter at the end (HDE)								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
S-type for HDE	20	7.0625	1.46268	.32707	6.3779	7.7471	5.90	8.85
H-type for HDE	20	11.5050	3.01146	.67338	10.0956	12.9144	5.90	14.75
C-type for HDE	20	14.3075	3.06797	.68602	12.8716	15.7434	8.85	20.65
Total	60	10.9583	3.96096	.51136	9.9351	11.9816	5.90	20.65

Table A8: Post Hoc tests against HDAE at 0.05 confidence interval

Multiple Comparisons						
Dependent Variable: Helix diameter at the end LSD						
(I) Coding HDE	(J) Coding HDE	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S-type for HDE	H-type for HDE	-4.44250*	.82907	.000	-6.1027	-2.7823
	C-type for HDE	-7.24500*	.82907	.000	-8.9052	-5.5848
H-type for HDE	S-type for HDE	4.44250*	.82907	.000	2.7823	6.1027
	C-type for HDE	-2.80250*	.82907	.001	-4.4627	-1.1423
C-type for HDE	S-type for HDE	7.24500*	.82907	.000	5.5848	8.9052
	H-type for HDE	2.80250*	.82907	.001	1.1423	4.4627
*. The mean difference is significant at the 0.05 level.						

Table A9: Mean value, SD, SE, LB, UP, MIN. and MAX value against HP for S, H, and C-type

Descriptive								
Hilex pitch								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
S-type for HP	20	17.9375	4.74838	1.06177	15.7152	20.1598	11.80	29.00
H-type for HP	20	.0000	.00000	.00000	.0000	.0000	.00	.00
C-type for HP	20	11.3575	3.21380	.71863	9.8534	12.8616	5.90	17.70
Total	60	9.7650	8.14930	1.05207	7.6598	11.8702	.00	29.00

Table A10: Post Hoc tests against HP at 0.05 confidence interval

Multiple Comparisons						
Dependent Variable: Hilex pitch LSD						
(I) Coding HP	(J) Coding HP	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S-type for HP	H-type for HP	17.93750*	1.04683	.000	15.8413	20.0337
	C-type for HP	6.58000*	1.04683	.000	4.4838	8.6762
H-type for HP	S-type for HP	-17.93750*	1.04683	.000	-20.0337	-15.8413
	C-type for HP	-11.35750*	1.04683	.000	-13.4537	-9.2613
C-type for HP	S-type for HP	-6.58000*	1.04683	.000	-8.6762	-4.4838
	H-type for HP	11.35750*	1.04683	.000	9.2613	13.4537
*. The mean difference is significant at the 0.05 level.						

Table A_{11}: Mean value, SD, SE, LB, UP, MIN. and MAX value against NC for S, H, and C-type

Descriptive								
No of coil								
	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
S-type for NCT	20	17.7000	5.05860	1.13114	15.3325	20.0675	8.00	26.00
H-type for NCT	20	23.8500	11.08472	2.47862	18.6622	29.0378	8.00	40.00
C-type for NCT	20	12.3000	5.86784	1.31209	9.5538	15.0462	3.00	22.00
Total	60	17.9500	9.02994	1.16576	15.6173	20.2827	3.00	40.00

Table A_{12}: Post Hoc tests against NC at 0.05 confidence interval

Multiple Comparisons						
Dependent Variable: No of coil LSD						
(I) Coding NC	(J) Coding NC	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
S-type for NCT	H-type for NCT	-6.15000*	2.46909	.016	-11.0943	-1.2057
	C-type for NCT	5.40000*	2.46909	.033	.4557	10.3443
H-type for NCT	S-type for NCT	6.15000*	2.46909	.016	1.2057	11.0943
	C-type for NCT	11.55000*	2.46909	.000	6.6057	16.4943
C-type for NCT	S-type for NCT	-5.40000*	2.46909	.033	-10.3443	-.4557
	H-type for NCT	-11.55000*	2.46909	.000	-16.4943	-6.6057
*. The mean difference is significant at the 0.05 level.						

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