Carotenoid Analysis and Evaluation of Phycocyanin Recovery and Biological Activity Using Different Methods of Extraction from *Arthrospira fusiformis* (Oscillatoriales, Cyanobacteria) Originated from Lake Chitu, Oromia, Ethiopia

Naod Hailu Dibaba

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 Applied Biology (Specialization in Biotechnology)

Office of Graduate Studies

Adama Science and Technology University

December 2022

Adama, Ethiopia

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Declaration

I hereby declare that this Master's Thesis entitled "Carotenoid Analysis and Evaluation of Phycocyanin Recovery and Biological Activity Using Different Methods of Extraction from *Arthrospira fusiformis* (Oscillatoriales, Cyanobacteria) Originated from Lake Chitu, Oromia, Ethiopia" is my original work. It has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of materials that are used for this thesis have been duly acknowledged through citation.

Name of the Student	Signature	Date

Recommendation

We, the advisors of this thesis, hereby certify that we have read the revised version of the thesis entitled "Carotenoid Analysis and Evaluation of Phycocyanin Recovery and Biological Activity Using Different Methods of Extraction from *Arthrospira fusiformis* (Oscillatoriales, Cyanobacteria) Originated from Lake Chitu, Oromia, Ethiopia'', prepared under our guidance by Naod Hailu submitted in partial fulfillment of the requirements for the degree of Master's of Science in Applied Biology (Specialization in Biotechnology). Therefore, we 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

We, the advisors of the thesis entitled "Carotenoid Analysis and Evaluation of Phycocyanin Recovery and Biological Activity Using Different Methods of Extraction from *Arthrospira fusiformis* (Oscillatoriales, Cyanobacteria) Originated from Lake Chitu, Oromia, Ethiopia" and developed by Naod Hailu, 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 Naod Hailu have read and evaluated the thesis entitled "Carotenoid Analysis and Evaluation of Phycocyanin Recovery and Biological Activity Using Different Methods of Extraction from *Arthrospira fusiformis* (Oscillatoriales, Cyanobacteria) Originated from Lake Chitu, Oromia, Ethiopia" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Applied Biology (Specialization in Biotechnology).

Chairperson	Signature	Date
Internal Examiner	Signature	Date
External Examiner	Signature	Date

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

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List of Abbreviations and Acronyms

ANOVA	Analysis of Variance
BETin	Bio and Emerging Technology Institute
CAGR	Common Annual Growth Rate
DH_20	Distilled Water
DPPH	2,2-diphenyl-1-picryl-hydrazyl
DW	Dry Weight
EE	Extraction Efficiency
EP	Extract Purity
FT	Freeze and Thaw
HB	Homogenization with Beads
HPH	High Pressure Homogenization
MAE	Microwave-assisted Extraction
MHA	Muller Hinton Agar
MP	Mortar and Pestles
PBPs	Phycobiliproteins
PC	Phycocyanin Concentration
PEF	Pulsed Electric Field
pH	power of Hydrogen
RSA	Radical Scavenging Activity
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SPSS	Statistical Package for the Social Sciences
UAE	Ultrasound-assisted Extraction
USD	United States Dollar
UV-Vis	Ultraviolet – Visible Spectroscopy

Abstract

Spirulina grows naturally in some alkaline lakes in warm tropical regions. Spirulina flourishes in saline-alkaline lake of the East African Great Rift Valley lakes, including Lake Chitu. Spirulina is famous and important source of phycocyanin pigment and various carotenoids with numerous applications. However, there are no investigations and clear scientific reports showing analysis of the total carotenoids and phycocyanin content of Spirulina isolated from Lake Chitu. Scientific reports have proven that various health benefits and applications of Spirulina in food, pharmaceutical, cosmetic and natural colouring industry does emerge from its high-quality protein, carotenoids, and particularly phycocyanin content. This study has aimed to conduct spectrophotometric evaluation of total carotenoid content of Spirulina culture identified and isolated from Lake Chitu and to extract and analyze phycocyanin pigment extracted using different extraction techniques from these cultures and then to evaluate the biological activity of partially purified lyophilized extracts at different concentrations. For this study, Arthrospira fusiformis containing samples were collected, identified and isolated, mass produced at laboratory scale, and then harvested and dried. Total carotenoid content was estimated using spectrophotometric analysis after extraction using 80% Acetone. Solvent type used for the study was optimized and crude phycocyanin extracts were obtained using classical mortar and pestle, freeze-thaw, homogenization with beads, ultrasound assisted extraction and microwave assisted extraction methods under same conditions of biomass to volume ratio of 1:50 w/v and solvent parameters. Extraction efficiency of each extraction methods employed and phycocyanin concentration, yield and purity were calculated using standard equations for analysis and comparison of the extracts. The presence of phycocyanin pigment was validated using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Partial purification of extracts was done using the ammonium sulfate precipitation and dialysis method. Antimicrobial activity of the partially purified lyophilized phycocyanin extracts at different concentrations was conducted against four different gram-positive and gram-negative bacterial strains. Antioxidant activity of these samples was also evaluated using the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay method. The total carotenoid content of 3.912 $mg.g^{-1}$ was present in the dry weight of Spirulina. 0.1M sodium phosphate buffer was found to have the highest extraction efficiency at 0.415 $mg.ml^{-1}$ phycocyanin concentration. The ultrasound assisted extraction method was found to give the highest yield of phycocyanin than the other methods at 36.35 $mg.g^{-1}$ with an extraction efficiency of 84.5 %. SDS-PAGE has validated the presence of α and β subunits of phycocyanin pigment in the samples. After purification, a food grade phycocyanin purity was achieved with the highest value of 0.98. All extracts have shown effective antibacterial activity against all pathogens tested with demonstrated dose dependency. The DPPH assay has shown effective antioxidant activity of samples ranging between 48.9 to 74.3 % of radical scavenging activity across the different samples and concentration. This study has revealed the potential of Lake Chitu's Spirulina as a good source of highly useful phycocyanin pigment compounds and carotenoids which have various applications in different sectors of industry and health.

Keywords: *Spirulina, Lake Chitu, Carotenoids, Phycocyanin, Extraction, Antibacterial, Antioxidant.*

CHAPTER ONE

1. Introduction

Microalgae are types of water organisms that have not yet been completely exploited, despite growing global interest (Dianursanti and Hafidzah, 2021). Many species of microalgae have been gaining popularity for several years as more people become aware of their value as a source of useful compounds in areas such as nutraceuticals, medical, feed, food, cosmetics, biofuels, and others. *Scenedesmus sp., Oedogonium sp., Botryococcus braunii, Arthrospira fusiformis, Chlorella vulgaris, Chroococcus sp., and Synedra sp.*, are few among the vast microalgae species commonly found microalgae in Ethiopia (Spolaore *et al.*, 2006; Asmare *et al.*, 2013).

A filamentous, spirally coiled cyanobacteria called *Arthrospira fusiformis*, also known as Spirulina microalgae is found in high abundances in some tropical soda lakes. It acts as the food web's key driver and forms almost monoculture unialgal blooms in soda lakes of the East African Rift Valley. This is attributed to their high levels of carbonate and bicarbonate content characteristics, as well as a pH exceeding up to 11 (Vareschi, 1978; Vonshak, 1997; Oduor and Schagerl, 2007; Kaggwa *et al.*, 2013). This particular microalga can also be found in large quantities in Ethiopia's Lake Chitu (Karssa *et al.*, 2018).

Spirulina has long been utilized as a dietary supplement for fish, shrimp, and poultry, and is increasingly being employed as a source of protein and vitamin supplements in aquafeeds. Due to significant research into the alga's growth environment and biology, artificial cultures have been optimized and production of this useful microalga is mainly done in most developed countries like the United States, Mexico, China, Japan, Thailand, and Taiwan (Herrera *et al.*, 1989). These days, it has grown in popularity in the human health food market, and it is utilized as a protein supplement and nutritious food in most Asian and many countries of the world (Habib *et al*., 2008).

High protein, polysaccharide, lipid, essential amino acids, fatty acids, important carotenoids, dietary minerals, and vitamin content of Spirulina makes it useful in agriculture, food industry, pharmaceutics, perfumery, and medicine. Phycocyanin, a protein-pigment complex from the phycobiliprotein family characterized by its strong blue color, is known to be present in the cell structure of Spirulina (Dianursanti and Hafidzah, 2021). Spirulina’s phycocyanin has a variety of applications in the food and medicinal

industries and it is the most commercially promising biochemical present in Spirulina (Herrera *et al.*, 1989). Not only in food industries such as in chewing gum, ice sherbets, soft beverages, and candies, but phycocyanin is also a blue natural colorant that is used in cosmetics such as lipstick and eyeliners (Chaiklahan *et al*., 2011). This pigment is mostly utilized in the food industry as a natural food colorant all over the world (Taufiqurrahmi *et al.*, 2017). Phycocyanin derived from Spirulina has been reported to have antimicrobial (including against virus and bacteria), anticancer (Romay *et al.*, 2003; Eriksen, 2008), metalloprotective (protection against heavy metal poisonings such as due to Cd, Pb, Fe, and Hg), immunostimulant (Herrera *et al.*, 1989; Silveira *et al.*, 2007), and antioxidant properties (Hosseini *et al.*, 2013).

According to Bhaskar *et al.* (2005), phycocyanin derived from Spirulina has a market worth of roughly 5–10 million USD per year as of that time and has been continuously increasing exponentially since then. In 2018, the global phycocyanin market was valued at over 18.5 million USD, with a compound annual growth rate (CAGR) of 8.6% for the years ahead, and global consumption was predicted to exceed 200 tons by 2025 (Global Market Insight, 2019). Later in 2020, the phycocyanin market was valued at 155.3 million USD, according to a market research analysis, and is expected to reach 409.8 million by 2030, with a CAGR of 9.6% for the coming years from 2021 to 2030 (Kamble and Deshmkuh, 2021). This rapid increase in market size and value is due to the increased consumer awareness of its various applications and benefits, such as the advantage it has over artificial colorants in terms of toxicity issues in food and beverages, which has boosted demand for naturally extracted colorants including phycocyanin (Global Market Insight, 2019). The phycocyanin market is segmented based on form, grade, application, and region it is produced. The market is divided into powder and liquid categories based on form. Further, the market is also divided into three grades of the product: food grade, pharmaceutical grade, and reagent/analytical grade (Kamble and Deshmkuh, 2021).

The ratio of purity, which is defined as the relationship between absorbance at 620 nm and 280 nm (A620/A280), determines the cost of phycocyanin products based on product grades. As Cisneros and Rito-Palomares, (2004) reported, food grade phycocyanin (purity more than 0.7) costs roughly 0.13 USD per mg, but pharmaceutical and analytical grade (purity greater than 4.0) costs up to 15 USD per mg (Bhaskar *et al.*, 2005). The ratio of purity and yield of phycocyanin is influenced by the extraction method, separation,

purification, and quality of the raw material used in the production process (Chaiklahan *et al.,* 2011).

As investigated in several studies, the concentration, yield and purity of phycocyanin obtained from Spirulina may vary depending on different microalga cultivation management strategies used and different extraction mechanisms employed which have a direct relation with the cost of production. Numerous studies have been done regarding the establishment of best practices for both cultivation and extraction techniques and still, there is an emerging interest to explore novel techniques in order to identify the best production efficiency (Soni *et al.,* 2006; Silveira *et al.,* 2007; Singh *et al.,* 2009; Chaiklahan *et al.,* 2011). Therefore, this study has aimed to explore the potential of phycocyanin production from locally isolated Spirulina strain using various extraction methods at a laboratory scale cultivation process. The study is also aimed to make an analysis of the overall carotenoid potential Spirulina from Lake Chitu and evaluate antimicrobial and antioxidant activity of its partially purified phycocyanin extracts. We believe that this is the first study utilizing Spirulina from Lake Chitu of Ethiopia for phycocyanin extraction and exploring its biological potential.

1.1. Statement of the Problem

Despite numerous studies on effective conventional methods of production, the price of phycocyanin products continues to be high because of high production costs and a limited supply on the current worldwide market. It is important to further improvise with the currently available sources and methods to identify effective extraction and cultivation processes in order to lower this high production cost. Many emerging nations find it impossible or extremely difficult to import such essential goods, either for personal use or for use in local industries since they have not yet been developed locally. Ethiopia is one of such nation. Ethiopia's Lake Chitu is still naturally endowed with the presence of Spirulina; however, no such commercial products are produced from it since little research has been done to assist projects, in particular, those aimed at generating phycocyanin locally. It has been a major loss of all its long-proven uses and advantages not to use this natural resource, which could have accompanied products made in many local industries and also for the global market. This contributes to the fact that this problem is one of the advantageous potentials of the country yet not utilized totally, which could have potentially aided in improving the health status of society as a whole and the use of natural goods for their superior benefits. Exploration of this natural resource is therefore necessary to start

using it in local industries. Additionally, the potential of Lake Chitu's spirulina to be exploited as a source raw material for the production of phycocyanin has not yet been investigated. This study also searches for responses to these inadequacies in the context of Ethiopia and the larger scientific community. In order to identify the potential of this local strain for use in local industrial production, the study was designed to investigate the phycocyanin production capacity of Spirulina from Lake Chitu at the laboratory scale as well as its distinctive biological activities.

1.2. Objectives of the Study

1.2.1. General Objective

The general objective of this study is to estimate total carotenoid content and to extract, quantify and measure biological activity of phycocyanin from culture of *A. fusiformis* isolates originated from Lake Chitu, Oromia, Ethiopia.

1.2.2. Specific Objectives

The specific objectives of this study are to:

- identify, screen, isolate, and grow culture of *A. fusiformis* in a laboratory from collected samples of Lake Chitu.
- determine total carotenoid content estimate in Lake Chitu's Spirulina.
- extract crude phycocyanin from Spirulina of Lake Chitu using different extraction techniques.
- partially purify crude phycocyanin extracts.
- quantify and analyze phycocyanin recovered from Lake Chitu's Spirulina.
- determine biological activity of phycocyanin extracts by anti-bacterial and anti-oxidant assays.

1.3. Significance of the Study

Global demand for phycocyanin is incredibly of huge interest and increasing as the awareness grows of its special industrial, health, and related advantages. To support this longstanding revolution, researchers from all over the world are conducting extensive studies to reveal different aspects of this useful compound. Locally, this study is useful by introducing phycocyanin for incorporation as a component with additional benefits in local food, pharmaceutical, and cosmetic industries. It is significant to undertake this research work to make a considerable indication of the potential of Lake Chitu's Spirulina which is found in mass quantity yet left out without proper utilization in Ethiopia despite having

huge potential to produce phycocyanin and other useful biomolecules such as zeaxanthin, γ-linolenic acid and sulfated polysaccharides. Scientifically, this study is aimed to investigate and establish a combination of local raw material (Spirulina) cultivation and different extraction methods for phycocyanin production and total carotenoid analysis. This may in turn play a vital role in the scientific challenge of establishing an effective process of phycocyanin production. Furthermore, this study may open a new window for a new product with better characteristics to enter the industrial sector that may contribute to the country's export capacity.

CHAPTER TWO

2. Literature Review

Microalgae, one of the most important organisms in the food chain of aquatic ecosystems, contain a variety of useful chemicals that are employed as nutritional food as well as active ingredients in drugs and cosmetics (Koyande *et al.,* 2019; Kusmayadi *et al.,* 2021). Bioactive compounds such as astaxanthin, β-carotene, lutein, omega-3 fatty acids, and glycerol sourced from different species of microalgae are some among the useful chemicals in different industries (Ng and Chew, 2022). Spirulina is one of the most common microalgae that has long been utilized for these and other purposes (García *et al.,* 2017). A huge volume of studies has been done on Spirulina's safety and toxicity, which has repeatedly demonstrated its positive potential, is an advantage of employing it as a source for these applications (Karkos *et al.,* 2011). Along with the characteristic high protein content buildup of Spirulina cells, Phycocyanin is another component of Spirulina that can be used in a variety of chemical and biological applications (Manirafasha *et al.,* 2018). Phycocyanin makes up about 15-25% of the dry weight of Spirulina which makes this microalga the best source of phycocyanin among other species of cyanobacteria (Sotiroudis and Sotiroudis, 2013; Dianursanti *et al.*, 2018).

2.1. Spirulina

Arthrospira fusiformis, a cyanobacterium often known commercially as Spirulina, is a primordial creature that evolved the capacity to use carbon dioxide dissolved in seawater as a nutrient supply for its reproduction and originated about 3.5 billion years ago. Spirulina is a photosynthesizing cyanophyte (blue-green algae) that thrives in bright sunlight, high temperatures, and alkaline tropical environments (Habib *et al.*, 2008; Anvar and Nowruzi, 2021).

2.1.1. Morphology and Taxonomy

Spirulina is symbiotic, multicellular, filamentous blue-green algae with symbiotic bacteria that can fix nitrogen from the atmosphere, according to its morphology. Under a microscope, the multicellular cylindrical mature trichomes are arranged in an open left-handed helix over the whole length, as shown in Figure 1 about the complete life cycle of the cells (Bhagwat *et al.,* 2021; Costa *et al.,* 2022; Rutar *et al.,* 2022). Along other components, Spirulina contains the two photosynthetic pigments carotenoids and

chlorophyll a. This microalga is autotrophic because it is photosynthetic and reproduces via binary fission, as do most algae. The filaments (or trichomes) have a helical form that is unique to the genus and can only be maintained in a liquid environment or culture broth medium. Floating mats are created by the existence of gas-filled vacuoles in the cells and the helical structure of the filaments. The trichomes are 50 to 500 μm long and 3 to 4 μm wide. Spirulina cells, like most cyanobacteria, have a peptidoglycan-based cell wall that is similar to Gram-negative bacteria but much thicker than most bacterial cells (Hoiczyk and Hansel, 2000; Palinska and Krumbein, 2001; Habib *et al.*, 2008; Armand *et al.*, 2015; Jung *et al.*, 2021).

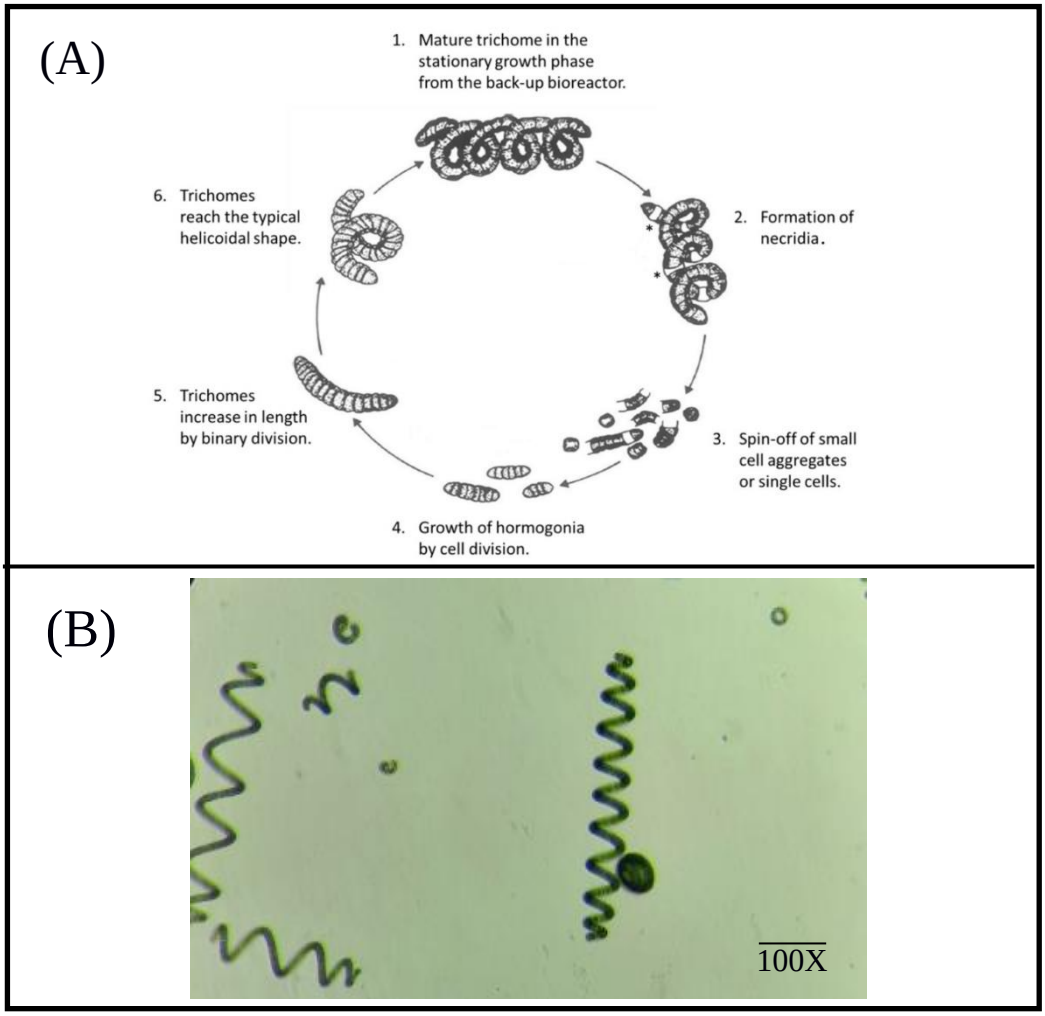

Figure 1. Life cycle (A) adapted from Jung *et al.* (2021) (the stars (*) indicates necridia) and morphology of Spirulina under microscope (100X) (B) obtained in this study.

Arthrospira is one of the 19 genera in the Microcoleaceae family of the order Oscillatoriales, according to taxonomic categorization (Nowicka-Krawczyk *et al.*, 2019). P. J. Turpin first isolated Spirulina from a freshwater sample in 1827. Later after many

years, in 1884, Wittrock and Nordstedt discovered Spirulina, a helical, septal, and blue-green microalgae near Montevideo, Uruguay (Ciferri, 1983; Sánchez *et al.,* 2003; Saranraj and Sivasakthi, 2014). The most well-known species of the genus *Arthrospira* are *Arthrospira fusiformis*, *Arthrospira maxima*, and *Arthrospira platensis*, which have taxonomic distinctions in filaments, vacuoles, and external cover or capsule regularity (Bai, 1999; Scheldeman *et al.,* 1999; Habib *et al.,* 2008). Studies made on this microalga have been conducted all around the world under the name "Spirulina," and changing this widespread designation among scientists and consumers has proven difficult. Blue-green algae were placed into the prokaryote kingdom after being proposed by Stanier and Van-Neil in 1962, and the microorganisms were given the name cyanobacteria. Bergey's Manual of Determinative Bacteriology has recognized and first published this nomenclature in 1974 (Stanier and Van-Niel, 1962; Bergey *et al*., 1974; Guglielmi *et al.,* 1993; Habib *et al.*, 2008).

There have been reports of two *Arthrospira* species coexisting in Lake Chitu which are *A. fusiformis* and *A. maxima* in different studies (Kebede, 1997; Li *et al.,* 2001; Karssa *et al.,* 2018). Only the degree to which the trichomes are tightly coiled in *A. fusiformis* and loosely coiled in *A. maxima* distinguishes the two species. Although it was debatable whether *A. fusiformis* and *A. maxima* were indeed distinct species, according to Li *et al.* (2001), investigation on molecular characterization analysis of Spirulina in Lake Chitu have determined that these species are the same. A 16S rRNA sequence comparison has revealed a 100% similarity between these two species.

2.1.2. Habitat Ecology

Spirulina cells inhabit natural lakes which have special physico-chemical qualities that allow them to maintain, grow, and reproduce. If the required compositional conditions are met, Spirulina can be found in soil, marshes, freshwater, brackish water, the ocean, and thermal springs (Habib *et al.*, 2008). Growth cycles are usually regulated by the restricted supply of nutrients. Upwelling from within water bodies, nutrient influxes from rivers, and pollution are all sources of new nutrients. When nutrients are depleted, the algae population expands rapidly, achieves a maximum density, and then dies off. When disintegrated algae release their nutrients or more nutrients flow into the lake, a new seasonal cycle begins. Spirulina thrives in alkaline, saline water (>30 g/l) with a high pH (8.5–11.0), especially where sun radiation is abundant at altitudes in the tropics (Habib *et al.*, 2008). Inside the Great Rift Valley, alkaline lakes of east Africa are good examples of

this. These lakes, which supported dense Spirulina populations, have an extremely high pH, reaching near pH 11 in certain cases, as well as very high salt concentrations, primarily sodium carbonate (Richmond and Soeder, 1986; Habib *et al.*, 2008). It is well reported that the Spirulina microalgae species thrives in very alkaline lakes in Africa and Mexico, where the cyanobacteria population is nearly monospecific. *Spirulina spp.* is more likely to predominate in water with a higher pH and conductivity. *Arthrospira fusiformis* has been isolated from waters containing between 85 g and 270 g of salt per liter, with optimum growth occurring between 20 and 70 g. This microbe's capacity to use ammonia as a nitrogen source at high alkaline pH values could be due to a comparatively high cytoplasmic pH (4.2 to 8.5) (Sasson, 1997).

2.1.3. Cultivation, Growth and Suitable Media Conditions

Spirulina, like most cyanobacteria, is an obligate photoautotroph, meaning it cannot grow in the dark on an organic-carbon-containing medium. It absorbs primarily nitrates and reduces carbon dioxide in the presence the light. Glycogen is the major assimilation product of Spirulina photosynthesis (Zaslavskaia *et al.*, 2001; Gershwin and Belay, 2007). In a study, the growth of Spirulina was found to be optimum at around 30°C, as compared between the range of 25 and 35°C (Soni *et al.*, 2019). However, under laboratory conditions, it was indicated in same study that Spirulina grows optimally at temperatures ranging from 25 to 37°C. Outdoors, it appears that a temperature increase of up to 39°C for a few hours has no harmful effect on this blue-green alga's photosynthetic capabilities. During the day, the minimum temperature at which Spirulina may grow is roughly 15°C though it can withstand relatively low temperatures at night. Spirulina also appears to have great resistance to UV radiation (Richmond and Soeder, 1986; Habib *et al.*, 2008).

For increased biomass output, cell productivity, specific growth rate, and protein content, Spirulina culture requires enough aeration, agitation, and suitable light intensity. The availability of nutrients in the media composition, pH, light, and temperature conditions influence Spirulina development and biomass yield both in the laboratory and industrial settings. Necessarily, a pH of 9.5 to 9.8 have to be maintained in the media preparation which also helps with inhibiting effect on most of other algae contaminants in a culture. For biomass growth, many photobioreactors and ponds have been constructed and modeled to meet different production efficiency (Belkin and Boussiba, 1991; Cornet *et al.*, 1992; Madkour *et al*., 2012; Soni *et al*., 2019). For many years, Zarrouk's medium has been used as the standard medium for Spirulina production (Zarrouk, 1966). Later, several modified

media alternatives using industrial effluents (Tanticharoen *et al.,* 1993), seawater (Faucher *et al.,* 1979), and sewage water (Saxena *et al.,* 1983) have been reported in different studies (Madkour *et al.,* 2012).

2.1.4. Biochemical Composition

Spirulina is a great source of natural food that has been used since earlier civilizations (Mathur, 2018). It is the main alga utilized as a food supplement (Karkos *et al.,* 2011). The nutritional and chemical contents of Spirulina microalgae have been analyzed since the beginning of applied phycology. Various investigations have been done and their principal bioactive components have piqued further interest. Protein, vitamins, fats, fibers, minerals, carbohydrates, chlorophyll, β-carotene, and some natural pigments were found to be abundant in them (Sánchez *et al.,* 2003; Wells *et al.,* 2017; Park *et al.,* 2018; Seghiri *et al*., 2019). In a study conducted on freeze-dried samples for functional composition, nutritional properties, and biological activities of Moroccan Spirulina microalga by Seghiri *et al.* (2019), the basic biochemical composition of *Spirulina* was summarized as in the Table 1 together in comparison with other species of microalgae. Spirulina was found to be rich in proteins than other microalgae species identified so far though it has low content of lipids. These key ingredients provide physiological and functional benefits, making it attractive for a variety of industries (Seghiri *et al*., 2019).

Table 1. Nutritional components analysis of Spirulina (% of dry matter).

Components	**Content Percentage of Spirulina**	**Content Percentage of Other Microalgae Species**
Moisture	12.66	< 9
Ash	14.56	7.4-10.4
Protein	76.65	3-71
Lipids	2.45	>5 - >60
Crude Fiber	4.07	1.36-25
Carbohydrates	6.46	10-68
Energy (Kcal/100g)	436.18	No Clearly detected

This data has been adapted from Seghiri *et al*., 2019.

Spirulina is a readily available source of natural pigments such as carotenoids and phycocyanin, which are commonly utilized as food supplements or natural additives (Park *et al.,* 2018). Consumer awareness of the value of natural colorants for their nutritional,

pharmacological, and health-related benefits has coincided with the use of spirulina. As a result, natural pigments and Spirulina as a source of these colors are becoming more widely used, particularly in the food and cosmetics industries (Chethana *et al.,* 2015).

2.2. Carotenoids in Spirulina

The presence of several bioactive pigments, such as carotenoids, chlorophylls, pheophytins, and phycobiliproteins, is connected to the majority of Spirulina's health advantages. Carotenoids are known to provide many health benefits due to their antioxidant and anti-inflammatory properties, as they can scavenge free radicals generated in the human body during stressful situations and lower inflammatory mediators (Costa *et al.*, 2022; Saini *et al.,* 2022).

Carotenoids are a group of lipid-soluble pigments found in a variety of plants and microorganisms that give them their red, yellow, and orange colors (Maoka, 2020). In most microalgae species including Spirulina carotenoids are synthesized and accumulated in plastids (Henríquez *et al*., 2016). They are naturally occurring and important for microalgae organisms because they serve as accessory light-harvesting pigments that support photosynthetic reactions and protect organelles from singlet oxygen-induced photo-oxidative damage (Pérez-Gálvez *et al.,* 2020). To animals, including humans, carotenoids are obtained from food because they are unable to synthesize them (Eggersdorfer and Wyss, 2018). Carotenoids are utilized in food, feed, as colorants and flavorings, and as a source of specialized biochemicals such as Provitamin A and Retinoids in nutritional supplements (von Lintig, 2012; Mezzomo and Ferreira, 2016). Specific dietary carotenoids are responsible for different protective effects. As proven by different epidemiological studies, dietary carotenoids have a number of vital roles in the human body, including fighting oxidative free radicals and reducing inflammatory stress-related disorders such as skin degeneration and aging, cardiovascular disease and neurological illnesses, certain types of cancer, and age-related eye diseases like macular degeneration and cataracts, among many others (Perera and Yen, 2007; Fiedor and Burda, 2014; Tan and Norhaizan, 2019; Mrowicka *et al.,* 2022). Spirulina's high levels of carotenoids, among other biomolecules, are one of the reasons for its commercial success. In Spirulina, carotenoids such as β-carotene, lutein, astaxanthin, zeaxanthin and cryptoxanthin are present (Park *et al.,* 2018). According to El-Baky *et al.* (2003), different species of *Arthrospira* accumulate varying content of carotenoids.

2.3. Phycobiliproteins in Spirulina

Phycobiliproteins (PBPs) are important photosynthetic pigments found in cyanobacteria and red algae that collect light while sheltering microalgae from damaging radiation. PBPs are categorized based on pigment colors as red – phycoerythrin, dark blue – phycocyanin and light blue – allophycocyanin. They are the principal accessory pigments in Spirulina, responsible for roughly 50% of light absorption, and located at the phycobilisomes, that are composed of hundreds of seemingly similar chromophore proteins and the major complex light harvesting antennae of photosystem II in cyanobacteria and red algae assembled on the thylakoids surface, as seen in Figure 2 (Fernández-Rojas *et al.*, 2014; Pez Jaeschke *et al.*, 2021). PBPs in Spirulina include C-phycocyanin (blue pigment), C-allophycocyanin (light-blue pigment), and phycoerythrin (red pigment) and these pigments vary in their spectral characteristics (Walter *et al.*, 2011; Mulders *et al.*, 2014). Spirulina's phycobiliproteins account for around 20% of the overall protein content of the cells. In recent years, they have been examined not just from a structural standpoint, but also for their possible pharmacological activities. Indeed, they have been found to contain antioxidant, antibacterial, anticancer, anti-inflammatory, and immune-modulatory characteristics, according to several reports (Kamble *et al.*, 2013; Stanic-Vucinic *et al.*, 2018; Costa *et al.*, 2022).

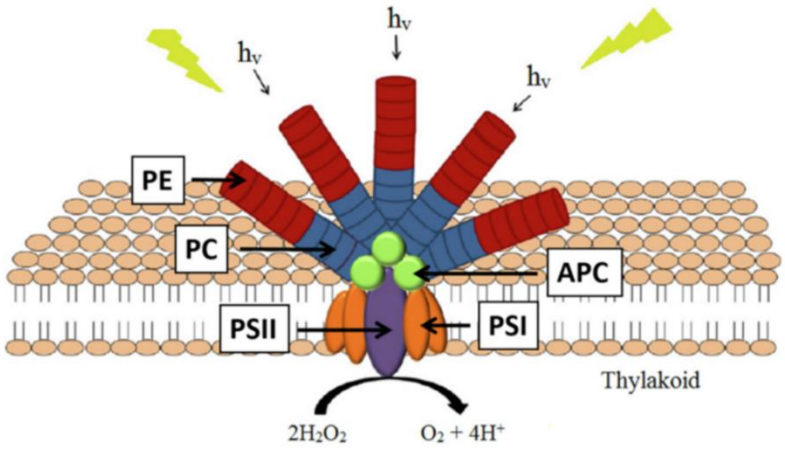

Figure 2. Phycobiliproteins structural organization on Phycobilisome. The schematic representation shows PE (phycoerythrin, red cylinders) to PC (phycocyanin, blue cylinders) and APC (allophycocyanin, green sphere) and reaction centers PSII (photosystems II, purple ellipse) and PI (Photosystems I, orange ellipses). Adapted and modified from Fernández-Rojas *et al.* (2014); Hsieh-Lo *et al.* (2019); and Pez Jaeschke *et al.* (2021).

2.4. Phycocyanin in Spirulina

With an estimated molecular weight ranging from 44 to 260 kDa, phycocyanin is a light harvesting, pigment binding protein which is present in liquid phase as mixture of several complexes; monomers, trimers, hexamers, and other oligomers in PBPs (Chaiklahan *et al.*, 2012; Weesepoel *et al.*, 2015; Buchweitz, 2016). All phycobiliproteins share the same structural theme including phycocyanin. It consists α and β protein subunits which both carry a different bilin-chromophore (isomeric linear tetrapyrrole prosthetic groups) differing in double bond arrangements (Morançais *et al*., 2018). Each subunit is typically composed of eight α-helices. The bilin groups are attached to the polypeptides through thioether bonds to specific cysteinyl residues which provide the foundation of the structure as shown in Figure 3. (Dianursanti and Hafidzah, 2021). Figure 2 shows how these pigments, including phycocyanin, form a cascade of energy transfer from phycoerythrin through phycocyanin and allophycocyanin, finally reaching photosystems I and II. Phycocyanin is the most prevalent pigment synthesized by the cyanobacteria Spirulina, accounting for up to 20% of the cell mass (Pez Jaeschke *et al.*, 2021).

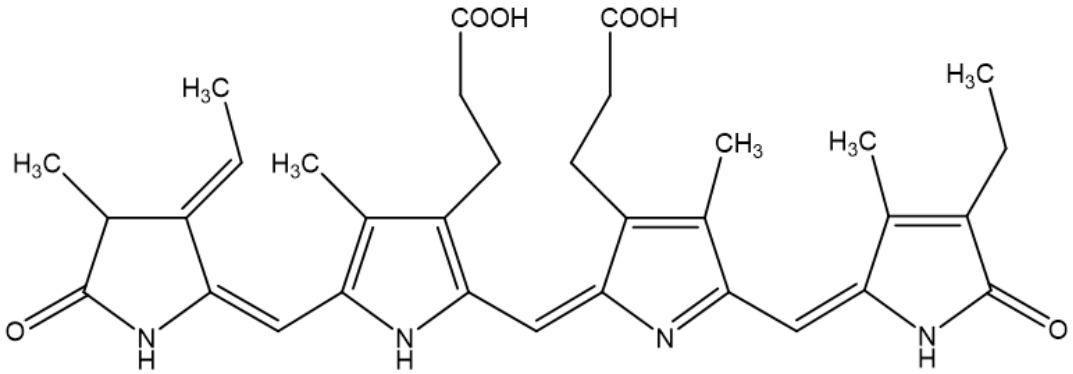

Figure 3. Phycocyanin chemical structure (Kim *et al*., 2018).

2.4.1. Extraction of Phycocyanin from Spirulina

Extraction is used to recover phycocyanin from Spirulina biomass. Cyanobacteria's cell walls are exceedingly robust (Siegelman and Kycia, 1978), though Spirulina is shown to have relatively fragile cell walls compared to others (Safi *et al.*, 2014). The extraction procedure used is critical for getting the most desired phycobiliproteins out of algae in their native state (Niu *et al.*, 2006). Cell rupture and the release of phycobiliproteins from within the cell are required for phycocyanin extraction. Thus, among other disruption methods, variations in osmotic pressure, abrasive conditions, chemical treatment, freezing-thawing,

and sonication are required for cell disruption. For large-scale operations, mechanical cell breakdown methods are usually preferred (Kula and Schütte, 1987).

Phycocyanin extraction can currently be done in a variety of ways in laboratories and industries. Several parameters influence the extraction outcome, including phycocyanin biomass content, size distribution, solvent type, extraction duration, temperature, mixing rate, biomass to solvent ratio and species stain. These factors influence the yield and also purity of phycocyanin during extraction (Bhattacharya *et al*., 2005; Moraes *et al.,* 2011; Prabuthas *et al.,* 2011; Taufiqurrahmi *et al.,* 2017; Dianursanti and Hafidzah, 2021). Optimization studies of phycocyanin extraction were developed for both the dried and wet biomass. These studies were reported in several publications using different methods of cell disruption as summarized in Figure 4 from Spirulina and various other cyanobacteria's (Moraes *et al.,* 2010; Silveira *et al.,* 2007).

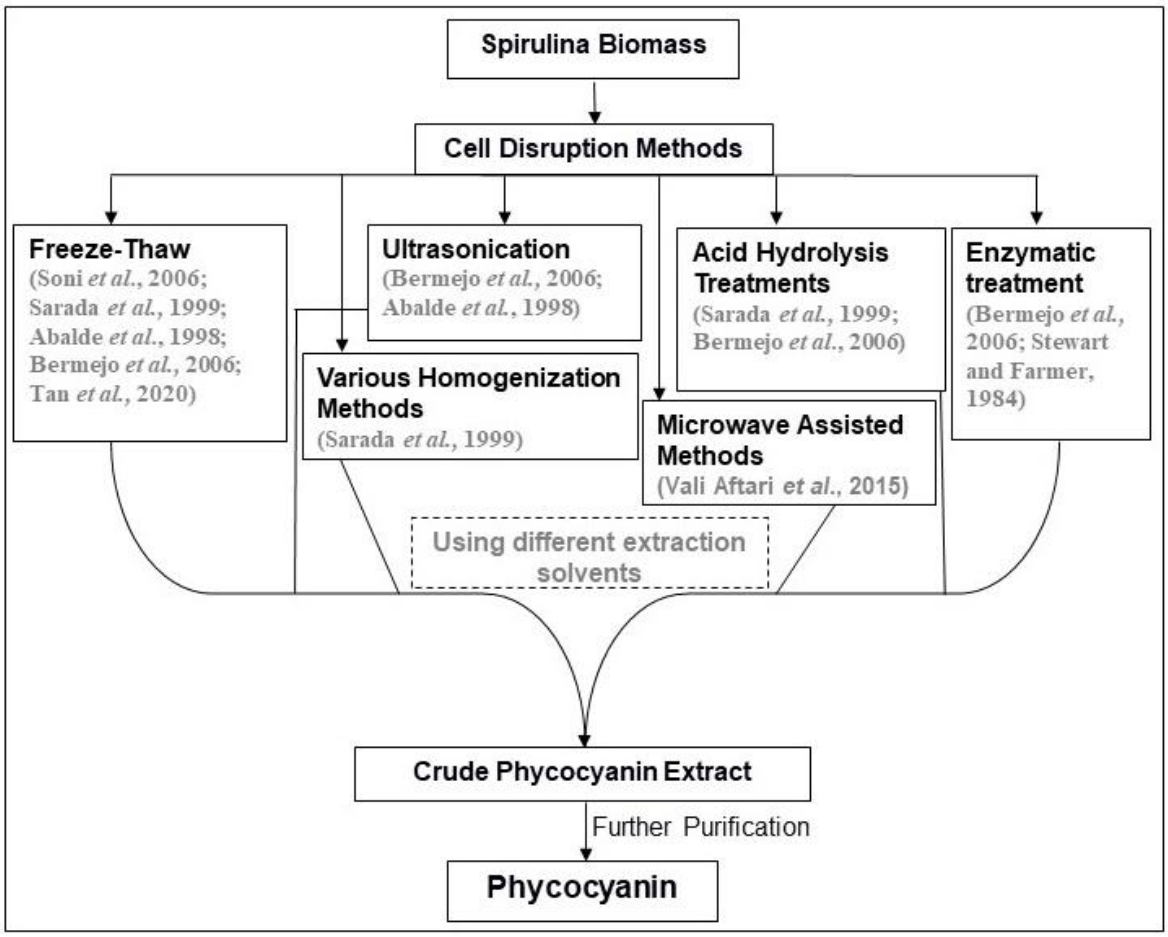

Figure 4. Summary of extraction process with different cell disruption techniques to extract phycocyanin from Spirulina cells.

2.4.2. Major Cell Disruption Methods for Phycocyanin Extraction from Spirulina

Manual Homogenization with Mortar and Pestle:

Cell disruption is done by crushing samples using a mortar and pestle in this basic homogenization procedure, which is more commonly known to be employed with plant materials. Solvents are introduced after the cells have been disrupted to extract the biological components. This approach has been used to extract phycocyanin from Spirulina cells in different studies (Moraes *et al.,* 2011; Horváth *et al.,* 2013).

In a study made by Viskari and Colyer, (2003), mortar and pestle extraction were found to be significantly more efficient than multiple freeze-thaw cycles of the sample dissolved in the extraction medium, but they also found that the grinding suffers from low reproducibility because it is done manually. Horváth *et al.* (2013) used a manual mortar and pestle homogenization for repeated cycles until no more phycocyanin was discovered in supernatants of cyanobacterial extract, allowing for five cycles of re-extraction, and reported that this improved the method's yield efficiency.

Extraction Using Freezing and Thawing Cycles:

At the laboratory scale, freezing and thawing cycles are routinely utilized to break the cyanobacteria cells (Moraes *et al.,* 2011; Tavanandi *et al*., 2018; Tan *et al.,* 2020). When the intracellular fluid freezes, ice crystals are formed that increases cell volume, which then is followed by cell contraction after thawing. Furthermore, due to variations in electrolytes concentration in certain areas of the cell, freezing causes changes in cell membrane pressure conditions and osmotic shocks, contributing to cell membrane disruption (Roquebert and Bury, 1993; Acker and McGann, 2003).

Repeated freezing and thawing cycles are time and energy-intensive, making the process best suited to the lab-scale analysis rather than industry, few authors have looked into the impact of the number of cycles on extraction yield and phycocyanin purity. According to Tavanandi *et al*. (2018), the optimal setting was four cycles of freezing and thawing, which allowed for high extraction yield and purity of phycocyanin. The release of contaminating proteins increases when the number of cycles is increased (5 and 6 cycles). Other researchers have also investigated the effects of 1-4 cycles on extraction yield and purity, have concluded that 3 cycles were optimal option (Saran *et al.*, 2016). This parameter may have an impact on extraction yield since it affects both cell disruption and phycocyanin stability; rapid extraction procedures are usually better studied to avoid degradation

processes. As a result, the number of cycles might be reduced to achieve higher extraction yields while still preventing phycocyanin degradation (Tavanandi *et al*., 2018; Pez Jaeschke *et al.*, 2021).

Although this method shows better performance than some of the other methods, according to Chittapun *et al.* (2020), the freeze-thaw technique showed better performance than Pulsed Electric Field (PEF) in terms of phycocyanin yield, while PEF was a better and rapid technology than freeze-thaw to obtain the product with high purity (Jaeschke *et al.,* 2019).

Extraction by Ultrasonication:

The transmission of ultrasonic waves (frequencies ranging from 20 kHz to 10 MHz) through target biological materials is known as ultrasonication, which basically works through the principle of propagation of ultrasonic waves in a medium that causes agitation, cavitation, shearing forces, shock waves, and the creation of free radicals, among other chemical and physical events (Chemat *et al*., 2017). Acoustic cavitation is the major driving factor when ultrasound is utilized to promote intracellular molecules extraction. The transmission of ultrasonic waves causes compression and rarefaction zones to emerge in liquids, resulting in the creation of bubbles. During the wave cycles, the volume of these bubbles shrinks and expands until they finally collapse. This occurrence is called acoustic cavitation (AshokKumar, 2011). On a microscopic level, the collapse of cavitation bubbles promotes localized high temperatures and pressures (up to 50 MPa), turbulence, collisions, and agitation. As a result, cavitation promotes cell membrane weakening and cell breakdown, resulting in higher extraction rates with an ideal solvent (Tiwari, 2015; Pez Jaeschke *et al*., 2021).

Ultrasound assisted extraction is widely utilized to break down cell walls, reduce particle size, and boost yield and mass transfer rate (Shirsath *et al.*, 2012; Dianursanti *et al.*, 2018). Although both ultrasound horn and ultrasound bath equipment can be used, the majority of extraction investigations are carried out utilizing an ultrasonic horn at low frequency (20–100 kHz) and high-power intensity (Ojha *et al.*, 2019; Tiwari, 2015). The cavitation process is favored in these conditions, resulting in increased extraction yields. High power intensities, on the other hand, cause strong and rapid membrane rupture, which may affect the purity of extracts. As a result, the major parameters that should be managed for ultrasound extraction are time and power intensity. Long exposure times and high-power

intensities might cause temperature rise, which should be avoided. Water jacket extraction chambers attached to a water bath or ice cubes can be used to manage temperature. Furthermore, Ultrasound horns can be used in a pulse mode to prevent sample overheating (Pez Jaeschke *et al.,* 2021).

Low extraction times of 2.5–16.2 minutes for ultrasound horns and 20–40 minutes for ultrasound baths are typical for Phycocyanin extraction, which is one of the technique's key advantages and result in considerable decrease in the overall cost of the operation (Hadiyanto *et al.,* 2016; Dianursanti *et al.,* 2018; Kumar *et al.,* 2020). In terms of ultrasonic intensity, moderate amplitude circumstances (40–60 percent) yielded higher extraction yields, but higher amplitude values did not improve phycocyanin concentration in the extracts (Tavanandi *et al.,* 2018). To facilitate Phycocyanin extraction, ultrasound bath can be also used in combination with glass beads (Moraes *et al.,* 2011; Ores *et al.,* 2016), homogenization (Pan-utai and Iamtham, 2019), or freezing and thawing (Patel *et al.,* 2005). In one such study, using ultrasound bath with glass pearls in combination, 56% higher efficiency than freeze-thaw technique was reported with extraction of 0.21 mg/g of phycocyanin concentration, which proved to be a promising efficient method (Moraes *et al.,* 2011).

Extraction by Bead Milling:

Bead milling is a mechanical cell disruption procedure that involves colliding biological cells with beads moving at a high speed. Shear forces created by collisions, friction between beads, or turbulence cause cell disruption (Nemer *et al.,* 2021). Bead milling is widely employed as a positive control in microalgae extraction investigations owing to its high disruption efficiency (Postma *et al.,* 2017). As a consequence, it has been used to determine the total amount of Phycocyanin found in cyanobacteria (Horváth *et al.,* 2013; Pott, 2018; Jaeschke *et al.,* 2019). Although no significant studies on bead milling optimization for Spirulina cells were discovered (Pez Jaeschke, *et al.,* 2021), the impact of processing factors such as cell disruption and bead collision frequency on other microalgae cell disruption was investigated to rationalize the process (Melendres *et al.,* 1991; Garrido *et al.,* 1994). Cell properties, medium viscosity, and flow rate all influence optimal circumstances. As a result, it is difficult to forecast how the parameters interact (Montalescot *et al*., 2015).

Glass, zirconium, ceramic, and steel are common materials that beads are made of (Yu, 2016). Small beads (0.2–0.6 mm) are chosen for cell disintegration since they increase the number of collisions (Garcia *et al.*, 2019; Zinkoné *et al.*, 2018). The impact of beads density on cell disruption is related to medium viscosity, according to Günerken *et al.*, 2015; low-density beads, such as glass beads, are better for low viscous medium, but high-density beads, such as zirconia beads, are better for high viscous medium. Furthermore, it has been shown that greater bead filling ratios cause higher disruption rates (Montalescot *et al.*, 2015; Postma *et al.*, 2015; Garcia *et al.*, 2019). The chamber capacity and geometry, agitation speed, and biomass content are also variables that can influence bead milling efficiency by influencing bead collisions (Postma *et al.*, 2015; Pez Jaeschke, *et al.*, 2021).

Although the effect of other factors such as biomass concentration is difficult to predict and debated among researchers (Doucha and Lívanský, 2008; Montalescot *et al.*, 2015) and its lack of selectivity necessitates additional purification steps to remove interference substances as fine cell debris (Günerken *et al.*, 2015). Overall, bead milling is a simple and fast disruption method, suitable for industrial-scale given the low energy input, high disruption efficiency and high biomass operation characteristics (Soto-Sierra *et al*., 2018; Pez Jaeschke, *et al.*, 2021).

Microwaves Assisted Extraction:

Microwave assisted extraction (MAE) employs microwave energy to rupture the cell membrane, releasing intracellular lipids into an organic solvent (Kumar *et al.*, 2021). Microwaves are non-ionizing radiation with frequencies ranging from 300 MHz to 300 GHz. Ionic conduction and dipole rotation generate heat as microwave passes through biological matrices, according to the general principle. Ionic conduction is the movement of ions under the influence of an electric field (Mandal *et al.*, 2009) and by causing friction between the ions and the medium, this movement increases the temperature. Dipole rotation is the alignment of dipolar molecules generated by an alternating electric field. The oscillation of the molecules creates collisions, which warms up the material (Vinatoru *et al.*, 2017). When compared to other approaches, this method uses a heating mechanism to greatly reduce extraction time (typically from a few seconds to half an hour) (Tatke and Jayswal, 2011). This has the benefit of preventing heat deterioration and oxidation. MAE also uses fewer solvents (which can be easily recovered), has a greater extraction yield and efficiency, is nontoxic, and can be used in larger quantities. MAE is one of the easiest and most effective ways of extracting biomolecules from microalgae among the various

approaches available. Nonetheless, the accompanying maintenance expenses are still substantial (Ventura *et al.,* 2017).

MAE can be carried out with or without the use of solvents. The solvent should be chosen for phycocyanin, a non-volatile chemical, based on its dielectric characteristics and phycocyanin solubility. When the biological material's dielectric loss tangent is greater than the solvent's, the substance may heat up faster, causing cell membrane breakdown. If the solvent has a larger dielectric loss tangent, however, the use of microwave may improve the diffusion process through the solvent (Vinatoru *et al.,* 2017). The partition coefficient, solubility of target chemicals in the solvent, dielectric characteristics of biomass/solvent, and mass diffusivity are all factors that influence MAE efficiency (Kumar *et al*., 2021). In a study made by Vali Aftari *et al*. 2015, comparisons have been made between ultrasonication and MAE methods for phycocyanin extraction and they have reported that the MAE system provides a significantly higher yield and purity of phycocyanin than Ultrasonication.

High Pressure Homogenization:

High pressure homogenization (HPH) is a mechanical cell disruption method in which biological suspensions are forced through a tight gap and subjected to high shear stresses. In narrow channels, a pressure drop (up to 2000 atm) is applied, resulting in high fluid velocities. Cell disruption is also achieved via the pressure drop between the nozzle and the environment, in addition to shear forces. The applied pressure, number of passes through the system, and temperature are the main factors that influence HPH efficiency. HPH does not require cell drying and can be employed for scaled-up processes. However, in order to achieve high-pressure conditions, this technology necessitates a large quantity of energy (Nemer *et al.,* 2021). Furthermore, low cell concentrations are commonly used, giving in greater specific energy consumption. Once cell debris is released into the medium, HPH is considered a non-selective extraction method, and purification steps are required after the extraction process (Carullo *et al.,* 2022).

Extraction through Pulsed Electric Field:

Pulsed electric fields (PEF) is seen as a promising technology for moderate and scalable cell disruption of wet biomass, avoiding the requirement for energy-intensive drying and the loss of labile chemicals that results (Carullo *et al.,* 2018). The technique involves repeatedly exposing fresh algae suspensions to high-intensity electric field pulses of short

duration (in microseconds) that cause cell membrane permeabilization via electroporation works by puncturing the cell membrane, allowing solvent penetration and selective release of intracellular matter without the formation of cell debris. (Carullo *et al.*, 2020). In batch mode, the PEF technique uses a moderate to high electric field intensity of 100–300 V/cm, and in continuous mode, 20–80 kV/cm (Ranjha *et al.*, 2021). PEF has recently been shown to increase the extraction yield by targeting intracellular compounds such as lipids, pigments, carbohydrates, and proteins from various microalgae and cyanobacteria, however, only a few studies have focused on Spirulina to date (Aouir *et al*., 2015; Martinez *et al.*, 2017; Jaeschke *et al.*, 2019; Carullo *et al.*, 2020; Ranjha *et al.*, 2021).

Carullo *et al*. (2020) recently reported that they were able to recover 73.7 % of the total phycocyanin utilizing aqueous wet microalgae suspension feedstock. They also stated that the PEF treatment allowed them to produce a higher purity phycocyanin extract than the HPH approach. According to their findings, using PEF in combination with mild heating could be a good way to recover water-soluble chemicals including phycocyanin from microalgal biomass efficiently and selectively.

All other approaches, except PEF, result in the non-selective release of intracellular chemicals, as well as the simultaneous dispersion of cell debris or other pollutants into the extraction medium, lowering the quality of the extracts and complicating downstream purifying processes (Aouir *et al*., 2015; Poojary *et al.*, 2016; Martinez *et al.*, 2017; Carullo *et al.*, 2018; Jaeschke *et al.*, 2019). However, the temperature at which PEF is applied has been shown to be an important factor in cell membrane electroporation. Though several studies have shown that raising the temperature of the system lowers the critical electric field required to trigger electroporation inside cells which enhances microbial inactivation and improving intracellular chemical extraction (Martinez *et al.*, 2017).

Chemical Treatment:

In this method, phycocyanin exudation occurs when certain chemicals disrupt the structure of the cell membrane and affecting its permeability. Chemical treatment is more suited to mass production of phycocyanin than freeze-thaw and lysozyme treatment methods, leaving less cellular debris than mechanical methods. On the other hand, the use of chemicals makes the purifying process more complex and can readily cause protein denaturation (Sarada *et al.*, 1999; Guan, 2016). Both organic and inorganic chemicals such

as HCl at different concentrations were studied and reported to successfully provide the release of the pigment (Moraes *et al.,* 2011).

Enzyme Digestion:

The naturally occurring enzyme, Lysozymes, which are glycoside hydrolase enzymes also known as muramidase or N-acetylmuramide glycanohydrolase are very useful for this cell disruption. By catalyzing the hydrolysis of 1, 4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins, these enzymes disrupt the cell walls of microorganisms. Egg white has a large amount of lysozyme, which can be used for cell disruption (Yoshimura *et al.,* 1988; Peters *et al.,* 1989; Hu *et al.,* 2013). Lysozymes gently and efficiently break down algal cell walls, making them potentially ideal for large-scale industrial extraction of phycocyanin. However, this procedure is not only costly but also produces certain unpleasant scents (Bermejo *et al*., 2006; Guan, 2016).

2.5. Factors Affecting Phycocyanin Extraction

2.5.1. Effect of Extraction Solvents

The influence of various types of solvents was evaluated by a number of studies (Saran *et al.,* 2016). Different researchers have evaluated distilled water (DH_2O), $CaCl_2$, double distilled water, citrate buffer, NaCl, potassium phosphate buffer, acetate buffer, sodium phosphate buffer, and also phosphate buffer saline (PBS) for phycocyanin extraction using different biomass/solvent ratios (Silveira *et al.,* 2007). In most cases, sodium phosphate buffer was considered the best solvent, leading to higher extraction yields. However, some studies have also achieved the highest concentration of phycocyanin extract yield and purity using double distilled water as extraction solvent (Tan *et al.,* 2020) while some others suggest DH_2O is a good solvent for phycocyanin extraction (Taufiqurrahmi *et al.,* 2017).

Beside the solvent types, the pH of the solvent plays a major role in the extraction recovery of total phycocyanin (Tan *et al.,* 2020). Because of the influence of solvent ionic strength on protein structure, pH has a direct impact on phycocyanin solubility. The best pH conditions were reported to be between 6 and 7 (Su *et al.,* 2014; Vali Aftari *et al.,* 2015), and phycocyanin is reported to be unstable at pH values below 5 and above 8 (Patil and Raghavarao, 2007; Su *et al*., 2014). An aqueous buffer solution is mostly utilized as a solvent to adjust the pH of the extraction medium within the pigment stability pH range.

Between the pH ranges of 6–8, sodium phosphate buffer is the most widely utilized solvent buffer solution. Some research was done to determine and optimize the best pH and solvent type for extracting phycocyanin from Spirulina (İlter *et al*., 2018; Silveira *et al*., 2007), however, the results were found to be contradictory (Pez Jaeschke, *et al.*, 2021).

2.5.2. Effect of Biomass/Solvent Ratio

The extraction ratio is another criterion that has received little attention. A wide range of ratio conditions (from 1:6 to 1:200) has been used due to a lack of optimization. As a result, not much is known about the influence of the ratio on extraction yield and extracts purity (Pez Jaeschke, *et al.,* 2021). The yield can be affected by differences in the biomass/solvent ratio used in the extraction process, which is also highly dependent on the type of solvent utilized. A higher yield of phycocyanin extraction can be achieved from the cells with more solvent. Every solvent has a maximum capacity limit in the amount of substance it can dissolve. The solvent can no longer dissolve once it has become fully saturated (Dianursanti and Hafidzah, 2021).

2.6. Applications of Phycocyanin

Phycocyanin is simple to get, safe, and non-toxic, making it an excellent candidate for further research and development with applications in the pharmaceutical, cosmetic, and food industries, and as a fluorescent dye (Eriksen, 2008). Several studies have been conducted on diverse applications and activities of phycocyanin from Spirulina. It has significant potential for development and utilization as a major constituent in different sectors as summarized in Figure 5.

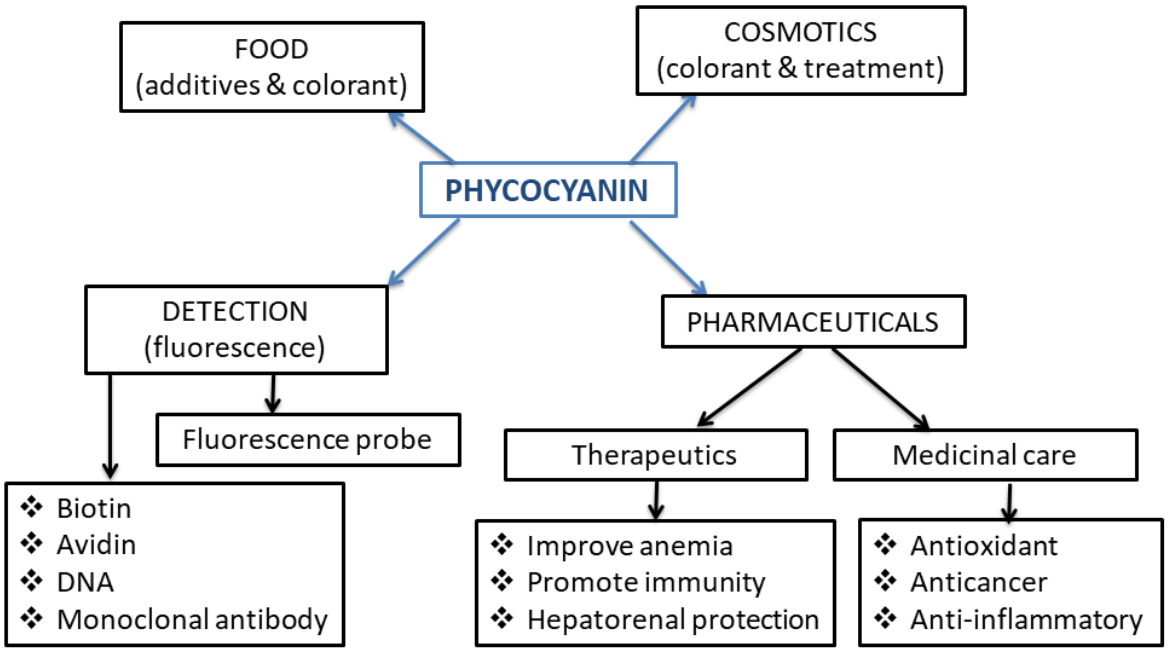

Figure 5. Key applications of phycocyanin.

In the Pharmaceutical sector, phycocyanin has numerous applications such as anti-inflammation (Romay *et al.*, 1998a; Romay *et al.*, 1998b), antioxidant (Renugadevi *et al.*, 2018), anticancer (Jiang *et al.*, 2017), immunological enhancement (Chen *et al.*, 2014), and hepatorenal protection (Ou *et al.*, 2010; Raghuvanshi *et al.*, 2020) benefits which have been reported in various studies (Liu *et al.*, 2016). Phycocyanin is also a nontoxic photosensitizer that can be employed in the photodynamic treatment of malignancies as adjuvant therapy (Bharathiraja *et al.*, 2016).

Spirulina-derived phycocyanin is widely utilized as a food ingredient both as a natural colorant and also has health benefits. It is also a famous colorant in the cosmetic industry having an added asset in the arena of skincare due to its free radical scavenging and anti-inflammatory properties which have gained significant attention (Khandual *et al.*, 2021; Ragusa *et al.*, 2021).

Because of its strong fluorescence, phycocyanin is being explored as a fluorescent reagent, fluorescent probe, and fluorescent tracer, which are used in medical diagnosis, immunology, biological engineering, and other research areas (Paswan *et al*., 2015; Liu *et al*., 2016; Zheng *et al*., 2018).

CHAPTER THREE

3. Materials and Methods

3.1. Description of Sampling Area

Samples of the Spirulina algae were collected from its natural habitat - Lake Chitu, where it is found in a mass quantity, 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 6. The area is found at an altitude around 1600m above sea level with annual rain fall ranging between 500 mm and 700 mm (Kebede *et al.* 1994; Assaye *et al.*, 2017). Lake Chitu is a pea-colored Crater Lake, covers 0.8 square kilometers of area with a maximum depth of 21 meters (Kebede *et al.* 1994). The lake's rich blue green alga provides food for more than 10 thousand Flamingos throughout a year and has high saline nature. Lake Chitu is the best source of the microalgae among other algae habituated lakes in Ethiopia for its suitable physicochemical nature for growth of Spirulina microalgae and in many literatures the species is proven to be found dominating this habitat in immense amount almost as a unialgal population (Kebede *et al.* 1994; Kebede, 1997; Ogato *et al.*, 2015; Assaye *et al.*, 2017).

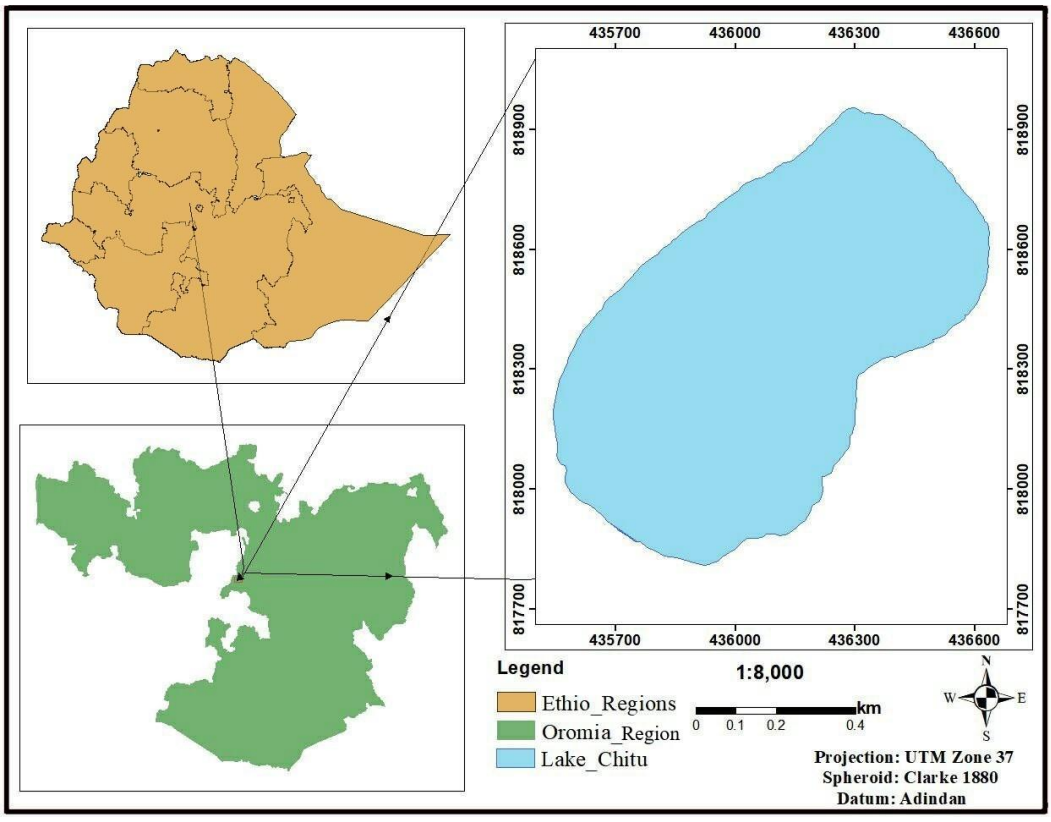

Figure 6. Geographical map of the study area made using ArcGIS (v10.8).

3.2. Sample Collection

Repres**e**ntative lake water samples with algal blooms containing suspended microalgae from five different locational site points were taken following standard methods described by Rout *et al*. (2013) and Karssa *et al*. (2018) using sample bottles from about 10 cm depths by manual sampling and each sampling site points were separated at least by 100 meters distance from each other. Lake water samples of 300ml were collected from each site using clean plastic bottles and then labeled accordingly with samples identification code. The pH values of the samples were measured using (pH Pen Tester, Bioevopeak Co. Ltd., China) and GPS coordinates of their respective sites were also recorded using 72H, Garmin GPS, U.S.

3.3. Growth Medium and Culture Conditions

Advanced automated microalgae culture growing setup (Figure 7), was used which automatically regulates 12:12 hours light/dark variations and temperature with air pump supply for continuous mixing and also equipped with CO_2 gas supply, at National Microalgae Biotechnology Laboratory, Bio and Emerging Technologies Institute (BETin), Addis Ababa, Ethiopia. Spirulina medium (SP Medium) recipe (See Appendix 3) adopted from Culture Collection of Algae and Protozoa (CCAP) catalogue (www.ccap.ac.uk) was prepared and used to grow the cultures.

Figure 7. Laboratory setup at National Microalgae Biotechnology Laboratory, BETin for growing algal cultures.

3.3.1. Identification

Samples were inoculated in test tubes containing 10mL of SP media for growth. Two milliliters of inoculant were injected in the medium and incubated at 30°C, mixed manually every 3 hours during the day for 20 days under 12 hours light and 12 hours dark variation with 3% CO_2 at 150 ml/min supplied every 10 minutes for 5 seconds (Kim *et al.*, 2018). Cells of Spirulina species were identified under microscope (Visiscope TL524PH, VWR Microscope, Italy) at 100X magnification by their morphological trichomes and further grown for screening and isolation.

3.3.2. Screening and Isolation of Pure Culture

Three best samples were first selected out of the first five group of grown sample cultures based on microscopic examination and further subjected to a serial dilution of 3 generations by inoculating 2ml, 1.5ml and 1ml in 10ml, 50ml and 100ml growth media to screen and obtain pure isolates of Spirulina by eliminating the contaminations. Frequent follow up microscopic investigation on the purity and the overall maintenance of cells were conducted.

3.4. Determination of Growth Rate and Growth Curve of the Isolates

To maintain effective growth of Spirulina biomass, a constant 10% of inoculum size was maintained on each batch by incubating for 20 days. Using growth conditions to yield optimal effective growth rate, growth curve was determined spectrophotometrically using UV-Vis instrument (Cary 60 UV-Vis, Agilent Technologies Inc., California, U.S.) by measuring absorbance at 680nm every 24 hours for 20 days. 4mL culture broth was transferred aseptically into cuvettes and measures were recorded for analysis. The data was also used to determine specific growth rate (μ) and doubling time (DT) using measurements at exponential phase of growth according to Göksan *et al*. (2007) through the following equations *(Eq. 1 and Eq. 2),* respectively.

$$\mu = \frac{lnX2 - lnX1}{t2 - t1} \qquad \textit{(Eq. 1)}$$

Where: μ= specific growth rate in mg/day; X1= biomass at time t1; X2= biomass at time t2; t1= initial time; t2= final time.

$$DT = \frac{ln2}{\mu} \qquad \textit{(Eq. 2)}$$

Where: μ= specific growth rate in mg/day

3.5. Biomass Harvest and Drying

Total dry biomass yield or dry weight (DW) of cultivation right at the time of harvest was determined according to Göksan *et al*. (2007); 50 mL of the algal suspension taken from log phase of the culture was filtered using a pre-weighed dry Whatman filter paper. The samples were thoroughly rinsed with DH_2O to remove the chemical load due to the culture medium from the biomass, then the filter paper with settled algal biomass was weighed after dried in a hot air oven at 105°C for 6 hours. The weight of total biomass was represented by the change in filter paper weight before and after filtering and drying which was simply calculated as in *Eq. 3* to find the dry weight of the biomass on the filter paper after drying then expressed in $g.L^{-1}$.

$$DW = X2 - X1 \qquad \textit{(Eq. 3)}$$

Where X_2 is total weight of filer paper after Drying and X_1 is Weight of filter paper

Cells of Spirulina were harvested from log phase cultures by suspending on a silk cloth for filtration and washed twice with DH_2O. Dry biomass was obtained by hot air oven (GX-65B Drying Oven, Huanghua Faithful Instrument Co., LTD., China) drying at 40 °C for 24 hours from the suspended wet biomass and then dried biomass was stored at room temperature for future steps (Desmorieux and Hernandez *et al.,* 2004).

3.6. Pigments and Total Carotenoid Estimation

It is basically understood that phytochemicals have a variety of biological properties that promote health and lower the risk of chronic diseases. One of the micronutrient phytochemicals with the most thorough research into their possible health benefits are carotenoids. It is important to note that particular types of carotenoids are responsible for various protective effects against particular diseases and disorders. Furthermore, numerous kinds of sources can be used to obtain each carotenoid (Milani *et al.,* 2017). It is crucial to investigate the potential total carotenoid content of Lake Chitu's Spirulina because the phytochemical carotenoid content of various sources can vary depending on the types and cultivation conditions of the source photosynthetic organism.

Extraction process was conducted to make withdrawal of desired constituents using selected solvent in which the desired substances can be dissolved. The solvent was selected based on its ability to dissolve the maximum amount of the active substance and the minimum for undesirable elements (Rinawati *et al.,* 2020). To determine total carotenoid,

10 ml of 80% acetone was added to a 0.5 g of dry and fine grounds of biomass was achieved by mortar and pestle manual grinding for 5 minutes according to the method developed by Yang *et al*. (1998). The solution was then centrifuged at 1,500 rpm for 5 minutes (TC4815D MICROSPIN Centrifuge, Eltek, Maalab, India) and the absorbance of the supernatant was measured using UV-Vis spectrophotometry at 663 nm, 646 nm and 470 nm using 80% acetone as a blank. Content of chlorophyll a, chlorophyll b and total carotenoids were calculated using the absorbance readings in equations (*Eq. 4, Eq. 5* and *Eq.6* respectively) as developed by Wellburn, (1994), established and adopted based on the solvent used for extraction and the spectral resolution range of the UV-Vis spectrophotometer instrument used which was 1.5 nm spectral bandwidth (Cary 60 UV-Vis, Agilent Technologies Inc., California, U.S.) and also according to Lichtenthaler, (1987) and Minchev *et al.* (2021).

$$Chlorophyll\ a\ (\mu g/ml) = 12.21\ Abs\ at\ 663\ nm - 2.81\ Abs\ 646\ nm \qquad (Eq.\ 4)$$

$$Chlorophyll\ b\ (\mu g/ml) = 20.13\ Abs\ at\ 646\ nm - 5.03\ Abs\ 663\ nm \qquad (Eq.\ 5)$$

$$Tot.Car.(\mu g/ml) = \frac{1000\ Abs470nm - 3.27(Chl\ a) - 104(Chl\ b)}{198} \qquad (Eq.\ 6)$$

Where: Abs is absorbance, Chl a is Chlorophyll a and Chl b is Chlorophyll b

3.7. Optimization of Solvent Type for Phycocyanin Extraction

To determine type of solvent which gives best result based on phycocyanin concentration, extraction of crude phycocyanin was done using 1:50 biomass/solvent ratio and extracted using classical mortar and pestle cell disruption method by manually grinding for 5 minutes according to Silveira *et al.* (2007). After 24 hours of extraction time crude phycocyanin extracts were obtained by centrifugation at 6000 rpm for 10 minutes (Taufiqurrahmi *et al.*, 2017) using different available solvents; DH_2O (pH 7), 1M phosphate buffer saline (PBS) (pH 7.4), 0.1M sodium phosphate buffer (SPB) (pH 7.1) and 1.5% $CaCl_2$. Spectrophotometric absorbance of the crude phycocyanin extract obtained at 615 nm and 652 nm were recorded. Comparisons were made based on analytically calculated phycocyanin concentration (PC) of each crude extracts using respective solvent types. Then the results were used to determine the type of solvent with the highest PC to be used for the next steps in this study. The phycocyanin concentration (PC) was estimated in

mg.ml^{-1} calculated from the optical density measures of crude extract at 615 and 652 nm, using the following equation (*Eq. 7*) (Bennett and Bogard, 1973):

$$PC = \frac{Abs615nm - 0.474(Abs652nm)}{5.34} \quad \text{(Eq. 7)}$$

Where: Abs is absorbance.

3.8. Crude Phycocyanin Extraction

For effective phycocyanin extraction 0.1M of SPB (pH 7.1) solvent was employed in 1:50 w/v for biomass/solvent ratio with 24 hours extraction time for each extraction method as optimized in the previous section of this study and as suggested by Taufiqurrahmi *et al.* (2017). Spirulina biomass was weighed on an electronic beam balance (LA 124, VWR, Italy), solvent was measured using graduated cylinder and cells were disrupted by five different methods. For all extraction processes using the five different cell disruption method, each sample was similarly centrifuged at 6000 rpm for 10 minutes at 4 °C using bench-top high-speed refrigerated centrifuge (TLG-16M, Drawell, China) after cell disruption. The pellet residues were discarded and the supernatants were carefully isolated and stored at 4 °C (Yong, 2018). Unless specified, all experiments were conducted at 4 °C under low-light/dark place to avoid heat and light protein pigment degradation (Khazi *et al.*, 2018). After extraction, the levels of the PC were determined using the spectrophotometric method for each extract using *Eq. 7* and the extraction yield in mg.g^{-1} of DW was calculated using *Eq. 8* (Silveira *et al.*, 2007).

$$Yield = \frac{PC \text{ X } V}{DB} \quad \textit{(Eq. 8)}$$

Where: Yield is the extraction yield of phycocyanin in mg of phycocyanin/dry biomass (g), V is the solvent volume (ml) and DB is the dry biomass (g).

Extraction Efficiency: For every given disruption and extraction process, the extraction efficiency was measured and calculated using equation *Eq. 9* according to Kilpatrick, (1985) and Viskari and Colyer, (2003), which was similarly used to calculate the amount of crude phycobiliprotein extracts. The absorbances of disrupted cell samples were measured at 545 nm and 750 nm and the absorbance of supernatant of the extract after centrifugation was measured at 545 nm for each extraction method. The measured absorbances were used in the following equation:

$$\%EE = \frac{Abs\ of\ supernatant\ at\ 545nm}{Abs\ of\ sample\ at\ 545nm - Abs\ of\ sample\ at\ 750nm} \text{ X } 100 \quad \textit{(Eq. 9)}$$

Where: %EE is percent extraction efficiency and Abs is absorbance.

Where Abs is absorbance, samples and supernatant are the stages at which the absorbance was measured and recorded for samples of disrupted biomass before centrifugation (entire cell material in the solvent) and supernatant of the extract after separation, respectively. The absorbance of the disrupted biomass sample at 750 nm is measured in the equation as a correction factor for incident light scattering in the sample due to cell debris.

3.8.1. Mortar and Pestle (MP)

Weighed dry biomass was added to a ceramic mortar and cells were disrupted by crushing and grinding using ceramic pestle manually for 5 minutes followed by the addition of solvent. The solution was transferred to a 50 ml falcon tube for centrifugation (Moraes *et al.,* 2011).

3.8.2. Freeze and Thaw (FT)

Dry biomass was weighed and dissolved in the solvent containing beaker and placed into deep freezer (ULF86 Ultra Low Temperature Freezer, Bioevopeak, China). Freezing for 2 hours at -80 °C and thawing at room temperature for 24 hours was conducted for 2 cycles (Tan *et al.,* 2020).

3.8.3. Ultrasound Assisted Extraction (UAE)

A prepared solution of weighed dry biomass and solvent in a beaker was treated with ultrasonication for 3 hours extraction time which was conducted in ultrasonic bath (RK106S Sonorex, Bandelin, Germany) at AAIT laboratory, Addis Ababa University (Minchev *et al.,* 2021).

3.8.4. Microwave Assisted Extraction (MAE)

A solution mix of solvent and weighed dry biomass in a beaker was treated at 100 °C flash irradiation for 10 seconds according to Juin *et al.* (2015) using Microwave oven (R-562CT(ST), Sharp, Japan) at BETin.

3.8.5. Homogenization with Beads (HB)

High speed homogenization of weighed biomass was achieved using High Speed Tissue Homogenizer (KZ-III, Servicebio, China) in the presence of 3mm steel beads for 1 minute grinding time and repeated for 3 cycles with a 15 second pause in between the cycles at 60Hz. The homogenized sample was mixed with solvent in a 50 ml falcon tube.

3.9. Partial Purification of Extracts

Since phycocyanin is a pigment-protein complex, the ionic strength and pH of the solution affect its solubility in water. When salt is added, globular proteins become more soluble by a process known as 'salting-in' and protein solubility often declines at increased salt concentrations, causing precipitation which on the other hand this phenomenon is known as 'salting-out'. The highest surface tension of water can be caused by salts that promote salting-out. Ammonium sulfate ($(NH_4)_2SO_4$) is a salt that is frequently employed for this purpose since it has a significantly higher solubility than any other phosphate salts. Due to its high solubility even at lower temperatures, pH versatility, low heat requirement of the solution, inexpensive cost, and overall salting-out effectiveness, ammonium sulfate is the preferred reagent for salting-out and this process is well known as ammonium sulfate precipitation which allows isolating proteins from other impurities in a solution. Briefly, proteins in aqueous solutions are highly hydrated, and when salt is added, the greater charge of the salt causes the water molecules to be more attracted to it than to the protein. The salt typically has an advantage in this hydration competition, which causes the proteins to interact, causing aggregation and consequently resulting in precipitation. After centrifuging the solution, the protein pellet can be redissolved in a low salt buffer to obtain the purified protein. Since each protein has its own unique properties, it frequently happens that they precipitate (or salt-out) at a specific salt concentration (Green and Hughes, 1955; Wingfield, 1998).

The ammonium sulfate precipitation and dialysis of each crude phycocyanin extracts for partial purification was conducted following a protocol designed based on the well-established principles and basic procedures adapted from Khazi *et al.* (2018) (see in Appendix 4). Partially purified samples were lyophilized against the extraction solvent and then stored at 4°C.

3.10. Pigment Protein Validation

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was employed to characterize and validate the extracted phycocyanin protein pigments in electrophoretic separation using 12% precast gels. The technique initially denatures the proteins then subject to electrophoresis that separates based on size. To make the bands visible, the gel was stained with Coomassie brilliant and destained with the destaining buffer for clear visualization and image capture (Patel *et al.,* 2005).

3.11. Biological Activity Test of Extracts

3.11.1. Antimicrobial Activity Test

Inoculum, media plates and culture preparation:

Four American Type Culture Collection (ATCC) bacterial strains, two belonging to Gram negative and two to Gram positive group, *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC27853), *Enterococcus faecalis* (ATCC79112) and *Escherichia coli* (ATCC25972), were obtained from Ethiopian Public Health Institute (EPHI), Addis Ababa, Ethiopia and used to measure antibacterial activity of each sample extracts using disc diffusion method along with an antibiotic positive control of commercial Ceftriaxone drug powder. All materials were autoclaved and Muller Hinton Agar (MHA) media was prepared according to Balouiri *et al.* (2016) then poured on Petri dish plates. Bacterial colonies were activated and prepared in nutrient broth media and incubated at 37°C. After 18 hours of incubation, newly grown fresh bacteria colonies with 10^{-7} colony forming unit (CFU) from the broth culture were inoculated onto the prepared MHA media Petri dishes by spreading technique using sterile swab.

Preparation and impregnation of discs, and incubation:

Lyophilized powder of sample extracts, 500 µg each, were added in 1ml of extraction solvent (0.1M of SPB) keeping it as a 100% maximum and then prepared at different concentrations as 50%, 25% and 12.5% which was 250 µg/ml, 125 µg/ml and 62.5 µg/ml respectively. A positive control (ceftriaxone 30 µg/ml) were prepared for each samples diluted using same 0.1M SPB which was used for extraction and the solvent alone was used as a negative control in the assay. Sterile 5 mm diameter paper discs were prepared and impregnated with samples of each concentrations including the positive and negative controls and then placed on each respective prepared bacteria inoculated MHA Petri dishes by separating each with quadrants on a single plate to fit each concentration of a single sample extract test against a single pathogen bacteria culture including the positive and negative controls. This was conducted in triplicate and a total 60 Petri dishes were labeled before incubation accordingly and investigated.

Determination of antibacterial activity by the disc diffusion method:

Antibacterial activity of each extracted phycocyanin samples against the four different human pathogenic bacteria were determined by measuring the clear inhibition zones observed on the culture plates around the placed discs in millimeters using ruler after incubation period of 24 hours at 37°C.

3.11.2. Antioxidant activity test

The free radical scavenging activities of the phycocyanin extracts in this study were measured by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay method. With this method, originally discovered by Goldschmidt and Renn (1922), then developed by Blois (1958), and later modified by studies of Brand-Williams *et al*. (1995) and Bondet *et al*. (1997), it is possible to determine the radical scavenging power of an antioxidant agent by measuring the decrease in the absorbance of DPPH at 517 nm spectrophotometrically. Resulting in a color change from purple to yellow, the absorbance decreases when the DPPH radical is scavenged by an antioxidant agent through the donation of hydrogen to form a stable DPPH molecule. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. This method is simple, quick, affordable, and popularly effective for determining a compound's capacity to act as a hydrogen donor or free radical scavenger in order to assess the antioxidant activity of different compounds (Kedare and Singh, 2011).

The DPPH radical scavenging assay for this study was conducted according to Renugadevi *et al.* (2018), with slight modification. 200 µg/ml stock solutions of each lyophilized sample were prepared and then aliquoted at different concentration of 100%, 50%, 25% and 12.5% for the test which was 200 µg/ml, 100 µg/ml, 50 µg/ml and 25 µg/ml respectively. 1 mL of each sample was added to 3 mL of a 0.1 mM methanolic DPPH and stored in a dark place for 30 minutes then absorbance was read at 517 nm. The methanolic DPPH solution was used as a control and activity of Ascorbic acid solution was used as a standard. The percentage radical scavenging activity was calculated according to the formula shown in Eq. 10.

$$\% RSA = \frac{A0-A1}{A0} \text{ X } 100 \qquad \textit{(Eq. 10)}$$

Where, %RSA is percent radical scavenging A_0 is the absorbance of the control and A_1 is the absorbance of sample.

3.13. Data Analysis

Computational equations enlisted in the methodology has provided the necessary data in the analytical procedures of this study. The statistical variance examining technique One-way and Two-way ANOVA were used for comparison of data obtained during the undertaking in computer statistical software SPSS (v26) at 95% confidence interval to see statistically significant differences among parameters (Tukey's test). Statistical values of *p*

< 0.05 were considered significant. In addition, mean values of data were tabulated and graphs were generated using Microsoft Excel.

CHAPTER FOUR

4. Results

4.1. Culture Maintenance and Biomass Analysis

Cells of Spirulina species from Lake Chitu were identified under microscope from collected samples by their morphological trichomes along with some contaminant organisms such as morphologically appearing Diatom species as shown in Figure 8 and then further screening and isolation of monoculture isolates were achieved through serial dilution as illustrated in Figure 9. Growth curve of Lake Chitu's Spirulina cells were obtained as shown in Figure 10. Specific growth rate was found to be 0.196 $mg.d^{-1}$ in the exponential phase of growth and dry weight at the harvest was found to be 0.364 $g.L^{-1}$ with a doubling time of 3.536 days. Dry biomass harvesting process steps are done as summarized in Figure 11.

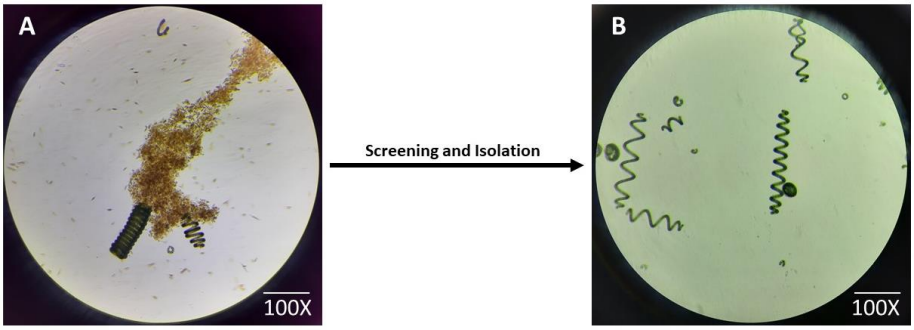

Figure 8. Microscopic images showing screening and isolation, captured sample before serial dilution (A) and pure culture sample after serial dilution (B).

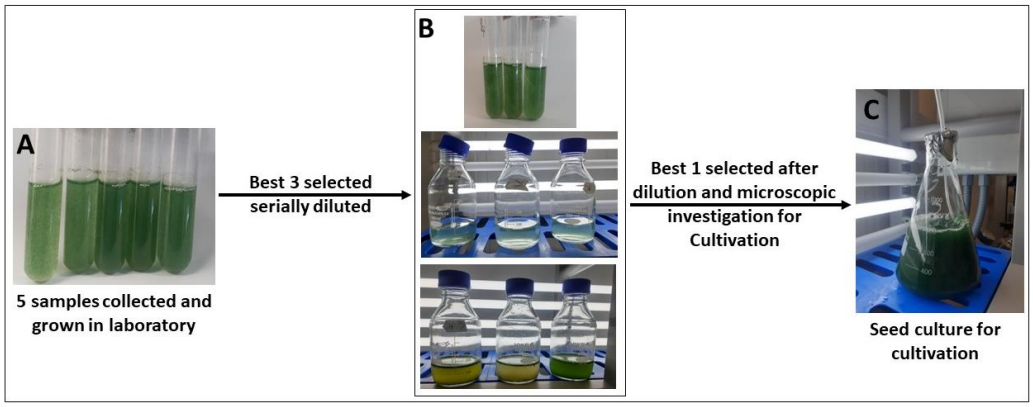

Figure 9. Serial dilution experiment for screening and isolation to obtain monoalgal culture. Samples collected from lake were grown in test tubes (A) and 3 best were selected based on microscopic investigation and serially diluted (B) to obtain 1 selected monoculture isolates of Spirulina (C) used for further cultivation.

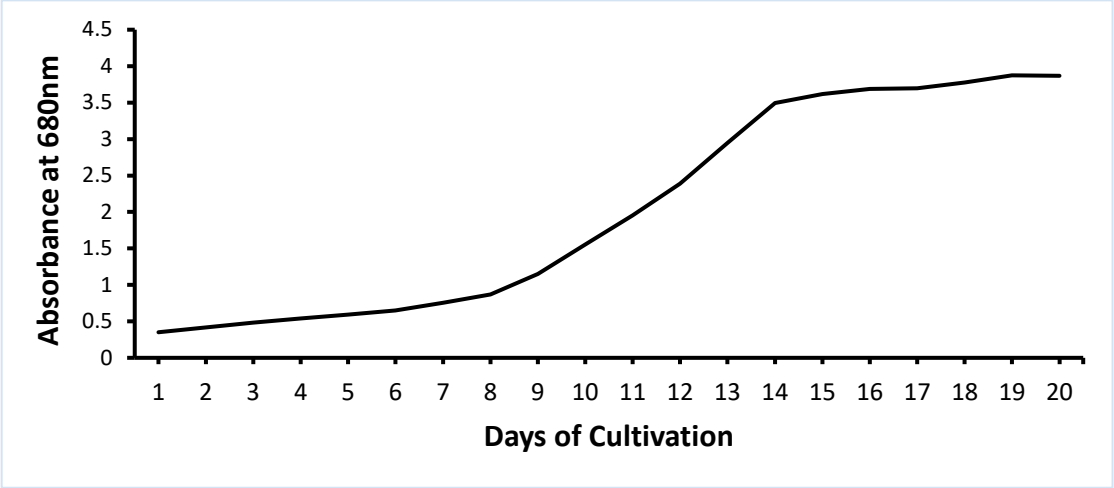

Figure 10. Growth curve graph of *Arthrospira fusiformis* isolated from Lake Chitu, Ethiopia.

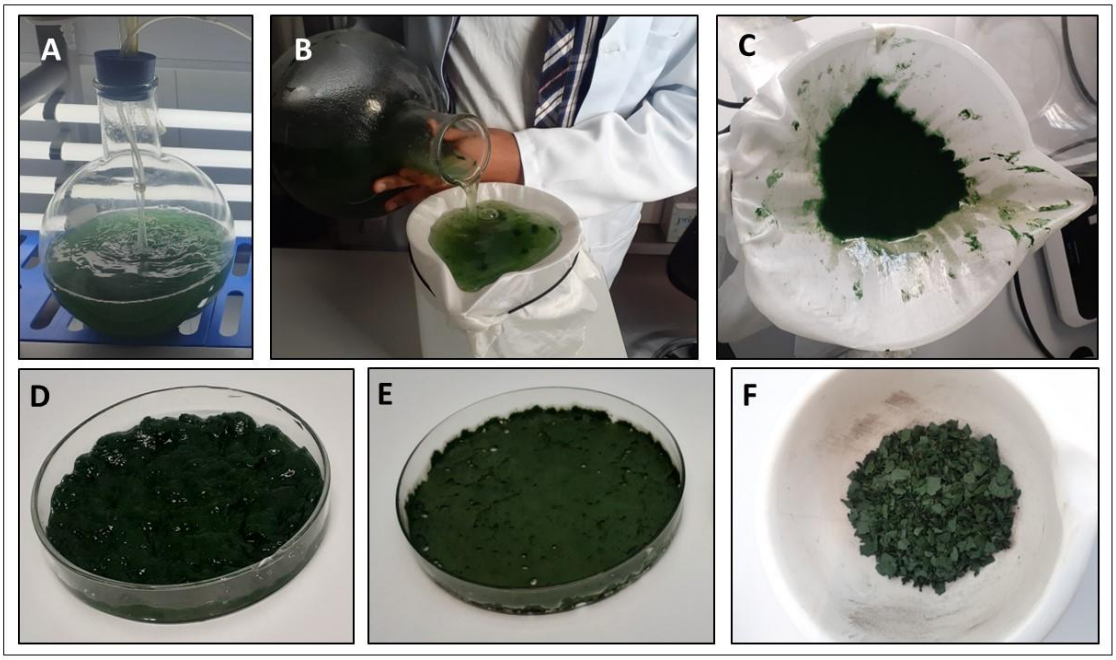

Figure 11. Biomass harvesting and drying process. Grown culture of Spirulina (A) was filtered (B) and washed (C) using a silk cloth and DH_2O. Suspended Biomass (D) was then dried (E) and collected (F) for next steps in the study.

4.2. Estimation of Total Carotenoids

Following the spectrophotometric method and equations analysis, Chlorophyll a content was found to be 470.3 $\mu g.ml^{-1}$ and Chlorophyll b content was found to be 153.21 $\mu g.ml^{-1}$. These values per DW content of Spirulina can be expressed as 9.406 $mg.g^{-1}$ and 3.0642 $mg.g^{-1}$, respectively. Calculated total carotenoid estimate of the samples cultivated was found to be 195.6 $\mu g.ml^{-1}$, which is equivalent to 3.912 $mg.g^{-1}$ DW of Spirulina.

4.3. Determination of Extraction Solvent Type

Standard extraction procedure has yielded crude phycocyanin using different solvents as shown in Figure 12 with different range of visible bluish-purple color. PC values of each extracts obtained using respective solvent types were calculated and the results are graphically illustrated in Figure 13. The solvent 0.1M SPB was found to yield higher level of PC (0.415 ± 0.024 mg.ml^{-1}) as compared to DH_2O (0.395 ± 0.026 mg.ml^{-1}), 1M PBS (0.39 ± 0.013 mg.ml^{-1}) and 1.5% $CaCl_2$ (0.407 ± 0.004 mg.ml^{-1}), respectively. Therefore, 0.1M SPB was selected to be used as an extraction solvent throughout the next experiments in this study.

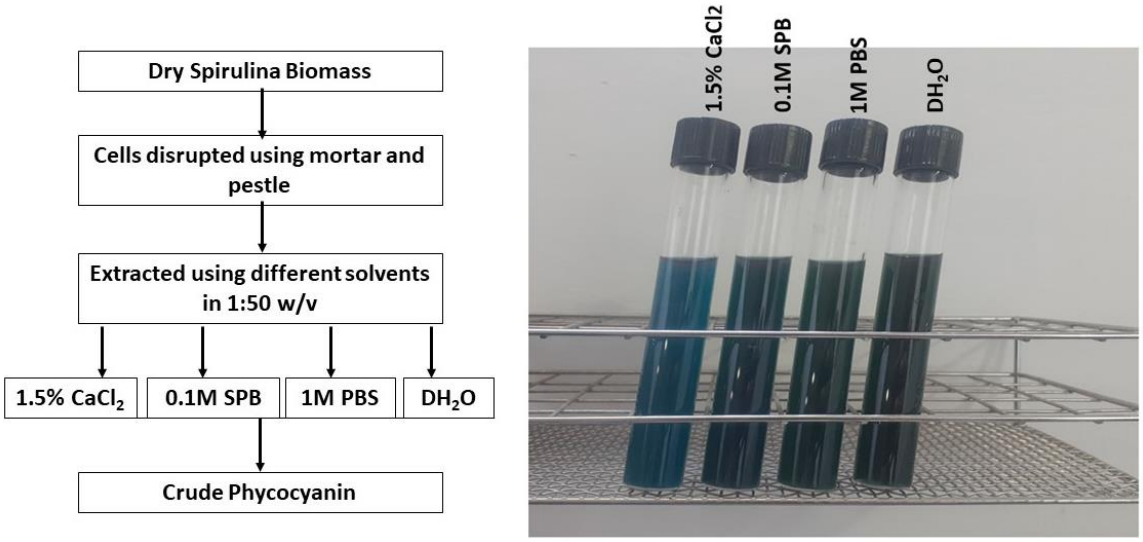

Figure 12. Solvent type determination process. Flowchart diagram (left) shows the steps involved in crude phycocyanin extraction using cell disruption method (mortar and pestle) and different solvents for the determination of solvent type. Results (right) obtained using the steps shown in the flowchart. SPB is sodium phosphate buffer and PBS is phosphate buffer saline.

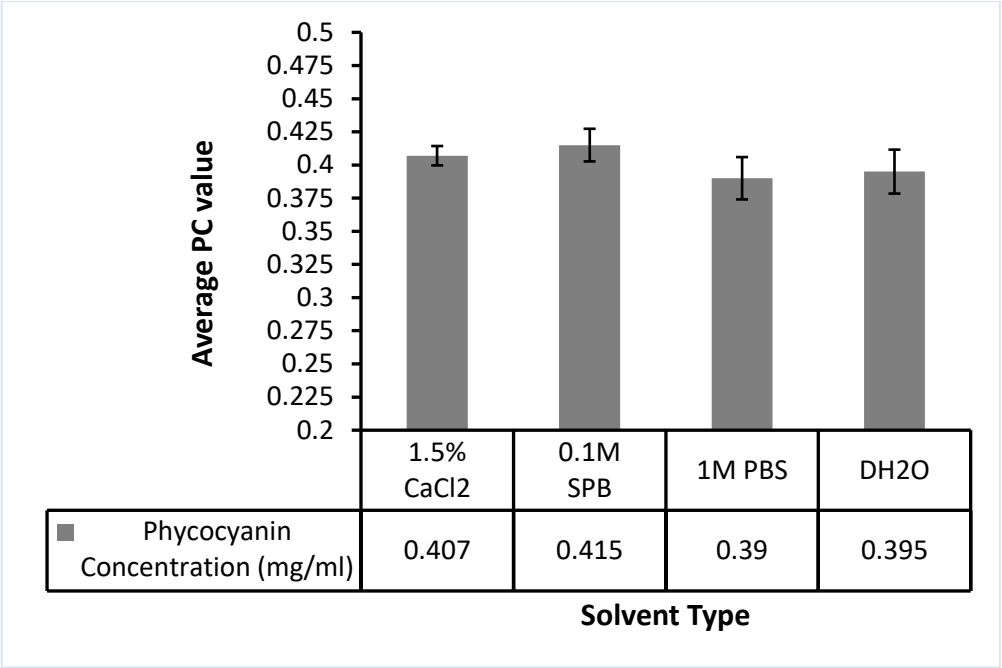

Figure 13. Phycocyanin concentration (PC) values of extracts obtained using different solvents. Extraction was done for the determination of solvent type. SPB is sodium phosphate buffer and PBS is phosphate buffer saline.

4.4. Crude Phycocyanin Extraction

The presence of phycocyanin pigment in the supernatant extracts was seen by the visible bluish-purple color of the solution in all extracts obtained using the five different techniques employed to extract crude phycocyanin from the Spirulina biomass as shown in Figure 14. PC values of each extract are presented in Table 2 in $mg.ml^{-1}$ with data provided in terms of mg of phycocyanin obtained per gram of DW of Spirulina, referred to as the extraction yield (Yield). Results for extraction efficiency in percentage were also obtained and presented for each extract.

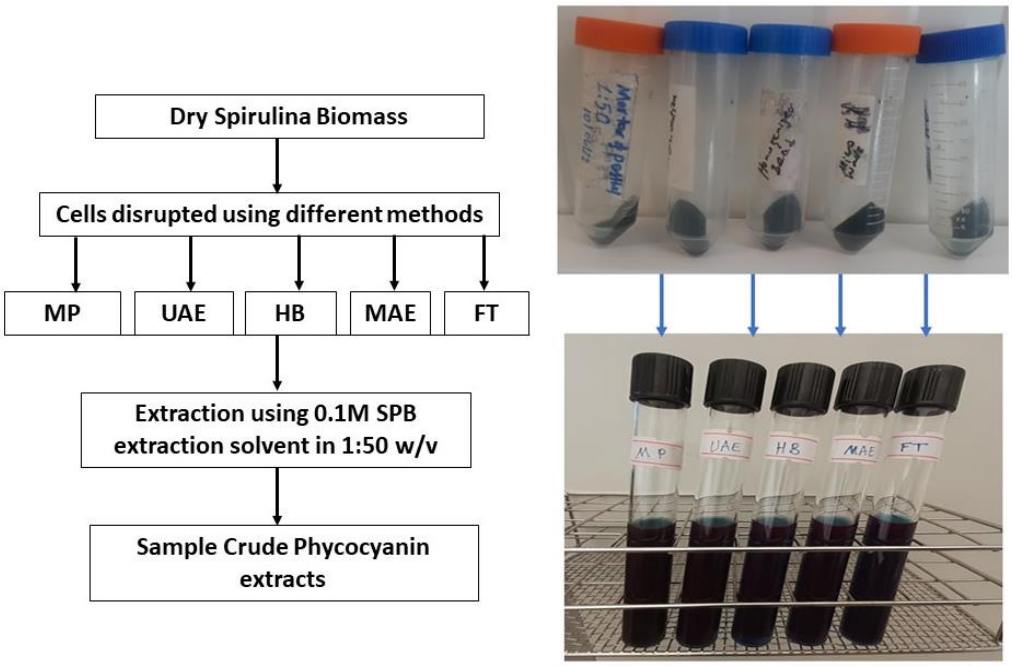

Figure 14. Crude phycocyanin extraction process. Flowchart diagram (left) shows the steps involved in crude phycocyanin extraction using different cell disruption methods. Results (right) obtained using the steps shown in the flowchart. MP is mortar and pestle, UAE is ultrasound assisted extraction, HB is homogenization with beads, MAE is microwave assisted extraction and FT is freeze and thaw method.

Table 2. Phycocyanin concentration (PC), extraction yields (Yield) and extraction efficiencies (EE) of crude extracts.

Extraction Method	PC ($mg.ml^{-1}$) ± SE	Yield ($mg.g^{-1}$) ± SE	EE (%) ± SE
HB	$0.622 \pm 0.006^{b,c}$	$31.1 \pm 0.31^{b,c}$	75.4 ± 2.03^{b}
FT	0.663 ± 0.008^{b}	33.15 ± 0.41^{b}	81.7 ± 1.11^{a}
UAE	0.727 ± 0.01^{a}	36.35 ± 0.49^{a}	84.5 ± 0.32^{a}
MP	0.424 ± 0.006^{d}	21.2 ± 0.31^{d}	66.4 ± 1.12^{c}
MAE	0.587 ± 0.015^{c}	29.35 ± 0.74^{c}	74.3 ± 0.46^{b}

[a] Differ significantly
[b,c,d] $p < 0.05$

4.5. Extract Purity

The crude extracts obtained in this study using the different extraction techniques were partially purified using ammonium sulfate purification and dialysis method as shown in Figure 15. The results obtained for determination of purity for crude and partially purified extracts are shown in Table 3. The purity of partially purified samples was found to be increased significantly in comparison with the crude extracts. The highest purity level of

the partially purified samples was found to be the same for FT and UAE samples (0.98). The purity of the remaining samples was 0.97 for HB, MP and MAE extracts. The partially purified samples were lyophilized as shown in Figure 16 for the next steps of this study.

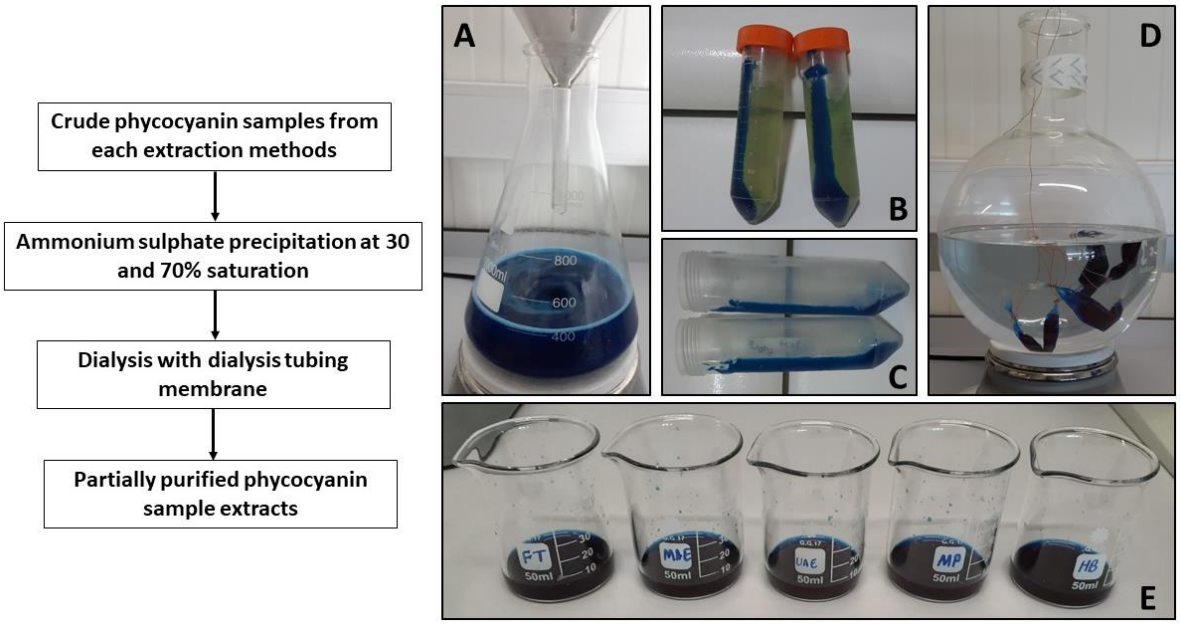

Figure 15. Partial purification process. Flowchart diagram (left) shows partial purification steps followed and image (right) shows addition of $(NH_4)_2SO_4$ salt to precipitate phycocyanin at a specific saturation on magnetic stirrer (A), precipitated phycocyanin from supernatant impurity after centrifugation (B), phycocyanin pellet (C), dialysis to remove the salt in dialysis membrane bags submerged in extraction solvent (D) and partially purified phycocyanin extracts of each extraction methods (E).

Table 3. Extract purity (EP) of crude and partially purified extracts.

Extraction Method	**EP $_{(A620/A280)}$ ± SE**	
	Crude	**Partially Purified**
HB	0.49 ± 0.003	0.97 ± 0.003
FT	0.53 ± 0.003	0.98 ± 0.003
UAE	0.54 ± 0.003	0.98 ± 0.003
MP	0.48 ± 0.003	0.97 ± 0.003
MAE	0.49 ± 0.003	0.97 ± 0.003

$p < 0.05$
EP is extract purity

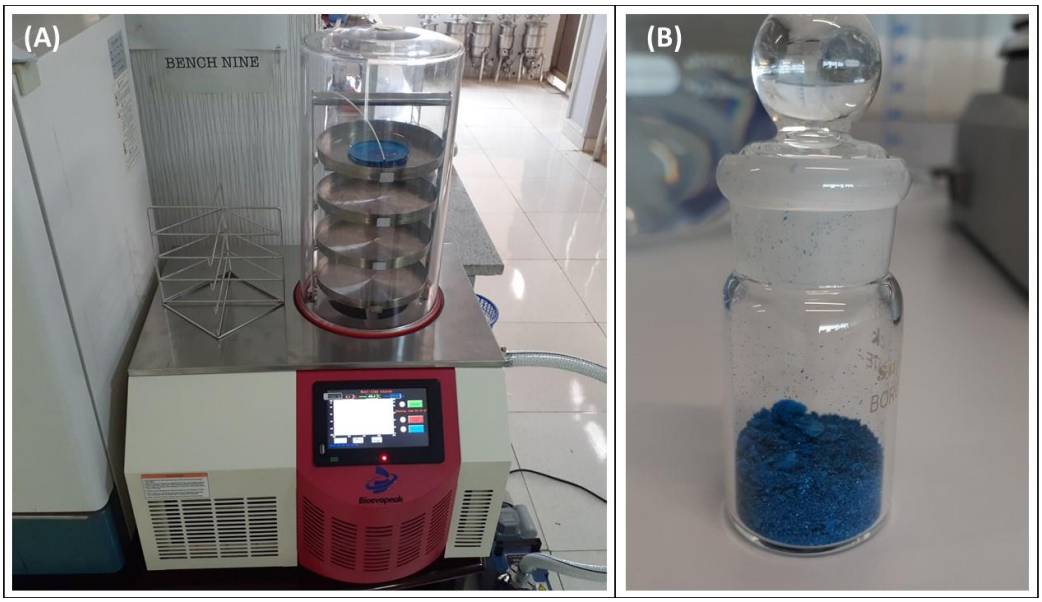

Figure 16. Lyophilization of samples using freeze dryer machine. Freeze dryer (A) and lyophilized sample of phycocyanin (B).

4.6. SDS-PAGE

The crude extract of Spirulina containing phycocyanin was subjected to electrophoretic separation by SDS-PAGE. The result shows two visible bands for every sample at the approximate corresponding molecular weight of 19 and 22 kDa as in Figure 17 (A). For the sake of comparison, gel image is shown from a previous report in Figure 17 (B) (Tantirapan and Suwanwong, (2014)).

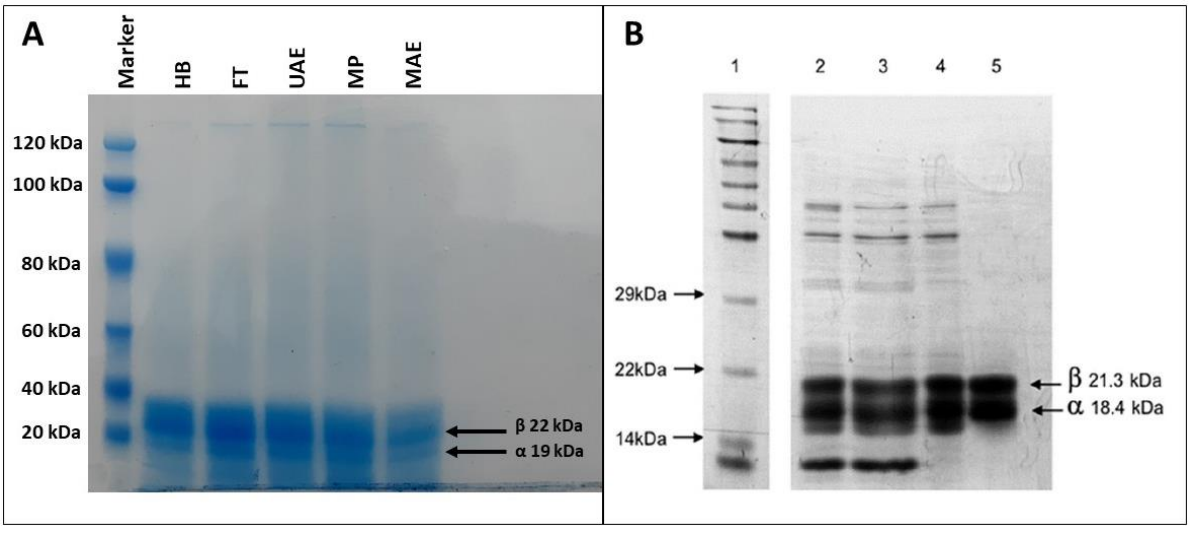

Figure 17. Polyacrylamide gel showing SDS-PAGE results for each extract in respective lanes loaded and bands observed in this study (A). SDS-PAGE result report of phycocyanin adapted from Tantirapan and Suwanwong, (2014) for comparison (B).

4.7. Antimicrobial Activity Test

Antimicrobial activity was examined using a disk diffusion assay at different concentrations of phycocyanin. All phycocyanin extract samples examined showed antibacterial activity against both Gram-positive bacteria (*E. faecalis and S. aureus*) and Gram-negative bacteria (*E. coli and P. aeruginosa*) at varied concentrations (500, 250, 125, and 62.5 µg/ml). According to the specific concentration used, each sample produced distinct zones of inhibition in a dose-dependent manner as can be seen visually in Figure 18 with varying diameter size values around the disks. These results were quantified by measurements of the disc size and presented in Table 4. The zone of inhibition data showed that most of the samples displayed a varied range of antibacterial activities (6 ± 0 to 25.3 ± 0.6 mm) against all of the tested bacterial strains as summarized in Table 4. The activity of each sample differs from one another only slightly across the different phycocyanin extract samples used for a particular bacterium. However, some degree of activity differences was notable between Gram-positive and Gram-negative bacteria, where Gram-negative bacteria were inhibited more than Gram-positive for all the phycocyanin extracts tested. It is noteworthy to observe that all the bacteria species showed dose-dependent inhibition, however, at lower concentrations Gram-positive bacteria (*E. faecalis and S. aureus*) showed no activity (denoted by NA in Table 4). The standard reference drug, Ceftriaxone, showed better activity in comparison with the activities of the samples which were found to be moderate albeit comparable to the standard.

Table 4. Antibacterial activities of each partially purified phycocyanin extract samples.

Samples	Concentration (µg/ml)	Mean Inhibition diameter (mm) ±SD			
		E. coli	***S. aureus***	***E. faecalis***	***P. aeruginosa***
HB	500	24.7 ± 0.6	17 ± 1	17.3 ± 0.6	25.3 ± 0.6
	250	20.3 ± 0.6	10.6 ± 1.2	10 ± 1.7	20.7 ± 0.6
	125	18 ± 1.7	NA	7 ± 0	16.3 ± 1.5
	62.5	12.3 ± 0.6	NA	NA	13.7 ± 1.2
	+ve Control 30 µg/ml	32.3 ± 0.6	32.6 ± 0.6	29.3 ± 0.6	33.7 ± 0.6
	-ve Control	NA	NA	NA	NA
FT	500	23.7 ± 1.2	17 ± 2	19 ± 1.7	24.3 ± 0.6
	250	19.7 ± 0.6	10.7 ± 2.3	11.3 ± 1.2	20.3 ± 0.6
	125	16.3 ± 0.6	8 ± 0	8.7 ± 1.2	16 ± 1
	62.5	13 ± 1	NA	NA	13 ± 1
	+ve Control 30 µg/ml	32. 3 ± 0.6	32 ± 1	30 ± 0	32.7 ± 0.6
	-ve Control	NA	NA	NA	NA
UAE	500	23.7 ± 0.6	16.7 ± 1.2	17 ± 0	25.3 ± 1.2
	250	19.7 ± 0.6	10 ± 0	9.7 ± 2.1	21.7 ± 1.5
	125	16.3 ± 0.6	NA	9.5 ± 2.1	18.3 ± 2.1
	62.5	11.3 ± 1.2	NA	NA	13. 7 ± 0.6
	+ve Control 30 µg/ml	33 ± 1	30. 7 ± 0.6	29.3 ± 0.6	32.7 ± 0.6
	-ve Control	NA	NA	NA	NA
MP	500	24. 7 ± 0.6	16. 7 ± 2.1	18 ± 1	23. 7 ± 1.5
	250	19. 7 ± 0.6	10. 7 ± 0.6	8. 3 ± 1.5	17.3 ± 3.8
	125	16.3 ± 0.6	6 ± 0	NA	16 ± 0
	62.5	12.3 ± 1.6	NA	NA	13 ± 0
	+ve control 30 µg/ml	32. 7 ± 0.6	31. 7 ± 0.6	28. 7 ± 0.6	30.3 ± 0.6
	-ve Control	NA	NA	NA	NA
MAE	500	24 ± 1	17.7 ± 2.1	19 ± 2.6	24.3 ± 0.6
	250	15.3 ± 3.5	11.3 ± 1.2	9 ± 1	20.3 ± 0.6
	125	11 ± 1.4	NA	NA	16 ± 0
	62.5	13 ± 0	NA	NA	13.3 ± 1.2
	+ve Control 30 µg/ml	33.3 ± 1.2	32.3 ± 1.2	30 ± 1	33 ± 1
	-ve Control	NA	NA	NA	NA

+ve = positive, -ve = negative, ±SD = Mean Standard Deviation and NA = No Activity

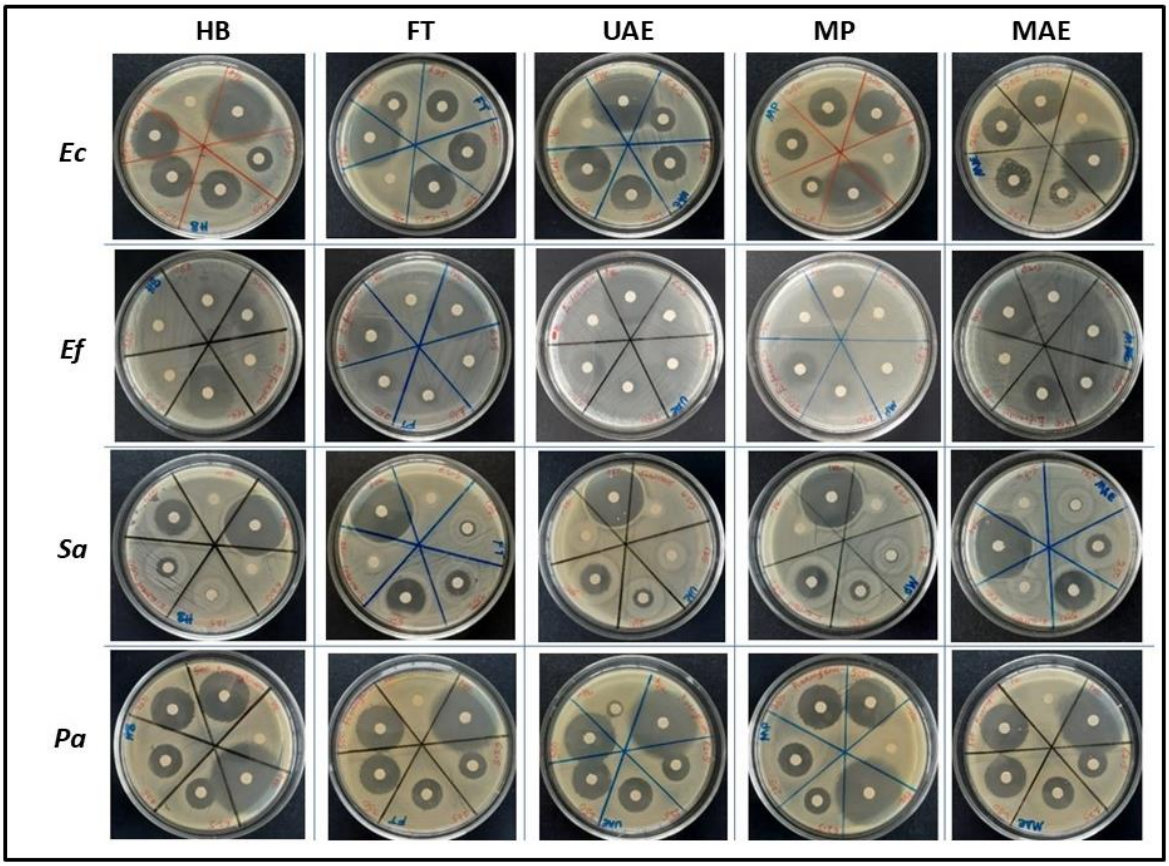

Figure 18. Antibacterial activity test assays representing trials of each sample. Each row of images shows test Petri dishes of different sample extracts against same bacteria type meanwhile each column of images shows test Petri dishes of same bacteria type against each sample extracts. *Ec, E. coli; Ef, E. faecalis; Sa, S. aureus and Pa, P. aeruginosa.*

4.8. Antioxidant Activity Test

The DPPH assay test for each extract have been conducted at different concentrations of each extract sample solution to determine the antioxidation potential of phycocyanin. The results were calculated as %RSA of the samples and reference standard ascorbic acid (Table 5). The absorbance of the control DPPH solution in the experiment which was used to calculate all the results was found to be 1.455. The antioxidant activity of samples extracted by different methods were found to be dose-dependent and increased with the increasing concentrations.

Table 5. Antioxidant activities (%RSA) of partially purified phycocyanin extract samples.

Concentration (µg/ml)	**HB**	**FT**	**UAE**	**MP**	**MAE**	**Standard**
25	48.9	50.7	51.5	49.3	48.9	90.3
50	56.6	58.1	58.3	56.8	56.6	91.2
100	64.8	64.2	66.4	63.6	65.6	94.4
200	73.1	74.2	74.3	72.9	73.7	96.7

CHAPTER FIVE

5. Discussion

This study was conceived, designed and executed to investigate the potential applications of the useful microalga, Spirulina, from Lake Chitu. Previous studies that show revealing evidences on Spirulina total carotenoid estimate and phycocyanin content are essential to draw the overall applicability of Spirulina from Lake Chitu in local and global industries.

The established laboratory cultivation system in this study was able to effectively grow Spirulina with the carefully carried out successful screening and isolation procedures of the cells to produce a monoalgal culture. The system was able to generate a harvest of 0.364 g of dry Spirulina per liter of media with a doubling time of growth of 3.54 days which was found in agreement with previous reports of 3.85 days (Costa *et al.,* 2002).

The estimation of total carotenoid content of Spirulina sample at laboratory condition has revealed that a total of 3.912 $mg.g^{-1}$ content of total carotenoid was present in the DW of Spirulina. This result was found to correspond with the previously reported total carotenoid content (1.97 to 4.31 $mg.g^{-1}$) of Spirulina in a study conducted on *in vivo* harvested Lake Chitu's Spirulina by Assaye *et al.* (2017). These lower and higher ranges of total carotenoid values in the study of Assaye *et al*. (2017) may be attributed to different seasonal variations unlike the present study which is solely based on standard laboratory cultivation.

Different extraction solvents investigated in this study have shown the potential to extract phycocyanin in a concentration range of 0.3946 to 0.415 $mg.ml^{-1}$. There was no significant difference in these values observed from ANOVA test ($p < 0.05$). The optimized extraction buffer with the highest PC value in this study was the one that have extracted the highest amount of phycocyanin per volume compared to others. With experimental value of 0.415 $mg.ml^{-1}$ PC, 0.1M sodium phosphate buffer (SPB) was found to be the efficient extraction solvent than the other solvents tested (PBS, 1.5% $CaCl_2$ and DH_2O). Same in other previous related studies, sodium phosphate buffer has been reported to show better efficiency than other solvents (Silveira *et al.,* 2007).

One of the most important requirements to obtain phycocyanin from cyanobacterium effectively is optimization of the extraction and purification steps. For extraction of cellular components, rupture of cells by the disruption of membrane is generally thought to

be an important and efficient step. Several studies have reported that the release of phycocyanin from the cells of spirulina is directly related to the cell rupture by different extraction methods. All the different methods of cell disruption used in this study to extract phycocyanin from Spirulina dry biomass have shown the presence of this molecule in the supernatant with bluish-purple color visible with the naked eye. With different values of extraction efficiency recorded ranging from 66.4 to 84.5%, the extraction yield data also showed that the five different methods of extraction investigated in this study have yielded different range of crude phycocyanin yield from 21.2 to 36.35 $mg.g^{-1}$. The highest yield of extraction with significant difference from others ($p < 0.05$) was obtained using ultrasound assisted extraction method (36.35 $mg.g^{-1}$) with an extraction efficiency of 84.5%. This agrees with some previous studies that cell disruption by ultrasonication yields higher amount of phycocyanin from the cells (Moraes *et al.,* 2011). This higher yield could be due to cavitation of cells because of the ultrasonic waves rupturing the cell walls to open effectively than other methods and favoring better release of phycocyanin from the cells compared to other mechanisms of cell disruption methods applied in this study. The other extraction techniques investigated here have also shown relatively effective extraction of phycocyanin in the following order FT (33.15 $mg.g^{-1}$) > HB (31.1 $mg.g^{-1}$) > MAE (29.35 $mg.g^{-1}$) > MP (21.2 $mg.g^{-1}$). The yields according to previous reports has been shown in the range of 2 – 48.88 $mg.g^{-1}$ (Table 6). Our results are found to be within the agreeable limits of this range. The differences in the extraction efficiency mainly arises across different studies due to various factors other than the cell disruption method employed which could be species type, cultivation conditions, extraction solvent type, substrate type (wet/dry biomass), extraction time, extraction temperature, pH, and etc.

Table 6. Phycocyanin yields reported in previous studies on Spirulina.

Treatment methods	Phycocyanin Yield ($mg.g^{-1}$)	References	Remarks
Homogenization	19.66	Sarada *et al.*, 1999	Using mortar and pestle
	21.2	This study	Using mortar and pestle
	31.1	This study	With beads
Freezing and Thawing	19.47	Sarada *et al.*, 1999	
	48.88	Khandual et al., 2021	
	35.31	Dianursanti and Hafidzah, 2021	
	33.15	This study	
Sonication	0.57	Moraes *et al.*, 2011	
	43.75	Moraes *et al.*, 2011	In the presence of glass pearls
	54.56	Khandual *et al.*, 2021	
	36.35	This study	
Microwave	29.35	This study	
Chemical	< 2	Moraes *et al.*, 2011	Using 2 M HCl, 4 M HCl and 1 M acetic acid
Enzymes	< 2	Moraes *et al.*, 2011	Using lysozyme

In the current study, partial purification of the crude phycocyanin extracts was achieved by a two-step fractional precipitation of ammonium sulfate protein purification technique at 30 and 70% saturation to salt out the protein pigment followed by the dialysis method to remove the salt which is commonly used in several studies such as Khazi *et al.* (2018), Kottuparambil and Thankamony (2018) and Khandual *et al.* (2021). The partial purification yielded samples that were significantly improved in their purity grade with an average of 92.9 % ($p < 0.05$). With this method, it was achieved to purify a food grade phycocyanin extract with purity > 0.7 with 0.97 and 0.98 purity values. The purification method used here was found to be advantageous because it can be used on a large scale,

requires fewer equipment, and is inexpensive. Moreover, it is convenient to recover partially purified phycocyanin for biological activity tests in this study.

Following extraction, the samples were confirmed by SDS-PAGE for protein validation. The two bands in each sample lane seen on the gel are with an estimation of molecular weight of 19 and 22 kDa indicate the presence of α and β subunits of phycocyanin pigment in the samples, respectively which is in agreement with the reported studies of Minkova *et al.* (2003); Saktihivel *et al*. (2009); Kamble *et al.* (2013); Seo *et al.* (2013); Song *et al.* (2013); Wang *et al.* (2014) and Hazra and Kesh (2017), respectively.

The antimicrobial activity tests of extracts have shown, in general, effective antibacterial activity for all samples with inhibition zones ranging from 6±0 to 25.3±0.6 mm and with dose dependency. This was found to be in agreement with previous studies of Ozdemir *et al.* (2004) and Muthulakshmi *et al*. (2012) conducted using phycocyanin extracts obtained from *Arthrospira platensis*. The moderate antibacterial activities of the extracted samples observed were only slightly different to each other between extracts. In comparison to activity of the reference standard commercial drug (Ceftriaxone), each sample has shown less activity than the positive control in all tests. The positive control has shown higher inhibition zones against each pathogen in every tests. Comparing the activity of different samples obtained using different extraction methods against each different species of bacteria, it was seen that there is no significant difference across the observed inhibition zones at each level of concentration. This may indicate that the partial purification process of phycocyanin conducted has produced same quality and concentration of partially purified phycocyanin compound. The highest inhibition zone was recorded at 500 µg/ml for HB and UAE extracts which was 25.3 ± 0.6 mm against *P. aeruginosa.* In general, all the samples have shown better antibacterial activity against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) as compared to their activity against Gram-positive bacteria (*E. faecalis* and *S. aureus*).

The antioxidant activity test of the sample extracts demonstrated the effective potential of each phycocyanin extract to scavenge free radicals. Just in agreement as reported in previous studies of Chu *et al.* (2010) and Chen *et al.* (2022) conducted using phycocyanin extracts obtained from *Arthrospira platensis*, the samples in the present study have also shown effective antioxidant activity. The DPPH assay method employed to test activity of the samples implied a range of activity between 48.9 to 74.3 %RSA across the different

extraction techniques and concentration. It was seen from the results that extracts obtained through different methods have displayed an increasing radical scavenging activity with an increase in concentration, as previously reported by Renugadevi *et al.* (2018) and Qiao *et al.* (2022). However, the lower range of the activity also require investigations for safe conclusion. As compared to the activity of standard ascorbic acid used for comparison, which has shown 90.3 – 96.7 %RSA at different concentrations, activity of samples at different concentrations were found to be comparable with approximately 30% less potential than ascorbic acid.

CHAPTER SIX

6. Conclusions and Recommendations

In general, the present study has revealed evidence that Lake Chitu's Spirulina is potentially a good source of highly useful phycocyanin pigment compounds and different phytochemicals which have various applications in different sectors of industry and health. So far in Ethiopia, Spirulina is only known well for its high content of protein and related health benefits, yet as can be stipulated from findings in this study it has been shown that there is more to benefit from this valuable cyanobacterium. Both the total carotenoid content and phycocyanin accumulation of Lake Chitu's Spirulina investigated have shown to be promising to assure the applicability of this microalga in the food, pharmaceutical, and cosmetic industries.

According to the findings of this study, the effective biological activity of Lake Chitu's Spirulina is an indicator that this microalga could be used as an effective domestic source of the therapeutic phycocyanin compound for pharmaceutical and cosmetics industries supported by continued studies.

It is important to disseminate this evidence and conduct more extensive studies to give proper attention to the various applications and commercialization opportunities that arise with this microalgae species. Therefore, based on the current findings, we present the following recommendations:

- In-depth studies of the carotenoids present and other metabolites of Lake Chitu's Spirulina are vital to assess their specific potential application in various sectors.
- More subsequent studies on the utilization of methods used in this study and other methods in combination could also yield a higher amount of phycocyanin recovery as also there is a global attention to advance on the field and improve industrial processes.
- As it is well known, the efficiency of the phycocyanin extraction process is affected by numerous factors, it is essential to make optimization studies to address these parameters specifically to the characteristics of Lake Chitu's Spirulina for better results.
- In addition, studies on further purification steps to the phycocyanin extracts would also result in higher biological activity.

- The potential economic benefits of Lake Chitu’s Spirulina possess must be studied in detail to demonstrate that it could add to the country's economy if it is produced industrially as a whole and/or for the production of phycocyanin.

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Appendices

Appendix 1: Pictures taken during investigation

Appendix 2: Major laboratory and field equipment materials used in this study

Figure: Image shows LYO60B-1S Benchtop Freeze Dryer, Bioevopeak, China (A); GX-65B Drying Oven, Huanghua Faithful Instrument Co., LTD., China (B); 1100L VWR Phenomenal pH meter (C); LA 124, VWR Electronic Beam Balance, Italy (D); TLG-16M, Drawell refrigerated Centrifuge, China (E); TC4815D MICROSPIN Centrifuge, Eltek, Maalab, India (F); Cary 60 UV-Vis, Agilent Technologies, California, U.S (G); RK106S Sonorex Ultrasonic Bath, Bandelin, Germany (H); KZ-III High Speed Tissue Homogenizer, Servicebio, China (I); MS-H280-PRO Magnetic Hotplate stirrer, DLAB, UK (J); Presoclave III Autoclave, J.P.Selecta, Spain (K); Visiscope TL524PH, VWR Microscope, Italy (L); ULF86 Ultra Low Temperature Freezer, Bioevopeak, China (M); pH Pen Tester, Bioevopeak, China (N); FS-50B Shaking Incubator, Huanghua Faithful Instrument Co., LTD., China (O); 72H, Garmin GPS, U.S (P); R-562CT(ST) Microwave, Sharp, Japan (Q).

Appendix 3: Spirulina media (SP media) preparation recipe used in this study

This medium is made up in 2 parts to reduce precipitation when autoclaving. For each part add the components to around 900 ml deionized water and then top up to 1 litre with DH_2O.

Parts	Components	Amounts
Part 1: to make up 1 Litre Stock in DH_2O	$NaHCO_3$	27.22 g
	Na_2CO_3	8.06 g
	K_2HPO_4	1.00 g
Part 2: to make up 1 Litre Stock in DH_2O	$NaNO_3$	5.00 g
	K_2SO_4	2.00 g
	NaCl	2.00 g
	$MgSO_4.7H_2O$	0.40 g
	$CaCl_2.2H_2O$	0.02 g
	$FeSO_4.7H_2O$	0.02 g
	$EDTANa_2$	0.16 g
	Micronutrient Stock solution	10 ml
	Vitamin Stock solution	5 ml

Autoclave part 1 and 2 separately at 121 °C and 15 psi for 15 minutes, allowed to cool then mixed in 1:1 ratio aseptically. Micronutrient and Vitamins stock solutions are prepared as in the following table and stored frozen at -20 °C.

Stock Solutions	Components	Amounts
Micronutrients 1 Litre	$ZnSO_4.7H_2O$	0.001 g
	$MnSO_4.7H_2O$	0.002 g
	H_3BO_3	0.010 g
	$Na_2MoO_4.2H_2O$	0.001 g
	$Co(NO_3)_2.6H_2O$	0.001 g
	$CuSO_4.5H_2O$	0.00005 g
	$FeSO_4.7H_2O$	0.70 g
	$EDTANa_2$	0.80 g
Vitamins 1 Litre	Biotin	0.0002 g
	Calcium pantothenate	0.02 g
	Cyanocobalamin	0.004 g
	Folic acid	0.0004 g
	Inositol	1.00 g
	Nicotinic acid	0.02 g
	Thiamine HCl	0.10 g
	Thymine	0.60 g

Appendix 4: Ammonium sulfate precipitation and dialysis procedure used in this study

Materials needed:

Ammonium sulfate, dialysis cellulose tubing membrane, magnetic stirrer, glass beakers, refrigerated centrifuge, and sodium phosphate buffer (0.1M, pH 7.1) (dialysate buffer).

Method procedure:

1. Place a beaker containing crude phycocyanin extract inside of a larger beaker containing crushed ice to maintain process temperature at 4 °C and then stir with a magnetic stirrer continuously and slowly.
2. Add a pre-weighed amount of ammonium sulfate while stirring slowly to attain a saturation of 30%, then agitate the mixture for almost two hours. Note that the white crystalline solid ammonium sulfate needs to be grinded in to a fine powder before adding if it contains any lumps. Amount of ammonium sulfate required to achieve the desired saturation percentage can be determined using a website calculator https://www.encorbio.com/protocols/AM-SO4.htm by entering starting volume of sample solution in ml, desired percentage saturation and starting percentage saturation of the sample.
3. Transfer the precipitated ammonium sulfate solution to conical centrifuge tubes (falcon tubes) and centrifuge at 10,000 rpm for 15 min at 4 °C then discard the pellet after carefully collecting the supernatant.
4. Slowly add a pre-weighed quantity of ammonium sulfate to the previously collected supernatant to reach a saturation of 70%, and then stir the mixture for six hours.
5. Transfer the ammonium sulfate precipitated solution to conical centrifuge tubes and centrifuge at 10,000 rpm for 15 min 4 °C then remove the supernatant carefully, and add a few milliliters of the extraction solvent sodium phosphate buffer to the pellet to resuspend the phycocyanin.
6. Using a pipette, transfer the phycocyanin solution precipitated by ammonium sulfate to the cellulose membrane of the dialysis tube. Complete dialysis will be accomplished by dialyzing overnight against 2 L of the dialysate buffer (0.1M of sodium phosphate buffer extraction buffer), which should be performed with high external volumes, enough stirring, and regular exchanges of the buffer.
7. Decant phycocyanin solution into centrifuge tubes and centrifuge at 10,000 rpm for 10 min to remove any undissolved material.

Appendix 5: SPSS output – One-way ANOVA analysis output of solvent type determination

Oneway

[DataSet0] C:\Users\Admin\Desktop\Nov 22\SPSS\One-Way Data.sav

Descriptives

Solvent - PC (mg/ml)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.5% CaCl2	3	.407200	.0073123	.0042218	.389035	.425365	.4005	.4150
0.1M SPB	3	.414733	.0413020	.0238457	.312134	.517333	.3732	.4558
1M PBS	3	.390133	.0221861	.0128092	.335020	.445247	.3711	.4145
DH2O	3	.394600	.0456521	.0263572	.281194	.508006	.3531	.4435
Total	12	.401667	.0298850	.0086271	.382679	.420655	.3531	.4558

ANOVA

Solvent - PC (mg/ml)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	3	.000	.355	.787
Within Groups	.009	8	.001		
Total	.010	11			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Solvent - PC (mg/ml)

Tukey HSD

(I) Solvent Type	(J) Solvent Type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.5% CaCl2	0.1M SPB	-.0075333	.0268814	.992	-.093617	.078550
	1M PBS	.0170667	.0268814	.918	-.069017	.103150
	DH2O	.0126000	.0268814	.964	-.073484	.098684
0.1M SPB	1.5% CaCl2	.0075333	.0268814	.992	-.078550	.093617
	1M PBS	.0246000	.0268814	.798	-.061484	.110684
	DH2O	.0201333	.0268814	.875	-.065950	.106217
1M PBS	1.5% CaCl2	-.0170667	.0268814	.918	-.103150	.069017
	0.1M SPB	-.0246000	.0268814	.798	-.110684	.061484
	DH2O	-.0044667	.0268814	.998	-.090550	.081617
DH2O	1.5% CaCl2	-.0126000	.0268814	.964	-.098684	.073484
	0.1M SPB	-.0201333	.0268814	.875	-.106217	.065950
	1M PBS	.0044667	.0268814	.998	-.081617	.090550

Homogeneous Subsets

Solvent - PC (mg/ml)

Tukey HSD[a]

Solvent Type	N	Subset for alpha = 0.05
		1
1M PBS	3	.390133
DH2O	3	.394600
1.5% CaCl2	3	.407200
0.1M SPB	3	.414733
Sig.		.798

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 6: SPSS output – One-way ANOVA analysis output of phycocyanin concentration obtained using different methods

Oneway

Descriptives

Phycocyanin Concentration (mg/ml)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
HB	3	.62200	.010817	.006245	.59513	.64887	.613	.634
FT	3	.66300	.014177	.008185	.62778	.69822	.652	.679
UAE	3	.72700	.017059	.009849	.68462	.76938	.708	.741
MP	3	.42400	.010817	.006245	.39713	.45087	.412	.433
MAE	3	.58700	.025710	.014844	.52313	.65087	.567	.616
Total	15	.60460	.106112	.027398	.54584	.66336	.412	.741

ANOVA

Phycocyanin Concentration (mg/ml)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.155	4	.039	139.567	.000
Within Groups	.003	10	.000		
Total	.158	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Phycocyanin Concentration (mg/ml)

Tukey HSD

(I) Extraction Methods	(J) Extraction Methods	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HB	FT	-.041000	.013599	.077	-.08576	.00376
	UAE	-.105000*	.013599	.000	-.14976	-.06024
	MP	.198000*	.013599	.000	.15324	.24276
	MAE	.035000	.013599	.149	-.00976	.07976
FT	HB	.041000	.013599	.077	-.00376	.08576
	UAE	-.064000*	.013599	.006	-.10876	-.01924
	MP	.239000*	.013599	.000	.19424	.28376
	MAE	.076000*	.013599	.002	.03124	.12076
UAE	HB	.105000*	.013599	.000	.06024	.14976
	FT	.064000*	.013599	.006	.01924	.10876
	MP	.303000*	.013599	.000	.25824	.34776
	MAE	.140000*	.013599	.000	.09524	.18476
MP	HB	-.198000*	.013599	.000	-.24276	-.15324
	FT	-.239000*	.013599	.000	-.28376	-.19424
	UAE	-.303000*	.013599	.000	-.34776	-.25824
	MAE	-.163000*	.013599	.000	-.20776	-.11824
MAE	HB	-.035000	.013599	.149	-.07976	.00976
	FT	-.076000*	.013599	.002	-.12076	-.03124
	UAE	-.140000*	.013599	.000	-.18476	-.09524
	MP	.163000*	.013599	.000	.11824	.20776

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Phycocyanin Concentration (mg/ml)

Tukey HSD[a]

Extraction Methods	N	Subset for alpha = 0.05			
		1	2	3	4
MP	3	.42400			
MAE	3		.58700		
HB	3		.62200	.62200	
FT	3			.66300	
UAE	3				.72700
Sig.		1.000	.149	.077	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 7: SPSS output – One-way ANOVA analysis output of phycocyanin yield obtained using different methods

Oneway

Descriptives

Phycocyanin Yield (mg/g)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
HB	3	31.1000	.54083	.31225	29.7565	32.4435	30.65	31.70
FT	3	33.1500	.70887	.40927	31.3891	34.9109	32.60	33.95
UAE	3	36.3500	.85294	.49244	34.2312	38.4688	35.40	37.05
MP	3	21.2000	.54083	.31225	19.8565	22.5435	20.60	21.65
MAE	3	29.3500	1.28550	.74218	26.1567	32.5433	28.35	30.80
Total	15	30.2300	5.30562	1.36990	27.2918	33.1682	20.60	37.05

ANOVA

Phycocyanin Yield (mg/g)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	387.159	4	96.790	139.567	.000
Within Groups	6.935	10	.694		
Total	394.094	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Phycocyanin Yield (mg/g)

Tukey HSD

(I) Extraction Methods	(J) Extraction Methods	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HB	FT	-2.05000	.67995	.077	-4.2878	.1878
	UAE	-5.25000*	.67995	.000	-7.4878	-3.0122
	MP	9.90000*	.67995	.000	7.6622	12.1378
	MAE	1.75000	.67995	.149	-.4878	3.9878
FT	HB	2.05000	.67995	.077	-.1878	4.2878
	UAE	-3.20000*	.67995	.006	-5.4378	-.9622
	MP	11.95000*	.67995	.000	9.7122	14.1878
	MAE	3.80000*	.67995	.002	1.5622	6.0378
UAE	HB	5.25000*	.67995	.000	3.0122	7.4878
	FT	3.20000*	.67995	.006	.9622	5.4378
	MP	15.15000*	.67995	.000	12.9122	17.3878
	MAE	7.00000*	.67995	.000	4.7622	9.2378
MP	HB	-9.90000*	.67995	.000	-12.1378	-7.6622
	FT	-11.95000*	.67995	.000	-14.1878	-9.7122
	UAE	-15.15000*	.67995	.000	-17.3878	-12.9122
	MAE	-8.15000*	.67995	.000	-10.3878	-5.9122
MAE	HB	-1.75000	.67995	.149	-3.9878	.4878
	FT	-3.80000*	.67995	.002	-6.0378	-1.5622
	UAE	-7.00000*	.67995	.000	-9.2378	-4.7622
	MP	8.15000*	.67995	.000	5.9122	10.3878

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Phycocyanin Yield (mg/g)

Tukey HSD[a]

Extraction Methods	N	Subset for alpha = 0.05			
		1	2	3	4
MP	3	21.2000			
MAE	3		29.3500		
HB	3		31.1000	31.1000	
FT	3			33.1500	
UAE	3				36.3500
Sig.		1.000	.149	.077	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 8: SPSS output – One-way ANOVA analysis output of extraction efficiency for different methods

Oneway

Descriptives

Extraction Efficiency (%)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
HB	3	75.4200	3.51168	2.02747	66.6965	84.1435	72.72	79.39
FT	3	81.6600	1.91392	1.10500	76.9056	86.4144	79.45	82.77
UAE	3	84.5433	.55076	.31798	83.1752	85.9115	84.01	85.11
MP	3	66.3500	1.94800	1.12468	61.5109	71.1891	64.98	68.58
MAE	3	74.2967	.79727	.46030	72.3161	76.2772	73.74	75.21
Total	15	76.4540	6.77453	1.74917	72.7024	80.2056	64.98	85.11

ANOVA

Extraction Efficiency (%)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	601.061	4	150.265	36.246	.000
Within Groups	41.457	10	4.146		
Total	642.519	14			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Extraction Efficiency (%)

Tukey HSD

(I) Extraction Methods	(J) Extraction Methods	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HB	FT	-6.24000*	1.66247	.024	-11.7113	-.7687
	UAE	-9.12333*	1.66247	.002	-14.5947	-3.6520
	MP	9.07000*	1.66247	.002	3.5987	14.5413
	MAE	1.12333	1.66247	.957	-4.3480	6.5947
FT	HB	6.24000*	1.66247	.024	.7687	11.7113
	UAE	-2.88333	1.66247	.457	-8.3547	2.5880
	MP	15.31000*	1.66247	.000	9.8387	20.7813
	MAE	7.36333*	1.66247	.009	1.8920	12.8347
UAE	HB	9.12333*	1.66247	.002	3.6520	14.5947
	FT	2.88333	1.66247	.457	-2.5880	8.3547
	MP	18.19333*	1.66247	.000	12.7220	23.6647
	MAE	10.24667*	1.66247	.001	4.7753	15.7180
MP	HB	-9.07000*	1.66247	.002	-14.5413	-3.5987
	FT	-15.31000*	1.66247	.000	-20.7813	-9.8387
	UAE	-18.19333*	1.66247	.000	-23.6647	-12.7220
	MAE	-7.94667*	1.66247	.005	-13.4180	-2.4753
MAE	HB	-1.12333	1.66247	.957	-6.5947	4.3480
	FT	-7.36333*	1.66247	.009	-12.8347	-1.8920
	UAE	-10.24667*	1.66247	.001	-15.7180	-4.7753
	MP	7.94667*	1.66247	.005	2.4753	13.4180

*. The mean difference is significant at the 0.05 level.

Homogeneous Subsets

Extraction Efficiency (%)

Tukey HSD[a]

Extraction Methods	N	Subset for alpha = 0.05		
		1	2	3
MP	3	66.3500		
MAE	3		74.2967	
HB	3		75.4200	
FT	3			81.6600
UAE	3			84.5433
Sig.		1.000	.957	.457

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 9: SPSS output – Two-way ANOVA analysis output of extract purity for different methods

Univariate Analysis of Variance

[DataSet1] C:\Users\Admin\Desktop\Nov 22\SPSS\Purity Two-way.sav

Between-Subjects Factors

		Value Label	N
Methods	1	HB	6
	2	FT	6
	3	UAE	6
	4	MP	6
	5	MAE	6
Purification Step	1	Crude	15
	2	Partially Purified	15

Descriptive Statistics

Dependent Variable: Purity

Methods	Purification Step	Mean	Std. Deviation	N
HB	Crude	.492833	.0116105	3
	Partially Purified	.970533	.0018148	3
	Total	.731683	.2617526	6
FT	Crude	.531233	.0033724	3
	Partially Purified	.983767	.0034675	3
	Total	.757500	.2478816	6
UAE	Crude	.543900	.0061612	3
	Partially Purified	.984133	.0053426	3
	Total	.764017	.2411809	6
MP	Crude	.480133	.0077164	3
	Partially Purified	.973800	.0027404	3
	Total	.726967	.2704420	6
MAE	Crude	.490633	.0017474	3
	Partially Purified	.968667	.0038553	3
	Total	.729650	.2618433	6
Total	Crude	.507747	.0265960	15
	Partially Purified	.976180	.0074494	15
	Total	.741963	.2389924	30

Levene's Test of Equality of Error Variances[a,b]

		Levene Statistic	df1	df2	Sig.
Purity	Based on Mean	1.929	9	20	.106
	Based on Median	.886	9	20	.554
	Based on Median and with adjusted df	.886	9	10.164	.567
	Based on trimmed mean	1.850	9	20	.121

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Dependent variable: Purity

b. Design: Intercept + Methods + STEP + Methods * STEP

Tests of Between-Subjects Effects

Dependent Variable: Purity

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	1.656[a]	9	.184	5878.429	.000	1.000
Intercept	16.515	1	16.515	527701.171	.000	1.000
Methods	.007	4	.002	57.990	.000	.921
STEP	1.646	1	1.646	52584.623	.000	1.000
Methods * STEP	.003	4	.001	22.320	.000	.817
Error	.001	20	3.130E-5			
Total	18.172	30				
Corrected Total	1.656	29				

a. R Squared = 1.000 (Adjusted R Squared = .999)

Estimated Marginal Means

1. Methods

Estimates

Dependent Variable: Purity

Methods	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
HB	.732	.002	.727	.736
FT	.757	.002	.753	.762
UAE	.764	.002	.759	.769
MP	.727	.002	.722	.732
MAE	.730	.002	.725	.734

Pairwise Comparisons

Dependent Variable: Purity

(I) Methods	(J) Methods	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
HB	FT	-.026*	.003	.000	-.033	-.019
	UAE	-.032*	.003	.000	-.039	-.026
	MP	.005	.003	.160	-.002	.011
	MAE	.002	.003	.536	-.005	.009
FT	HB	.026*	.003	.000	.019	.033
	UAE	-.007	.003	.057	-.013	.000
	MP	.031*	.003	.000	.024	.037
	MAE	.028*	.003	.000	.021	.035
UAE	HB	.032*	.003	.000	.026	.039
	FT	.007	.003	.057	.000	.013
	MP	.037*	.003	.000	.030	.044
	MAE	.034*	.003	.000	.028	.041
MP	HB	-.005	.003	.160	-.011	.002
	FT	-.031*	.003	.000	-.037	-.024
	UAE	-.037*	.003	.000	-.044	-.030
	MAE	-.003	.003	.416	-.009	.004
MAE	HB	-.002	.003	.536	-.009	.005
	FT	-.028*	.003	.000	-.035	-.021
	UAE	-.034*	.003	.000	-.041	-.028
	MP	.003	.003	.416	-.004	.009

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: Purity

	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Contrast	.007	4	.002	57.990	.000	.921
Error	.001	20	3.130E-5			

The F tests the effect of Methods. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

2. Purification Step

Estimates

Dependent Variable: Purity

Purification Step	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
Crude	.508	.001	.505	.511
Partially Purified	.976	.001	.973	.979

Pairwise Comparisons

Dependent Variable: Purity

(I) Purification Step	(J) Purification Step	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
Crude	Partially Purified	-.468*	.002	.000	-.473	-.464
Partially Purified	Crude	.468*	.002	.000	.464	.473

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: Purity

	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Contrast	1.646	1	1.646	52584.623	.000	1.000
Error	.001	20	3.130E-5			

The F tests the effect of Purification Step. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

3. Methods * Purification Step

Dependent Variable: Purity

Methods	Purification Step	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
HB	Crude	.493	.003	.486	.500
	Partially Purified	.971	.003	.964	.977
FT	Crude	.531	.003	.524	.538
	Partially Purified	.984	.003	.977	.991
UAE	Crude	.544	.003	.537	.551
	Partially Purified	.984	.003	.977	.991
MP	Crude	.480	.003	.473	.487
	Partially Purified	.974	.003	.967	.981
MAE	Crude	.491	.003	.484	.497
	Partially Purified	.969	.003	.962	.975