Plasma Assisted Extraction of Biological Substances from *Spirulina maxima* to apply for biomedical applications

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Janvi Mistry

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Principal Supervisor: Prof. Krasimir Vasilev

Co-Supervisors: Dr. Andrew Hayles

& Thi Giang Tuyet Pham

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Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University: and to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

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Date: 31st October 2024

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LIST OF ABBREVATIONS

Abbreviation	Full name
C-PC	C-phycocyanin
ATCC	American Type Culture Collection
PAE	Plasma assisted extraction
TCP	Tissue culture plate
PBS	Phosphate Buffer solution
ROS	Reactive oxygen species

Abstract

In recent years, emphasis has been made on the investigation of natural compounds for incorporation as active components or nutraceuticals with medicinal uses. Currently the demand for naturally occurring bioactive compounds is growing rapidly in pharmaceutical and biological industries. This has led to the development of extraction techniques that embrace green chemistry which provide higher efficacy and sustainability. *Spirulina spp*., and other microalgae, are good sources of bioactive nutrients such as antioxidants, anti-inflammatory, antibacterial, and immunomodulatory compounds. Traditional extraction techniques, solvents extraction in particular, can be limited by extraction yield, adverse effects on the environment and degradation of biomolecules during the process. The restrictions make it challenging to develop the scientific approach to the conservation of bioactivity of natural compounds along with advanced, eco-friendly technologies for subsequent extraction for pharmaceutical and other industrial uses. Thus, atmospheric plasma treatments can be considered as a promising solution for improving the extraction yield without employing toxic solvents.

This work aims to evaluate the use of atmospheric plasma in different gas environments: Nitrogen, Argon and Compressed Air and treatment periods on the bioactive compounds of *Spirulina spp*. for possible use in medicine. These results suggest that the different plasma treatments in terms of the used gas types did not show any influence to the C-phycocyanin, protein concentration, and protein purity profiles; hence, the type of gas does not have an impact on the efficiency of the extraction process. Extraction process was affected by plasma treatment duration. An optimal concentration and purity of C-PC were achieved within a 5-minute treatment, even though its protein content was still high, which points to the fact that plasma-assisted extraction is best done within a five-minute time period. The results also showed that both short and long treatment durations negatively affected yields of bioactive compounds, emphasizing the importance of accurately controlling plasma exposure time.

The plasma exposure did not influence the antioxidant capacity of *Spirulina*, as evaluated using antioxidant activity, which remained unaffected in terms of Trolox equivalents. The cytotoxicity of tested compounds on THP-1 macrophages and HaCaT keratinocytes was evaluated by MTT assays concerned with cell viability. Compared to the control, the relative cell viability of THP-1 macrophage cells was lower, revealing cytotoxicity of samples at higher concentration of plasma treated *Spirulina* extract. On the other hand the viability of HaCaT

cells was not significantly affected and it was observed that *Spirulina* extracts are not toxic to human keratinocyte cells. Thus, these findings suggest that the role of drug dosage and administration must be viewed within a biomedical framework. The tests on antimicrobial activity against Staphylococcus aureus revealed no substantial levels of bacterial inhibition after plasma treatment, which confirmed the need for additions to improve the antimicrobial functions.

In conclusion, the findings of this study suggest that atmospheric plasma might be considered an environmentally sustainable extraction method for acquiring bioactive compounds from *Spirulina spp*. The parameters of plasma treatment, especially the time, are critical to the efficacy of extraction. The current study aims at developing a new strategy of extraction of bioactive molecules for the use in biological and pharmaceutical industries whereby it demonstrates that the Atmospheric Plasma treatment is a more sustainable approach to addressing the above constraints. Future work should be directed towards optimizing plasma parameters in order to increase biocompatibility and antibacterial efficacy.

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CHAPTER 1

1. Introduction

The existence of bioactive components, namely, bioactive compounds, is the defining characteristic of all bio-sources including sea algae, and many others, with significant differences in their structure and role. Microalgae biomasses are a great source of several bioactive substances, including vitamins, phenolics, polysaccharides, lipids, carotenoids, and phycobiliproteins.(Soni et al., 2017) Tocopherols, tocotrienols, carotenoids and many other organic active ingredients are the most necessary and important substances due to their properties. Antioxidant, anti-inflammatory, and anti-bacterial flavonoids tocopherols, carotenoids, and phenolic compounds derived from microalgae are beneficial to cardiovascular, ocular, and immunological health. While phycobiliproteins such as C-phycocyanin guard against oxidative damage and promote liver and kidney health, polysaccharides improve immunity and gut health (Citi et al., 2024).Several studies have been completely performed around natural compounds and these compounds have been applied as functional ingredients or nutraceuticals for diverse types of medical purposes as time has continued its advancement.(Sorrenti et al., 2023) In the past decade, there has been a sharp increase in the interest of ingredients of biomedical, chemical, pharmaceutical, cosmeceutical and nutraceutical products and their active compounds(Wassie et al., 2021). Despite the way natural resources have been admitted into various industries, the real question remains as to, which extraction approach is the best and standard, to achieve industrial requirements hence, the need for the correct extraction method has been advocated for.

One such promising source is nutrient-rich microalgae like *Spirulina spp*. In addition to essential fatty acids (y-linolenic acid), polyphenols, photosynthetic pigments such as c-phycocyanin (C-PC), chlorophyll, and carotenoids mainly ß-carotene, vitamins (B1, B3, etc.), minerals (magnesium, calcium, phosphorous, etc.), and carbohydrates are found in Spirulina spp., despite its high protein content (Thevarajah et al., 2022). The bioactive compounds derived from *Spirulina spp*. that have been found are noteworthy due to their combination of anti-inflammatory, antioxidant, and antibacterial properties as well as immunomodulatory properties and many as mentioned in Figure 1.1. (Maddiboyina et al., 2023) . They are attractive for use in biological applications because of these qualities. *Spirulina*-containing consumables

provide several advantages, including as enhanced antioxidant qualities, changed physical attributes (such color and texture), and better stability during storage. (Zhang et al., 2022)

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Figure1.1: Therapeutic Functions of Bioactive compounds derived from *Spirulina maxima* (Fernandes et al., 2023)

The process of extraction is significant in the generation of new bioactive products and can have both positive and negative ecological impacts. Solvents are commonly used in extraction using methods including Soxhlet extraction, maceration, liquid-liquid extraction (LLE), and solid-liquid extraction (SLE) take a lot of time and discharges a large number of harmful solvents (Martins et al., 2023). Frequently, these techniques lead to a low extraction yield and do not produce the desired biologically active molecule that is well characterized. Another assertion which has been put forward about them is that they are harmful to the environment because they have to do with the use of toxic compounds that could spoil the preservation of ecological system(Chemat et al., 2012).

Plasma-assisted extraction (PAE) is a novel technique which utilizes unique features of plasma and as such provides more selective and effective bioactive component extraction. Plasma which unlike other extraction techniques is made of gases carrying charged particles and reactive species has several advantages over them.(Choi et al., 2023). The advantages are higher extraction yields, shorter processing times, and the technology can be used to extract materials without the use of solvents(Pina-Pérez et al., 2022). PAE provides a way to optimize the extraction of desirable bioactive compounds by utilizing the inherent characteristics of plasma.

The goal of this review is to give a detailed summary of PAE research progress and medicinal applications of the extracts of bioactive substances from *Spirulina*. It is going to present the historical background and basic principles of PAE which will describe both its pros and cons. In addition, the review will assess the current research in the area, comprising research designs, methodologies, and findings. It will also be able to point out areas demanding further research and demonstrate the research needs. Overcoming these gaps might result in the creation of PAE

systems that are functional and scalable, and selective for the extraction of beneficial bioactive compounds from *Spirulina*, thereby opening the door for its use in medicine and biotechnology.

1.1 Microalgae -Spirulina

1.1.1 Spirulina maxima

Recently, Microalgae have received much attention across the globe due to their huge potentials and uses. Properties that can be applied in renewable energy, biopharmaceuticals, and nutraceuticals. Microalgae offer countless opportunities in biofuels, bioactive medicinal substances, and food production, and it has been so due to its source of renewable energy, sustainable, and low cost (Mahata et al., 2022; Siddhnath et al., 2024). Microalgae species have been investigated to be used as value addition products with outstanding pharmacological and biological features(Khan et al., 2018). Microalgae are submerged phototrophic microorganisms with a multitude of exceptional and complex physiological and metabolic qualities. They have the potential to convert atmospheric CO_2 to a valuable substrate like carbohydrates and lipids and other bioactive substances(Cheng et al., 2017; Farooq, 2022) . Bioenergy and biopharmaceuticals are possible prospects for microalgae, but not enough is known about their future potential. Algae are classified based on their size into two main categories: microalgae and macroalgae. The microorganisms referred to as microalgae are those single unicellular organisms that are either eukaryotic, like those from the Chlorophyta group, or prokaryotic, such as the Chloroxybacteria. (Alias et al., 2022). On the other hand, macroalgae are macroscopic and multicellular. Additionally, algae can be further classified into three major groups based on their pigmentation: Brown algae (Phaeophyta), red algae (Rhodophyta), and green algae (Chlorophyta). This classification takes into account their pigments which are responsible for both their characteristic colours and physiological processes(Hachicha et al., 2022)

About 3.6 billion years ago, the first photosynthetic life form on Earth, Cyanobacteria generated the atmosphere of our planet with oxygen(Henrikson, 1989). The evolutionary link between bacteria and green plants is made up of blue-green algae(Prasanna & Kaushik, 2010) . It was in 1519 when a researcher and conqueror from Spain named Hernando Cortez made his first discovery of *Spirulina* (Soni et al., 2017). When Cortez visited the Aztecs of Lake Texcoco in the Valley of Mexico, he discovered that Spirulina was being served with all the meals. The blue-green microalgae Spirulina which is a spiral cellular structure and has an

amazing ability to live in environments much harsher than those that would kill most other algae (Ismaiel et al., 2016). Large freshwater lake in Africa, North America and Mexico, are the habitats where algae *Spirulina* grows abundantly. *Spirulina* also grows extensively in the Pacific Ocean (Ghaeni & Roomiani, 2016)

1.1.2 Spirulina Components

Spirulina is a powerhouse food packed with nutrients. As mentioned in figure 1.2 dried powder of Spirulina It is mostly protein (55-70%), with some carbohydrates (15-20%), fats (5-8%), minerals (6-8%), and a small amount of moisture (3-5%). Despite being low in fat overall (only 1%), *Spirulina* is a good source of plant-based fats like Poly Unsaturated Fatty acids (PUFAs), especially linolenic acid (36% of its fat content) (Fernandes et al., 2023). This superfood is also rich in minerals like manganese and magnesium. Several B vitamins (B1, B2, B3, B6, B9, B12), vitamin C, vitamin E, and pigments like zeaxanthin, canthaxanthin, chlorophyll A, phycocyanin, and beta-carotenes are all found in *Spirulina*.(Koli et al., 2022)

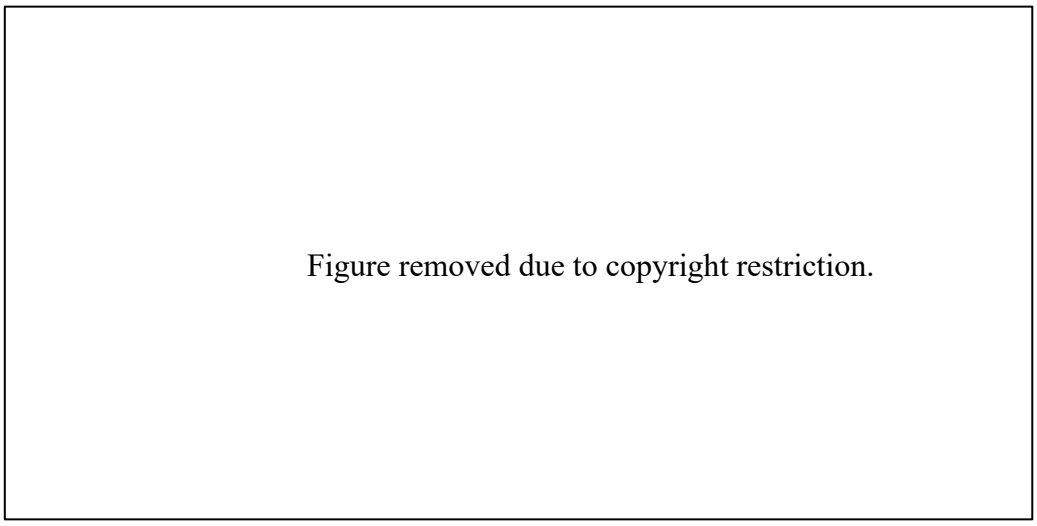

Figure 1.2: *Spirulina maxima* Structure and Spirulina Components(Fernandes et al., 2023)

The food industry's desire for natural ingredients is being fulfilled by an innovative Rival: microalgae. These small wonders are full of bioactive compounds like pigments and health-boosting nutraceuticals, and the market for them is rapidly expanding. C-phycocyanin(C-PC), a stunning blue pigment from microalgae is already a huge market with an estimated worth of $409. 8 million by 2030.(Thevarajah et al., 2022)

In addition to their rich pigmentation, microalgae possess elevated protein levels. Spirulina is remarkable as a particular variant comprises 60-70% protein by weight. Approximately 47% of Spirulina's protein composition is comprised of C-phycocyanin .(Thevarajah et al., 2022) The combination of the precious proteins and the highly demanded C-phycocyanin pigment makes *Spirulina* a multi-faceted star for big production projects. It is a single place for these so-called valuable compounds which are used in various industries.

Many fields are now exploring the special composition of *Spirulina*; functional foods, nutraceuticals, and even medicines are curious about its possible benefits. *Spirulina maxima* stand out among microalgae due to its elevated levels of bioactive compounds, especially C-PC, which demonstrate considerable antioxidant and anti-inflammatory properties. The ease of cultivation and broad availability enhance its appeal as a sustainable resource. However, the successful extraction of these bioactive substances is very important for it to be able to reach its full potential. The main challenge is to find a way to get all of them without damaging their functionality or their natural features.

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Figure 1.3: Phycobilisomes structural organization. Phycoerythrin (PE), phycocyanin (PC), and allophycocyanin (APC) make up this complex, which is arranged to efficiently transport energy (hv) unidirectionally to the reaction centre. In photosystems (PS) II and I, the cascade starts with PE and proceeds to PC and APC before arriving at the reaction centre. (Fernandes et al., 2023)

Since this pigment is released because of cell membrane disruption, the choice of an appropriate extraction method is vital in the process of obtaining pure and stable C-phycocyanin. Cyanobacteria get half of their light energy through the phycobiliproteins (PBPs) that are their main accessory pigments also known as PBPs. As shown in Fig 1.3., these pigments are located at the surface of thylakoids in the Phycobilisomes. PBPs are separated into three categories based on the colour of the pigment: CAPC (light blue), C- PE (Red), and C-PC, dark blue. These pigments in a successive manner, receive and transfer energy starting with C-PE, then C-PC, then C-APC finally reaching the photosystems I & II as depicted below (Jaeschke et al., 2021). However, the major pigment among the PBPs synthesized by cyanobacteria is C-phycocyanin, which may account for up to 20% of the dry cell weight (Jaeschke et al., 2021)

1.2 Extraction process used for bioactive compound of Spirulina maxima

There are a number of methods for cell disruption which include freeze and thawing technique, bead milling technique and mechanical disintegration process including mixing and homogenizing technique(Nunes et al., 2023). Although extracts with a rather high degree of purity can be obtained using the extraction process based on freezing and thawing, it is a tedious process that cannot be easily optimized for application in large-scale industries.

Using traditional extraction methods including maceration, liquid-liquid extraction (LLE), solid-liquid extraction (SLE), and Soxhlet extraction to extract bioactive compounds from *Spirulina* presents a number of significant problems (Lee et al., 2010). Including the following: These methods are hazardous to the environment. Although microalgae have a variety of cell wall structures, the most prevalent kind is stiff cell wall, which makes it difficult to extract solvent and impedes the process's rate-determining phase(Barba et al., 2015). The majority of

traditional extraction techniques also call for the use of large amounts of solvents, and at temperatures higher than 100 °C, there is still a chance that the necessary molecules may be changed or denatured (Wang & Weller, 2006). Not to mention, bulk transmission can be expedited by heat.

Temperature significantly affected the extraction since higher temperatures made the extraction process easy due to increased diffusion coefficient and solubility of useful chemical substances in shorter time of extraction (Manzan et al., 2003). But it is crucial to note that high temperatures can sometimes cause the degradation of important nutrients such as protein and vitamins.(Ahmad et al., 2020; Rädecker et al., 2021) These restrictions have made scientists look for other methods that are effective, resource friendly and in compliance to the green chemistry principles.

Another extraction method that differs from the previous one is Enzyme-assisted extraction, also known as EAE. The *Spirulina* cell walls are decomposed by EAE enzymes, which makes it easier for the bioactive chemicals to be released (Coelho et al., 2020). It has been evident that through the use of EAE, better extraction rates, short processing time as well as ability to extract particular target molecules are some of the gains made over traditional methods. However, because enzymes used in this process are relatively costly, they are likely to get denatured along the process and additional purification steps will be needed to isolate the required molecules in their purest form; using EAE at a larger industrial production may pose some challenges (Das et al., 2021).

In the Microwave assisted Extraction (MAE) technique, the solvent and sample is subjected to microwave irradiation which accelerates the rate of diffusion of the target molecules (Delazar et al., 2012). Still, this is a unique method that has over conventional methods certain benefits, for example shorter extraction times, less solvent consumption and higher rates of extraction (Routray & Orsat, 2012). However, there are some disadvantages of MAE such as the heat sensitive chemicals as they might be destroyed and, in addition, microwaves penetrate shallow depths only(Veggi et al., 2012).

There are other extraction methodologies that have also been explored in the extraction of *Spirulina*; they include; Ultrasound-Assisted Extraction (UAE), Supercritical Fluid Extraction (SFE), and Pressurized Liquid Extraction (Martí-Quijal et al., 2023; Rodrigues et al., 2018) Undoubtedly, using the methods mentioned above has several advantages such as a minimum

of solvent utilized in the process, shorter time required for the extraction process, and increased selectivity. However, in some cases, the requisite instruments and optimisation processes themselves, has some constraints (Dey & Rathod, 2013).

1.3 Plasma Assisted extraction methods

Studies have also been conducted in attempts to develop new techniques for extraction including plasma assisted extraction (PAE) to overcome the challenges. That is why the application of plasma methods was begun to attract a lot of interest for obtaining valuable products, including bioactive compounds from microalgae. It is equally important and effective to use plasma in the extraction of bioactive compounds due to its ability to disrupt the microalgal matrix. As for the technologies that may be most promising in terms of delivering efficient bioactive substances extraction, plasma can be considered as one of such possibilities, to optimize extraction efficiency. (Misra et al., 2016; Tendero et al., 2006)

Langmuir named the substance as plasmas which constitute 97% of the universe and are at times called the fourth state of matter (Merche et al., 2012). It is composed of electrons, radicals, molecules, and ions. If energy is introduced into a gas, then one may purposely make plasma – ionized gaseous structures that contain positively charged ions and negatively charged electrons. The main concepts related to plasmas are excitation, ionization, and dissociation of gaseous molecules which create reactive species such as electrons, ions, radicals, and electromagnetic waves.(Turkoglu Sasmazel et al., 2021)

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Figure 1.4 After solid, liquid, and gaseous forms, plasma is the fourth form of matter and functions as an ionized gas

These free ions are driven, when electric field is impressed across plasma gas, where they may collide with atoms of the gas or the electrode. Therefore, during the excitation process, the

energy using the translation technique increases the translational energy and increases the internal energy of the gas atoms to another level. Ionization is that form of emission of the atom during the phase of excitation when enough energy is supplied to remove the most loosely bound electrons. When electrons, ions, neutral particles, and radiation collide with a material, it releases energy that can power excitation as well as ionizing reactions. On the other hand, there is dissociation which occurs in case a molecule collides with an electron, ion or photon in a non-typical interaction. (Turkoglu Sasmazel et al., 2021)

Usually, there is a vacuum pump employed to generate the plasma within the plasma generator chamber but producing the atmospheric pressure plasma does not entail using a vacuumed device. The elements produced by atmospheric plasma, which cause surface interaction leading to surface activation, cleaning, etching, and functionalization. They can also disrupt cells and membranes, and therefore facilitate the recovery of desired molecules from biological materials such as microalgae(Merche et al., 2012).

These approaches employ a number of techniques to generate reactive species such as ionized gas plasmas, radicals, electrons, ions, electromagnetic radiation, etc. For instance, dielectric barrier discharges, DBDs, are a technique that enables one to achieve thermal and non-thermal operation by developing plasma between two electrodes that are subsequently shielded by a dielectric material(Brandenburg et al., 2023). Other methods of generating non-thermal plasmas include Atmospheric pressure plasma jets (APPJs) and Radio frequency (RF) capacitive discharges, which utilizes RF power or expels the plasma through a nozzle. Corona discharges are commonly used in surface treatment and are high voltage discharges between electrodes divided by an insulating dielectric material. Thermal plasma sources are continuous DC plasma comparable with the transferred arcs and plasma torches. When gases are exposed to microwaves, electrode-less systems known as microwave-induced plasmas (MIPs) are created that ionize gas to form plasma. Like pulsed DBD, pulsed discharge produces non-equilibrium plasma suitable for treatment of materials by the application of pulsed power supply. (Hoffmann et al., 2013)

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Figure 1.5: Diagrammatic representation of three cold atmospheric pressure plasma devices. (Pham et al., 2023)

Furthermore, PAE can minimize extraction times and assist in the elimination or substantial reduction of organic solvents, which also aligns with the sustainability and green chemistry concepts. It also assists to set up standard and efficient extraction techniques of bioactive compound from natural sources, for instance*, Spirulina* and it equally solves some ecological problems(Kirchhoff, 2005)

1.4 Atmospheric Plasma Treatment

In the area of plasma-assisted extraction (PAE) the present research has focused on evaluating various plasma sources for efficient extraction of potent bioactive compounds from *Spirulina,* especially C-phycocyanin. Therefore, extensive research has been conducted in the recent cold atmospheric plasma (CAP) systems and atmospheric pressure plasma jets (APPJs). For instance, Pham et al. established how an atmospheric plasma jet system could be utilised to deposit thin bioactive films from biomass such as *Spirulina*, thus explaining how APPJs could be used to extract compounds like C-phycocyanin (Pham et al., 2023). Atmospheric pressure plasma systems like using CAP sources for the extraction of phytochemicals has been highlighted.(Heydari et al., 2023)

Some of the plasma sources are reactive oxygen species, charged particles and UV radiation, which are created by different plasma and are one of the key factors in the approach and the design of the experiment for plasma assisted extraction processes. These reactive species are able to and capable of effectively damaging and permeabilizing the cell walls and membranes of microalgae to release intracellular products such as the C-phycocyanin. Moreover, the type of plasma to be used (DBD, APPJ, or CAP) and its parameters: power, gas flow rate, exposure time, type of solvent, pH, and temperature, pre-treatment of biomass (fresh, dried or disrupted) (Heydari et al., 2023) and the post- extraction processes like purification and stabilization also influences the extraction efficiency and selectivity. (Munekata et al., 2020)

The existing literature shows that *Spirulina* and some of the byproducts especially C-phycocyanin have several uses. Some of the products that may be produced from it includes nutraceuticals, functional foods, cosmetics, personal care items, and in the biomedical fields like anticancer, wound healing, antioxidant properties. Thanks to their distinctive characteristics and documented biological activity, these compounds can be effectively applied in different conditions.(Maddiboyina et al., 2023)

As it has been made clear in the discussion up to this point, plasma-assisted extraction techniques have various advantages over conventional extraction techniques. Advantages of extraction includes higher yields and rates of extraction, the option of choosing what to extract and against what, shorter time and energy to be spent in the whole process, and the option of solvent-less or 'Green' extraction. However, some drawbacks include the following: some compounds used in the process may degrade at higher temperatures such as some of the compounds mentioned in the above methods, plasma settings may not be optimized for the specific microalgae species and the chemical one wishes to capture, and it is also possible to experience some challenges when scaling up, and the methods require certain specialized equipment. (Heydari et al., 2023)

1.4.1 Mechanism of Plasma treatment

Microalgal species including *Spirulina maxima* can take advantage of plasma therapy since this technique is effective in enhancing the bioavailability of intracellular compounds. *Spirulina* has a cell wall that is composed primarily of peptidoglycans, polysaccharides, and proteins. This cell wall is comparatively rigid, and it could restrict the bioavailability of active molecules such as C-phycocyanin. (İlter et al., 2018) Coming in direct contact with the structural components of the cell wall, the non-thermal ambient plasma contributes a comprehensive array of reactive species; in effect, owing to the reactions mediated by such plasma, there are several beneficial incidences. They are the reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are generated by plasma and indicate the onset of `oxidative stress at the cell wall that in turn forms temporary pores to pass out other intracellular chemicals. (Yusupov et al., 2013)

Another potential effect that plasma treatment can have been the presence of events that are like electroporation. The electric fields that are generated by plasma have the capacity to make the cell membrane become permeable for a short while thus making it easy for substances like intracellular C-phycocyanin to evacuate from the cell(Ogawa et al., 2005). Plasma therapy is a procedure that is as gentle as well as effective in enhancing the bioavailability. Plasma treatment parameters can be optimized to deliver adequate energy for cell wall permeabilization while maintaining sensitivity to prevent harm to the fragile intracellular components.

In addition to the use of plasma therapy, reactive oxygen species ROS appear which cause lipid peroxidation. (Chauvin et al.) This in turn interferes with physical barrier that is the cell

membrane, and which is tasked with maintaining the integrity of internal molecules. Likewise, the oxidation of the protein bound to the membrane triggers the breakdown of the selectivity of the cell membrane, thereby releasing extra molecules including C-phycocyanin.(Ji et al., 2019) This selective disruption ensures rational delivery: the bioactivity of the compound is not affected as may be observed in extraction processes which may use chemical and physical means that are very aggressive.(Colla et al., 2007; Ji et al., 2019)

This is one of the most contentious and discussed aspects of plasma-assisted extraction methods since it is assumed that the reactive plasma species should produce oxidative reactions that will denature and reduce the efficiency of the extracted bioactive compounds (Zocher et al., 2016). Although plasma can penetrate the algal cell wall and membrane in order to extract C-phycocyanin, it also has the drawback of increasing the likelihood of unwanted oxidation of other components like proteins, carotenoids and C-phycocyanin itself during the process of extraction. This trade-off between extraction efficiency and possible target molecule degradation has gained attention from several research(Sommer et al., 2021)

It will be crucial to identify the most suitable plasma conditions in terms of extraction efficiency of the target molecule and avoiding its degradation, which constitutes a significant knowledge gap currently hindering the plasma-assisted extraction of bioactive chemicals from microalgae, such as *Spirulina*. In this work, it was demonstrated that the efficiency of the extraction of the chemical in question and its structural properties may be significantly influenced by plasma operation parameters such as power, gas flow rates, and exposure time. Determining the performance of these features is essential in the creation of extraction techniques that are both selective and efficient. Several research studies focus on improving plasma conditions especially for the recovery of valuable products from *Spirulina*, including C-phycocyanin; however, there is still extensive literature review needed to develop guidelines for a range of target compounds and microalgal species.

Some of the other gaps, which are rather obvious, are the lack of the simple understanding of many processes that occur during the whole extraction process, for instance, between the microalgal cells of species such as *Spirulina* and plasma. While some of the reactive species generated in plasma may cause degradation of cell walls and membranes, and the release of intracellular chemicals further studies need to be conducted for the better understanding of the mechanism of cell damage by plasma and how plasma species interact with biomolecules in

the incorporated matrix of microalgae. Further understanding of these processes could aid in the design of better extraction methods, enriched with more selectivity for the target species of microalgae, or the metabolite of interest.

However, more research is needed to ascertain the biological effect of the remained components when exposed to the plasma reactive environment and the interaction of the various extracts of *Spirulina*. The use of plasma-assisted extraction will contribute towards increasing the extraction yield of various bioactive compounds present in *Spirulina* like C-phycocyanin. Further research should focus on consequences related to the restructuring of the extracted proteins, such as their functionality and applicability to the creation of biomedicines or functional food. Hence, this research gap needs to be filled to ascertain the viability and safety of the extracted bioactive compounds from plasma-assisted approaches.

1.5. Summary of the State of Research

High demand for the naturally occurring bioactive compounds - especially those that are of microbial origin such as *Spirulina* microalga have been made evident in this literature review. *Spirulina* is known to contain various active and beneficial chemical compounds such as C-phycocyanin, carotenoids and proteins which can be used in biomedical, pharmaceutical and nutraceutical related industries. However, the sustainability, selectivity and efficiency of the extraction methods known in traditional chemistry is often jeopardized. Nevertheless, prior research has predominantly concentrated on traditional extraction techniques, which are energy-demanding and require substantial solvent utilization. The scalability and efficiency of plasma-assisted extraction (PAE) for bioactives are still inadequately investigated.

In this respect, the plasma-assisted extraction (PAE), as following the tenets of green chemistry has emerged as an innovative and potential approach. Besides, PAE is known to be capable of compromising the microalgae cell walls and membranes that are rigid to facilitate the process, through using reactive species from plasma sources It makes possible to obtain valuable compounds inside the cell membrane. Existing reports reveal the capability of PAE in the release of C-phycocyanin and other biomolecules from Spirulina by varying the type of plasma sources and working parameters.

Nevertheless, from the literature assessment, the following areas have been found to have some major research limitation in the studies currently being conducted. It is still difficult up to date

to determine the best plasma conditions that would increase the extraction yield while at the same time avoiding reduction of the target bioactive compounds. However, there is comparatively less understanding of how plasma species act on microalgal cells and other species such as *Spirulina*. Maybe, explanation of these systems will result in development of extraction techniques that will not just be effective but will also be selective. PAE has demonstrated potential in food processing applications; however, its capability to enhance the extraction of bioactives from microalgae, such as Spirulina maxima, remains to be fully explored. This research seeks to fill this gap.

To fill these knowledge gaps, the following work aims to undertake the following: Carry out an extensive literature review on the effects of plasma on the extraction of C-phycocyanin from *Spirulina*. The purpose of the study is to identify optimal plasma operating parameters, examine the interaction processes of plasma with microalgae, and investigate the biocompatibility and bioactivity of extracted C-phycocyanin by using computational analysis and experimental data.

1.6 Aims and Hypothesis

The following are the aims and Hypothesis of the project; The main purpose of the project is to acquaint methods that shall enable the setting up of efficient and reliable Plasma assisted extraction techniques that can assist in the isolation of worthwhile bioactive compounds from natural sources.

1.6.1 Hypothesis

Hypothesize that atmospheric plasma treatment enhances the extraction efficiency of bioactive compounds from *Spirulina maxima* by disrupting its cell wall, with the optimal conditions dependent on treatment duration and carrier gas type.

1.6.2 Specific Aims

Aim 1: Optimize the conditions (carrier gas and duration of treatment) for extraction of bioactive compounds from *Spirulina maxima*

Aim 2: Investigate the effects of atmospheric plasma treatment on the cellular components and biochemical composition of the biomass of *Spirulina maxima*

Aim 3: Investigate the effects on antibacterial activity of atmospheric plasma treated extract of *Spirulina maxima*

Aim 4: Study the morphology of *Spirulina maxima*

CHAPTER 2

Material and Method

Spirulina maxima biomass was purchased from OXYMIN® SPIRULINA. Bovine serum albumin (Sigma); Trolox (Sigma); DPPH (Sigma); *Staphylococcus aureus* (ATCC 25923). Beside this, other reagents and chemicals used were purchased from chemical supplier.

2.1 Sample preparation for plasma treatment

Spirulina maxima biomass was purchased from OXYMIN® SPIRULINA.

Spirulina maxima biomass powder was obtained from OXYMIN® and weighed 2gm of powder for plasma treatment.

2.1.1 Plasma chamber design

The following figure 2.1 displays the design of the plasma chamber that utilized to treat the microalgae power. Overall, the chamber consists of the quartz tubes, TPU seal blocks, tungsten electrode. The main sample container space made from the gap between two quartz tubes whereas the outer tube is 22 mm diameter, and the inner tube is 10 mm. Both tubes are in the same length of 15 mm. In the core of the inner quartz tube, 20mm pure tungsten electrode was placed. Outside of the outer quartz tube, there was a copper-aluminium alloy wire twisted around. This set-up creates the dielectric barrier, which will allow the plasma discharge to go through the sample in the experiment. The TPU seal blocks act as the stopper to keep the sample in place. During the plasma treatment process the chamber was tightly sealed to accommodate stable gas flow and prevent external contamination. A gas outlet was created in one seal block to stabilize chamber air pressure and eliminate extra gas. The temperature of the chamber was monitored to ensure the sample was not overheated during the process.

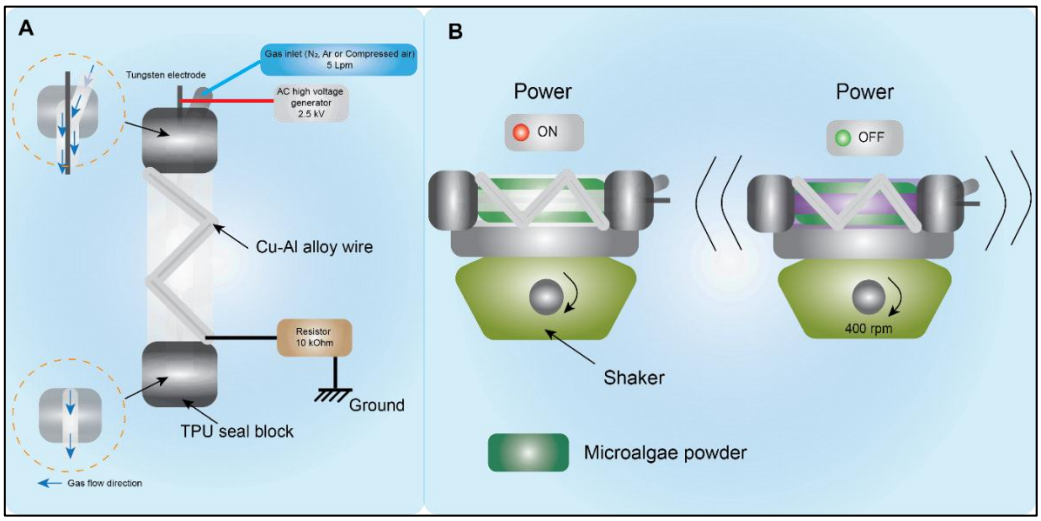

Figure 2.1:(A) The design of the plasma chamber. The chamber operated with consistent voltage at 2.5kv, the air flow rate at 5 lpm under atmospheric pressure. (B) The schematic of the experiment.

2.1.2 Atmospheric plasma treatment of the Spirulina maxima

As described in figure 2.2 The plasma was generated using specific gas like Argon, Nitrogen and Compressed Air at a flow rate of 5litre/minute with constant voltage of electricity is 2.5 kV were applied on the electrode. The chamber was kept at atmospheric pressure, and the gas was continuously fed into the chamber to sustain plasma generation during the treatment process.

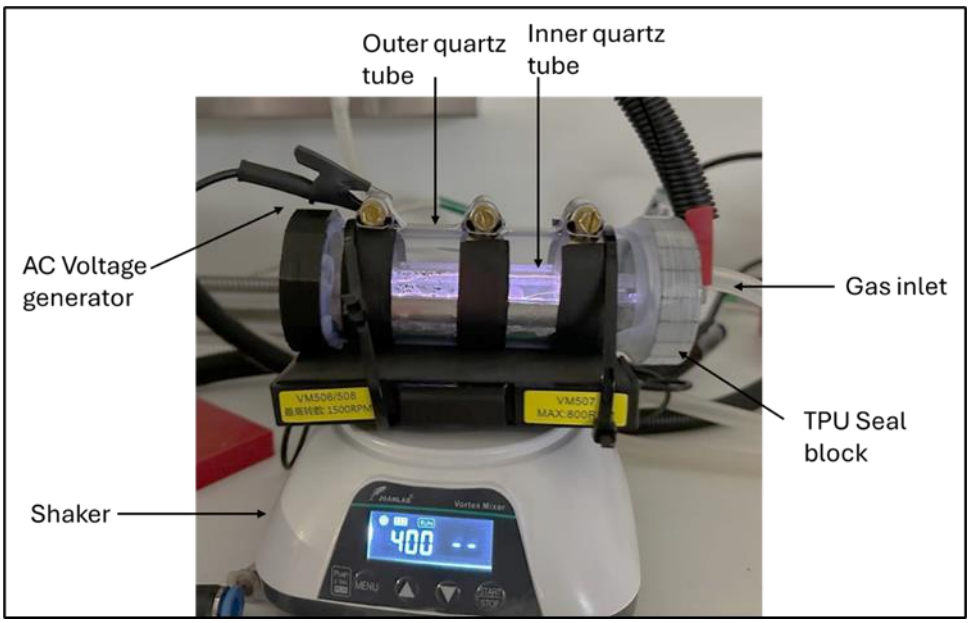

Figure 2.2 Experimental setup for the plasma treatment to *Spirulina maxima*

The plasma system was shaked under precise speed 400 rpm to guarantee the exposure of the particles to plasma. The sample was heated intermittently, and the temperature of the sample was maintained frequently to avoid any heat damage to the cells of *Spirulina*.

To find out how different plasma exposures influenced *Spirulina*, the length of the plasma treatment was altered. There was no exposure time for the control (no treatment) to increased exposure from 1 to 7 minutes. Moreover, the treatment was expanded with additional intervals at 10 and 15 minutes. These treatment times range enabled an investigation of the effects that varying plasma exposure times had on the *Spirulina* samples.

2.1.3 Post-Treatment Analysis

Following plasma treatment, the *Spirulina* samples were removed from the chamber right away and examined according to several characteristics. One of the pigments used in photosynthetic processes, phycocyanin, was assessed using spectrophotometry. In addition, the biochemical reaction of the plasma therapy was evaluated by measuring the amount of oxidative stress biomarkers and the release of intracellular chemicals.

2.2 Sample Preparation for Quantification

Simple extraction of the C-phycocyanin was done by the incubation and treatment with the water. Weighed 10 grams of the Plasma treated powder and dilute with 10ml of the Distilled

water followed by the incubation at Room temperature for 10 minutes. Afterward Centrifuge at 4000rpm for 10 minutes to get the supernatant of extracted C-PC. Filter the supernatant by using 0.45um fillers and freeze dried the solution for 48-72 hours.

After following above method, results were not expected so there were modifications in above method. Weighed 10 grams of the Plasma treated powder and dilute with 10ml of the Distilled water followed by the incubation at room temperature for 10 minutes. After that Filter the supernatant by using 0.45um fillers in centrifuge tube for the further quantifications.

Each experiment was performed in triplicate to guarantee the reproducibility and precision of the results. Samples of untreated Spirulina maxima served as negative controls for all analyses, encompassing C-PC quantification and antibacterial assays.

2.3 Biochemical Quantification

2.3.1 C-phycocyanin release from Spirulina

C-PC concentration and Purity were determined by measuring the absorption spectrum of the sample at different wavelength 280nm, 452nm,620nm,652nm using LAMDA 365 UV/VIS spectrometer of PERKIN ELMER. For the measurement, 1mg of extracted sample were diluted with 10ml of distilled water. C-PC concentration and purities were calculated using below Equations.(Kuhnholz et al., 2024) (İlter et al., 2018).

$$[1].\ PC\ (mg/ml) = (A_{615} - 0.474 * A_{652})/5.34$$

$$[2].\ Purity = A_{620}/A_{280}$$

where C-PC concentration in the extract; A_x is absorption of the final extract at the specific wavelength 615 and 652. Purity of C-PC is C-PC in the extract calculated as the ratio of absorptions at 620 nm and 280 nm.

2.3.2 Total protein Release from Spirulina

Total protein concentration in the samples was assessed from the Pierce™ BCA Protein Assay Kit (ThermoScientific) according to the instructions provided by the manufacturer. In this assay proteins convert Cu^{2+} ions from copper (II) sulfate to Cu^{1+} ions in an alkaline environment, the degree of conversion being directly related to the amount of protein present in a sample. The

Cu^{1+} ions are then allowed to interact with bicinchoninic acid (BCA), which produces a highly chromogenic purple complex with an optical density at 562 nm.(Scientific, 2013)

For the assay, weighed 1 mg of freeze-dry sample, which was dissolved in 1 mL of distilled water. From this solution, 150 µL of the sample extract was added in triplicates in a 96-well plate. An equal volume 150 µL BCA reagent was then added to each well was an equal volume of BCA reagent. The plate was incubated at 37°C for 2 hours for the colorimetric reaction to occur. Optical density was subsequently determined at 562 nm in a SYNERGY-HTX multi-well plate reader. The protein densities were determined by normalizing the reported absorbance values relative to a standard curve from known protein concentrations.

2.3.3 Antioxidant Activity

C-phycocyanin (C-PC) sample was also tested for antioxidant activity using DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical assay. The DPPH assay depends on the radical quenching capability of antioxidants that react with the DPPH radical which has a violet colour owing to the presence of the odd electron. The DPPH radical reacts with antioxidants by accepting a hydrogen atom, in which the antioxidant transforms to DPPH from violet colour to yellow. This change is determined Spectro photographically at wavelength 517 nm.(Baliyan et al., 2022)

To make up the working solution 10ml of DPPH stock solution was diluted with 45ml of methanol. For the assay, 15 µL of the sample extract was added into 96 well microplate in triplicates and 285 µL of DPPH working solution was added to each of the well. The plate was then covered from light and left to incubate at room temperature for 24 hours to allow for reduction of the DPPH radical. The absorbance was determined at 517 nm using a SYNERGY-HTX multi-well plate reader: the lower absorbance values signifying higher antioxidant activity.

2.4 Biocompatibility assay

2.4.1 cytotoxicity assay

Cytotoxicity assays are tests that must be applied to allow one to determine a potential toxic effect of a substance or a compound on cells. In cytotoxicity experiments the assessment of the effects of the compound on the ability to live or the survival of the cells is used. Phorbol myristate-acetate (PMA) differentiated from a human monocytic leukemia cell line (THP-1)

monocytes into macrophage like pheno-type from (THP-1) and human keratinocyte cell line is acquired from adult human skin (HaCaT) showing strong adhesion to tissue culture plastic (Berger et al., 2017) (Citi et al., 2024). In terms of morphology, antigens and receptor expression, and cytokine production, these cells are true monocyte-derived macrophages in culture. Earlier studies have utilised the THP-1 and HaCaT to examine in vitro drug cytotoxicity and material biocompatibility (McCanna et al., 2015).

2.4.2 Seeding of THP-1 cell

THP-1 cells (TIB-202, ATCC, Virginia, USA) were grown with RPMI 1640 (Gibco) containing 10 % heat inactivated fatal bovine serum (FBS) (Gibco- BRL), 1 % streptomycin/penicillin (Gibco-BRL), 5 % CO_2 and incubated at 37C. More specifically, 0.1µg/mL of phorbol 12-myristate-13-acetate (PMA) (Gibco-BRL) was used and for THP-1 cells cultured in 75-cm^2 growth flasks (Corning). It became necessary to treat them for 48 hours at 37˚C in a humidified CO_2 incubator in order to produce cells that resembled THP-1 macrophages. Cells were trypsinzed using 0.25 % Trypsin-EDTA (1X) (Gibco-BRL) when they had grown into 80–85%confluence. (Berger et al., 2017)

2.4.3 Seeding of HaCaT Cell

Hamburg Cancer Cells which are the immortalized human keratinocyte cell line is acquired from adult human skin (HaCaT) was seeded and grown in human epidermal keratinocyte line 300493 cell line services from Eppelheim, Germany. The medium employed was Dulbecco's modified eagle's medium (Sigma) with added 10% heat inactivated fetal bovine serum (Gibco), and streptomycin/penicillin (Gibco-BRL) at 1%. They were tripsinized using 0.25% Trypsin-EDTA (1X) from Gibco-BRL when the cells grew to about 80-85% densities to harvest cell for treatment. Cells were incubated with the sample in 24h. After that, cell viability was analysed by the MTT assay (Sigma)(Hayward et al., 2005) .

2.4.4 MTT assay

Cells were incubated with the sample with two treatment which include 40% treatment (40 µl of sample and 60µl of media) and 60% treatment (60µl of sample and 40µl of media) in 24h. After that, cell viability was analysed by the MTT assay (Sigma) according to manufacturer instructions. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma) was harvested and filtered in PBS at 5 mg/mL from some batches of MTT containing a small

amount of insoluble material. MTT solution 10µl and 100 ml medium was added on all wells of an assay at the times and plates were incubated at 37°C for 4 hours. To dissolve the dark blue crystals, DMSO purchased from ChemSupply was added to every well carefully mixed. The plates were then read on a SYNERGY-HTX multi- well plate reader by Bio-Tek at 570nm test wavelength after a few minutes at room temperature so that all the crystals were dissolve completely. The percentage cell viability was obtained by dividing the data by the control cells cultured in presence of only the culture media. Wells with culture medium only as 100% cell viability.(Mosmann, 1983)

2.5 Antibacterial ability of Spirulina

2.5.1 Colony Forming Units assay

The colony-forming unit (CFU) assay was applied to evaluate the bacterial growth inhibition by extracts from Spirulina maxima treated with atmospheric plasma for 0 and 5 minutes.

2.5.2 Sample treatment for CFU

A bacterial suspension was prepared from Sterile Tryptic Soy Broth (TSB) at a starting concentration of 1×10^5 Colony Forming Units per millilitre (CFU/mL). This suspension was used as the inoculum to determine the effect of Spirulina maxima extracts exposed to plasma on the survival of the bacteria. For the experiment, bacterial suspension was mixed with the plasma treated Spirulina maxima extracts equally, where samples were taken at 0 minute (untreated) and 5 minute of plasma treatment. In order to facilitate any interaction between the bacteria and the plasma treated Spirulina, each extract-bacteria culture was incubated at 37°C for 24hrs. After 24 hours of incubation, the combination was further diluted with TSB to obtain various dilutions which could be utilised to determine viable bacteria. To ensure a wide range for precise CFU counting, the dilutions were made from 10^{-2} (1:100) to 10^{-8}(1:100,000,000). Using an aseptic technique, a Tryptic Soy Agar (TSA) plate was inoculated with 10 µL of bacterial-extract combination. For more control and to ensure that the colonies are well distributed and isolated, the droplets were placed at the correct position around the agar. To minimize the chances of errors, each sample was streaked out at least three times on the different Petri dishes. TSA plates were then incubated for a period of 24 hours at a temperature of 37°C. It is over this period that the live bacterial cells could grow to maturity and produce clear and distinct colony systems. Petri plates that corresponded to the dilution procedure were

observed to count bacterial colonies after 24 hrs of incubation. To calculate the correct CFU conform the dilution scheme only counting plates with between 30/300 colonies. This range ensures it does not grow beyond the limit needed for appropriate colony counts or even below the limit needed for colony counts. Using the formula, one may estimate the CFU per million of the initial bacterial suspension. The following formula was used to compare the CFU value for the plasma treated Samples with that of the control un-treated sample to find out the log reduction of bacteria.

$$\text{CFU/mL} = \frac{Number\ of\ Colonies \times \text{Dilution Factor}}{\text{Volume Plated (mL)}}$$

$$\text{Log Reduction} = \log 10\left(\frac{\text{CFU control}}{(\text{CFU plasma} - \text{treated}}\right)$$

2.6 Morphology study of Spirulina maxima plasma treated and non-treated

2.6.1 Epifluorescence Microscopy

In bioscience study, epifluorescence microscopy is one of fluorescence microscopy methods that is extensively employed to visualize the cellular and subcellular structures besides morphology of the cells. It employs a wavelength selective bandpass filter, to create desired light for instance ultraviolet, blue or green light that is sourced out of a multispectral light source. This light is reflected on to the specimen through a dichroic mirror, while the two — the excitation light and emitted fluorescence go through the same objective lens. Epifluorescence microscopy is similar to fluorescence microscopy, but in the later there is a barrier filter between the excitation and emission paths. It's even more beneficial for thicker specimens because of better penetration depth and high SNR in comparison to classical brightfield microscopy.(Webb & Brown, 2013)

The Spirulina maxima pellet after treating with plasma was spread at the concentration of 0.1 mg/ml on clean glass slides and left to dry at room temperature for four hours. After that, the slides were stained with a 0.01% calcofluor white (Sigma-Aldrich, Inc., Saint Louis, USA) for 5 min and potassium hydroxide was added to increase the sensitivity of microalgae visualization. The cell walls of Spirulina maxima Control and Plasma treated) were observed and analysed through Olympus IX83 Inverted Microscope.(Wei et al., 2015)

CHAPTER 3 Results

3.1 Effect of Different Carrier gas treatment on Spirulina maxima

A variety of carrier gas treatments of plasma to *Spirulina* were investigated in Figure 3.1 (A) to determine how they affected the release of C-phycocyanin. The concentrations of C-phycocyanin (C-PC) did not exhibit any significant variations across any of the three gas treatments, which were nitrogen, argon, and compressed air separately. Since the concentrations varied from 0.010 mg/ml to 0.012 mg/ml across all of the gases, it can be concluded that the type of gas does not have a major impact on the extraction of C-PC components.

With purity values ranging from 0.25 to 0.30, the extracted C-PC did not exhibit any significant differences between the gases as shown in Figure 3.1(B). This was likewise the case with the analysis of the purity. The conclusion that the kind of gas does not have a substantial impact in the quality of C-PC extraction is strengthened as a result of this circumstance.

To evaluate the effect of plasma treatment on the protein content of extracted from *Spirulina*, the total protein content of plasma treated, and non-treated extracts was assessed as a mg/ml in dry weight. As demonstrated in Figure 3.1(C), Protein concentrations remained similar throughout all three types of gas, with levels ranging around 0.004 mg/ml to 0.005mg/ml. This is comparable to the findings obtained from the C-PC procedure. Regardless of the gas that was utilized, there were no discernible variations in the concentration of proteins that were found.

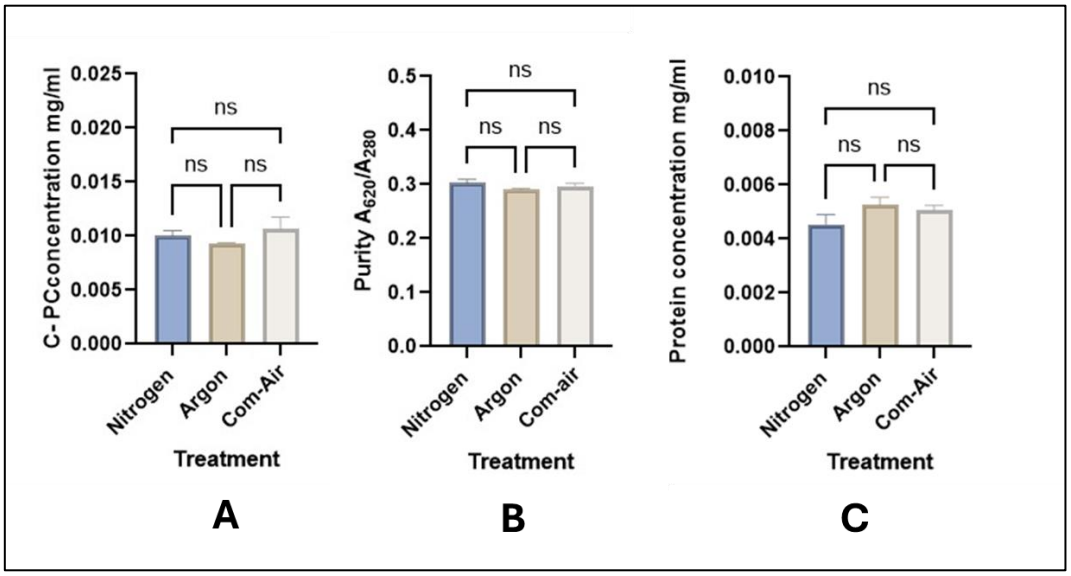

Figure 3.1: (A) Effect of different gas treatments on Release of C-phycocyanin (C-PC). Spirulina Was treated with Different gas Nitrogen, Argon, Compressed Air and C-PC concentration (mg/ml) was measured after exposure to each gas treatment. (B): Effect of different gas treatments on Purity of released C-PC (C): Effect of different gas treatments on Protein Content of Spirulina after Different gas treatment (ns = not significant), error bars present (mean ± standard deviation, n = 3).

3.2 Effect of Different Treatment Durations on Extract of Spirulina maxima

The release of C-phycocyanin from the plasma-treated and non-treated extract was quantified and expressed in terms of mg/ml of dry weight during a period as demonstrated in Figure 3.2(A). Within 5 minutes of plasma treatment, the concentration of C-PC increased to 0.08 mg/ml, this indicated improvement from the previous concentration. In this case, the concentration of C-PC was much higher than the control; the control had a significantly higher concentration ($P < 0.01$). The concentration of C-PC was found to be reduced after 10 minutes and even more to 0.025 mg/ml after 15 minutes suggesting that C-PC attain lesser concentration after being exposed to plasma for a longer time.

The purity of the extracted C-PC was calculated and as mentioned in Figure 3.2(B) the purity significantly increased to its maximum value after plasma treatment for 5 min. Following that, the purity of the C-PC reduced after 10 min of treatment and remained low after 15 min, suggesting that extracting samples at long exposure durations affects the purity of the extracted C-PC.

The total protein content in both plasmas treated and nontreated extracts were quantified in mg/ml using dry weight as shown Figure 3.2(C). This was done in order to see whether the plasma treatment affected the protein levels of extract of *Spirulina*. The protein concentrations also revealed a similar trend over the period of time and there was marked significant increase within the concentration at 5 minutes 0.4 mg/ml. The concentrations of protein decreased 0.22mg/ml to 0.20 mg/ml once 10 and 15 minutes respectively had elapsed.

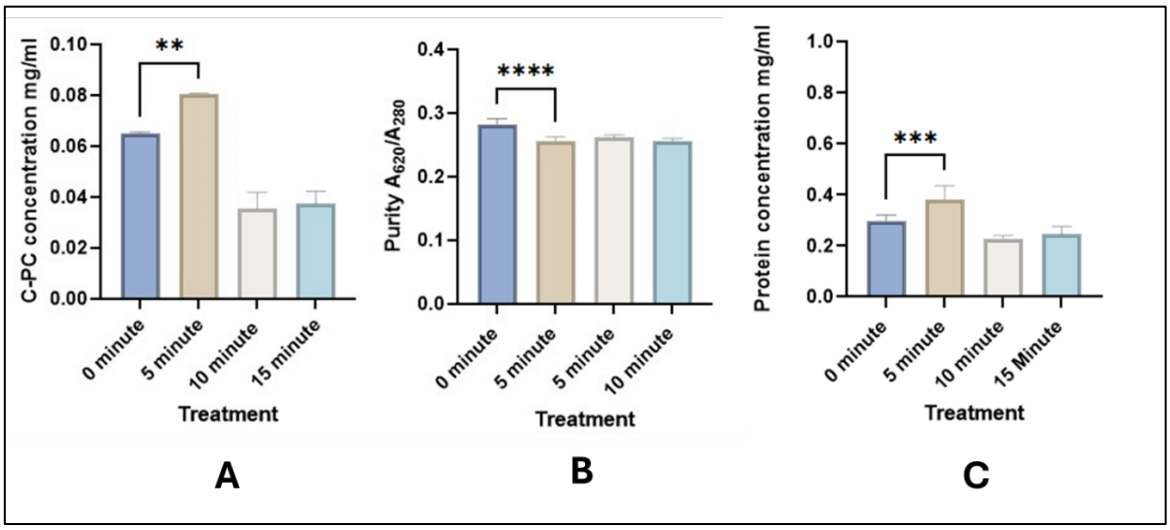

Figure 3.2: (A) Effect of different Time treatments on release of C-phycocyanin (C-PC). C-PC concentration (mg/ml) was measured after exposure to four different time period of 0,5,10 and 15 minutes. (B): Effect of different treatment duration on purity of released C-phycocyanin(C): Effect of different treatment duration on protein content of *Spirulina*. Protein concentration (mg/ml) was measured after 0, 5, 10, and 15 minutes of treatment. Error bars represent the standard deviation (mean ± standard deviation, n = 3) and, as indicated by "**" $p<0.01$, "***" $p < 0.001$, "****" $p < 0.0001$.

3.3 Further Investigation into the Effect of Treatment Duration of Spirulina maxima

A follow up investigation of the treatment duration over time was done in order to optimize it more thoroughly. The duration of the treatment, which was considered to be ranging between 0 and 7 minutes, was included in this investigation.

C-PC was found to be present at the beginning of the plasma treatment at a concentration of 0.22 mg/ml, was observed in Figure 3.3(A). After one minute of treatment, a considerable drop was seen, which demonstrates that there was an instantaneous decrease in C-PC concentration following plasma therapy. This is determined by the fact that the concentration immediately drops. This is supported by the measurements that were taken. Within the ensuing 2 to 3 minutes, the measurement was consistent, with the concentrations of C-PC remaining relatively low at roughly 0.02-0.03 mg/ml.

After 4 minutes, the concentration starts to rise, and it doesn't reach its peak point until 5 minutes have passed. This must be the most favorable length of plasma exposure for C-

PCextraction because the concentration starts to rise after 4 minutes. Despite this, the concentration begins to decline once more with treatment duration of 6 and 7 minutes, and at approximately 7 minutes after that, it reaches a concentration of 0.20 mg/ml. It is possible to draw the conclusion from these findings that plasma treatment not only increased the extraction efficiency of C-PC but also raised the possibility that C-PC would degrade even if it was exposed to plasma treatment for a period of time that was longer than five minutes.

Purity of C-PC is performed on all samples as shown in Figure 3.3(B) that was generated for the purity of the C-PC at each of the time points was measured. The untreated sample with 0 minute has a purity of 0.25 of the C-PC. It reduces the purity after exposure to plasma for 1 minute. At the same time, the purity stabilizes in the range of 0.2 to 0.3 from 2 to 7 minutes after plasma treatment, which indicates that while the initial plasma treatment affects C-PC purity, further exposure does not have a significant effect on its level. This is a decline in purity which happens after the first sharp decrease has been noted to have taken place.

Around 0.25 is the overall purity of C-PC at 7 minutes which is fairly similar with 5 minutes and above time intervals. This means that there is no more deterioration or additional purifying action that occurs after 1 min, the membrane reaches a new steady state.

To determine the impact that plasma treatment has on the amount of protein found in extract of *Spirulina maxima*, Figure 3.3(C) the total protein content of dry weight extracts that were treated with plasma and those that were not treated was measured in mg/ml. A high protein concentration of approximately was seen in the sample that has not been treated at the time of

0 minutes. After 1 minute of plasma treatment, there was a slight decrease, which indicates that plasma exposure has an immediate impact on the amount of protein present in extract.

Beginning at 2 minutes, the protein concentration does not change, and it continues to fluctuate between 0.14 mg/ml and 0.16 mg/ml during the remaining time points up to 7 minutes. The findings of this study indicate that although plasma treatment has the potential to initially lower protein concentration, extended exposure ranging from 2 to 7 minutes does not result in substantial further protein degradation, hence maintaining the protein concentration at the same level.

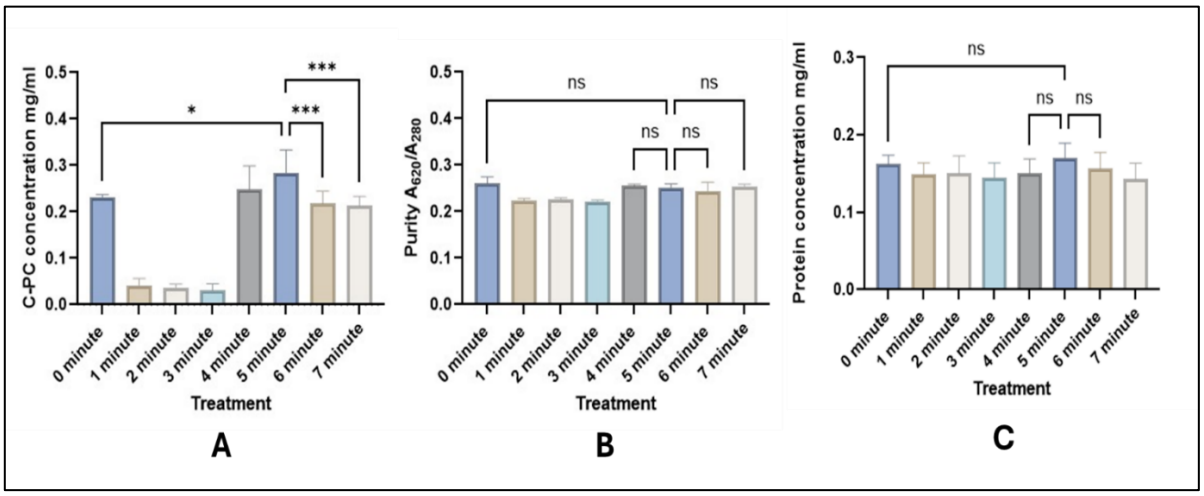

Figure 3.3: (A) Effect of different Time treatments on release of C-phycocyanin (C-PC). C-PC concentration (mg/ml) was measured after exposure to seven different time period of 0 to 7 minutes. (B) Effect of different Time treatments on total content of protein in Spirulina. Protein concentration (mg/ml) was measured after exposure to seven different time of 0 to 7 minutes. (C): Effect of different Time treatments on purity of released C-phycocyanin: purity was measured after exposure to seven different time of 0 to 7 minutes. Error bars represent the standard deviation (mean ± standard deviation, n = 3). "*" = $p<0.05$, "***" = $p<0.001$ and "ns" = non-significant

3.4 Antioxidant activity of the Spirulina maxima after plasma treatment

The antioxidant activity of C-phycocyanin (C-PC) extracted from plasma-treated *Spirulina maxima* was measured (figure 3.4) at different time points: 0 minutes, 5 minutes, 10 minutes. The trend shows a decrease in antioxidant activity with longer treatment durations, expressed in µM TE/mL. At 0 minutes, the C-PC exhibited the highest antioxidant activity, with a value of approximately 20 µM TE/mL. This reflects the maximum potential of C-PC to neutralize free radicals before the plasma treatment. At 5 minutes, non-significant antioxidant activity is observed, with a value of 20 µM TE/mL, indicating a moderate antioxidant potential. At 10 minutes, the antioxidant activity further declines to 15 µM TE/mL, suggesting that extended plasma exposure continues to degrade the antioxidant properties of C-PC.

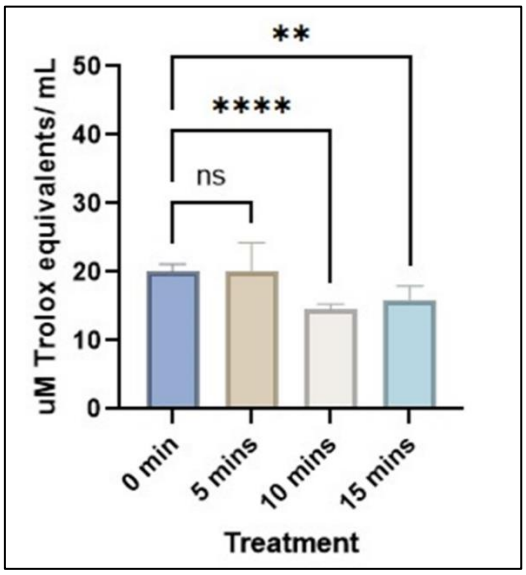

Figure 3.4: Effect of different treatment duration on Radical scavenge activity of the *Spirulina.* Concentration of antioxidants measured in µM Trolox equivalents/mL at different time durations like 0, 5, and 10 minutes. error bars present (mean ± standard deviation, n = 3). ns = not significant

3.5 Cytotoxicity study of plasma treated Spirulina maxima extract

The MTT assay was used to determine the effect of extract of plasma treated *Spirulina maxima* for five minutes had on the viability of the HaCaT keratinocyte and THP-1 macrophage cell lines (Figure 3.5). Both treatment concentrations 40% treatment (40 µl of sample in distilled water and 60µl of media) and 60% treatment (60µl of sample in distilled water and 40µl of media) were employed It was observed that the viability of THP-1 macrophage cells was, on an average of 55% when extract was plasma treated and was present in a concentration of 40% and 60% respectively. Based on these observations, the plasma-treated extract of *Spirulina maxima* does not show a significant effectiveness in modulating the viability of THP-1 cells in both concentrations. This was because the cell viability that was attained in both treatments was quite similar.

Similar findings were observed with the HaCaT keratinocyte cells where the viability rate was approximately 100% in both the treatments concentrations for 5-minute plasma treated *Spirulina maxima* extract. Under these conditions, the current results showed that plasma-treated extract of *Spirulina maxima* has a moderately non-toxic impact on these cell lines' viability.

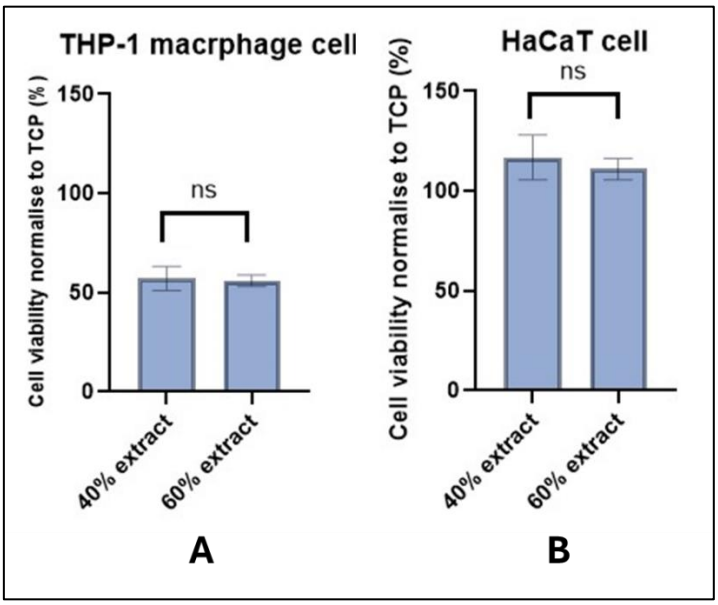

Figure 3.5: Effect of different time treatments on cell viability of *Spirulina* extract. Cell viability % normalized to Tissue culture plate (TCP) was measured after exposure to 5-minute plasma treatment, under two treatments 40% and 60%. (A) cell viability measured using THP-1 macrophage cell line (B) cell viability measured using HaCaT cell line Error bars represent the standard deviation (mean ± standard deviation, n = 3).

3.6 Antibacterial activity of the plasma treated extract of Spirulina maxima

The Figure 3.6 indicates the log reduction in colony-forming units (CFUs) of *Staphylococcus aureus* subjected to plasma-treated extract of *Spirulina maxima* at two intervals: 0 minutes and 5 minutes. The untreated control CFU count served as the baseline for log reduce, hence representing normal bacterial growth without intervention. The results show that there appears to be no difference between the outcomes at 0 minutes and 5 minutes plasma treatment in terms of log CFU counts because of both conditions eliciting equally similar decrease to the control. Therefore, plasma treatment has no impact on the antibacterial property of *Spirulina* extract because the counts of the colony forming units remained unchanged following plasma exposure. Therefore, the amount of bacterial colony formation obtained after the plasma treatment for 0 min and 5 min do show that the plasma treated extract did not possess a higher antibacterial effect than the untreated extract.

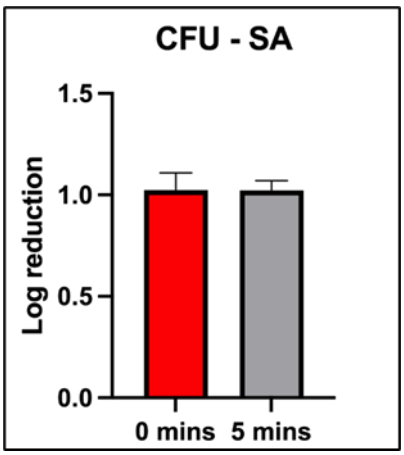

Figure 3.6: Log reduction in colony forming units (CFUs) of *S. aureus* following treatment with plasma-extracted C-PC from *Spirulina maxima* at two time points: 0 minutes and 5 minutes. Error bars represent the standard deviation (SD).

3.7 Morphology study of Spirulina maxima

Fluorescent microscopy was employed to analyze and compare the ultrastructure of *Spirulina maxima* under the effect of atmospheric plasma. The initial morphology of *Spirulina maxima* was around 100µm in length, as was illustrated in Figure 3.7(A) so that the change in morphology after plasma treatment could be examined. In order to cover the whole length of the structure, the multicellular cylindrical trichomes are positioned in the form of an open left-handed helix. For instance, when exposed to atmosphere plasma for 5 minutes over the surface area of 1 cm^2, the *Spirulina maxima* broke into smaller sizes of about 20 µm fractions. A large number of small pits formed on the surface of the microalage as described in Figure 3.7 (B).

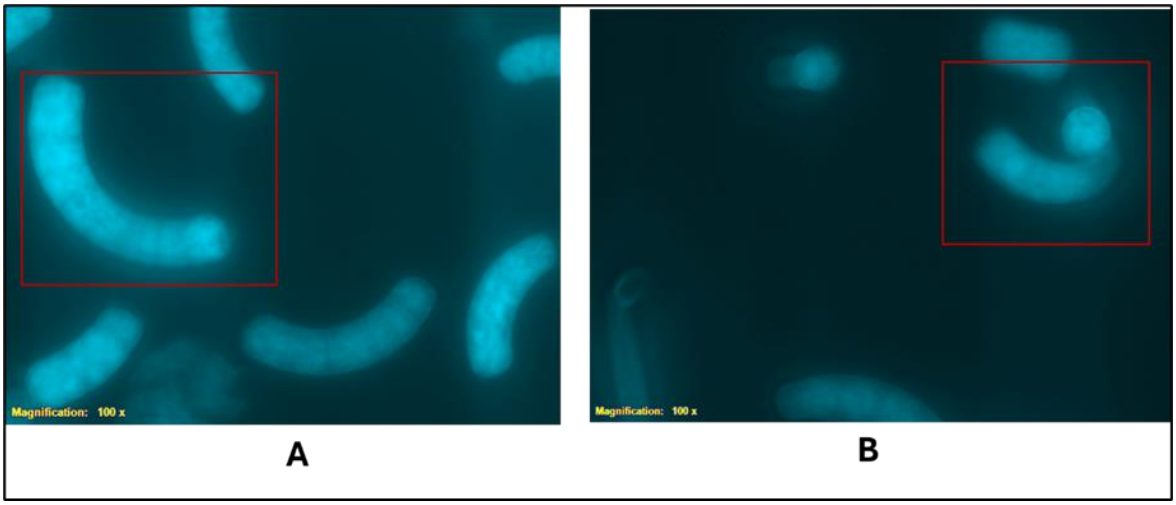

Figure3.7: Morphology of *Spirulina maxima* was Demonstrated by epifluorescence (A) shows the original morphology of *Spirulina maxima*. (B) showed after being treated with atmospheric plasma for 5 minutes, *Spirulina maxima* was fragmented into smaller fractions. Magnification 100X was used in epifluorescence images.

CHAPTER 4 DISCUSSION

4.1 Effect of Different Carrier gas treatment on extract of Spirulina maxima

The results indicate in Figure 3.1 that the extraction of C-Phycocyanin (C-PC) from *Spirulina maxima* exhibited negligible variation across the three distinct plasma gas treatments: The three gases that were used include Nitrogen, Argon and Compressed air. This shows that the kind of gas used in plasma treatment may not significantly influence the level of C-PC released/ ensnared, despite the formation of different reactive species by each gas.

The reactive species generated by each gas used in plasma treatment is unique and may affect the permeabilization of cell membrane and release of intracellular molecules like C-PC. Nitrogen as carrier gas in plasma gives predominantly reactive nitrogen species (RNS) whereas Argon, being an inert gas, forms a lesser number of reactive chemical species and principal control being physical effects of ionization. Compressed air consists of oxygen and nitrogen and consequent generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)(Sklias et al., 2021). However, there is no variation in the quantity of C-PC and Protein content identified for each of the gases used in the plasma treatments; The foregoing therefore suggests that the effectiveness of the plasma in permeabilising *Spirulina* cell membranes was generally similar without a corresponding effect of degradation of or limitations to structural alteration of C-PC. This finding accords with studies suggesting that plasma treatment, regardless of the type of gas, effectively weakens cell membrane barriers to release bioactive compounds such as C-PC(Bekeschus et al., 2022). The data indicate that although the selection of plasma gas affects the types of reactive species generated, its impact on the ultimate yield of bioactive chemicals may not differ greatly under controlled conditions. This suggests that the nature of the gas type in plasma assisted extraction plays only a minor role of the secondary nature, while the extraction efficiency is dominated by the treatment duration and intensity rather than the reactive species produced from the gas.(Miebach et al., 2022)

The findings also demonstrate that C-PC concentrations are not significantly affected by different gas treatment types, which shows how flexible plasma-assisted extraction is for a wide range of biomedical applications because the extraction behavior is consistent across gas types. This implies that rather than the extraction rate, the procedure can be modified based on the gas's needed utility. In addition, the carrier gas exhibited no change, thus demonstrating the need of employing nitrogen as a carrier and the most economical option.

The advantage of the biological use of any of these gas kinds is the ability to so use them without affecting the C-PC yield or quality in actuating delivery systems and treatment of diseases such as cancer and antioxidant treatments. *Spirulina maxima* subjected to plasma may act as a source of bioactive compounds for formulation of drugs or as a therapeutic entity in functional foods with constant doses of C-PC regardless of type of plasma gas used during extraction. This flexibility improves the effectiveness of plasma-assisted extraction in large scale and applicability of the technique in industries and research on human health and diseases.

4.2 Effect of Different Treatment Durations on Extract of Spirulina maxima

As shown in figure 3.2, the significant increase of C-PC concentration after plasma treatment for 5 minutes reflects the fact that plasma treatment for 5min is the most optimal since it provides the right balance in terms of the disruption of the cell wall and the preservation of C-phycocyanin integrity(Puač et al., 2018). Reactive particle species including ROS and RNS are known to effectively permeabilize cell membranes during this time which would enhance the release of intracellular molecules including the C-PC (Yan et al., 2017). This finding concurs with emerging studies on plasma assisted extraction that reveal that brief plasma treatments increase the extraction rate due to the creation of channels in the cell membrane, without negatively impacting the targeted analytes.(Sommer et al., 2021)

It is important to note that the C-PC concentration reduces after 10 minutes. This is maybe because of continuous plasma exposure that causes oxidative destruction. When the reaction time is extended to 10 minutes, the sustained generation of ROS and RNS can cause oxidative damage to sensitive biomolecules thereby lowering the yield of C-PC.(Thirumdas et al., 2015) This means that whereas the plasma is capable of penetrating cell walls, there is a certain duration beyond which further exposure has a negative impact on the stability of the bioactive compound(Misra et al., 2016).

Like C-PC, total protein reaches the highest concentration at the 5-min time point, which suggests that this time period is optimal not only for the release of C-PC, but also for the extraction of protein as a whole. During plasma treatment, the cell membrane of *Spirulina* gets damaged and proteins leaching out into the media containing the cell. This decrease in protein concentration at 10 and 15 minutes is in agreement with the theory that extended exposure leads to protein oxidation and subsequent breaking down of proteins and therefore a general decrease in total protein concentration.(Takai et al., 2012)

Previous research has also revealed similar discoveries, precisely, plasma-assisted extraction for short durations enabled the efficient liberation of proteins, but the treatment made for long periods causes oxidative stress and, thus, the breakdown of the proteins(Thirumdas et al., 2015) This goes to support how critical it is to only allow a certain amount of time for plasma to be treated to make high yields while at the same time ensuring that the extracted compounds are of the best quality.

The purity of C-PC has been characterized, and it was seen to change over time. It reached its highest level after 5 minutes of plasma treatment and then dropped. This means that plasma can be used to easily collect both C-PC and proteins within 5 minutes. However, the longer the proteins stay in plasma, the more damaged proteins and other contaminants they pick up, making the C-PC less pure(Oh et al., 2017). Because of the need to get very pure C-PC, it can be used in many areas of biological technology, for example as an anti-inflammatory, an antioxidant, or even a possible cancer drug. (Romay et al., 2003)

Isolating very pure C-PC is very important for uses in the biological fields. In cancer therapy, C-PC has been shown to cause apoptosis in cancerous cells. The type of compound that is removed determines how well the treatment works. This study shows that plasma exposure for 5 minutes increases the yields and purity of extracts to their highest level. This means that it can be used in situations that need a lot of high-quality bioactive chemicals. Additionally, because C-PC can work as an antioxidant by getting rid of free radicals, it is important to keep its integrity in order to successfully fight the effects of oxidative stress in antioxidant therapies.

4.3 Further Investigation into the Effect of Treatment Duration of Spirulina maxima

It is demonstrated from the figure 3.3, that the ideal time for effective extraction occurs 4 to 5 minutes after the plasma treatment, when the C-PC concentration reaches its highest point. One possible explanation from these results is that besides the standard mechanisms attributed to reactive oxygen species and reactive nitrogen species (Pham et al., 2023), modification in the surface morphology of the cell wall induced by plasma may represent an additional source for the enhancing effect on *Spirulina maxima*. The plasma treatment can potentially alter the physical cellular matrix; therefore, this might allow a release of C-PC in a different controlled manner(Beyrer et al., 2020).Due to its nature, this structural change might result in the increase of a stiff or cross-linked cell wall matrix that could act as an initial C-PC trap and prevent from

further dislocation upon long-term exposition. There may be a reason why shorter periods e.g., 1–3 min fails to fully permeabilize cells.

It is noted that the C-PC concentration has an unusual inverse behavior where higher levels are detected in the control samples at 0 minutes and the lowest at 1–3 minutes, and then it rises again at 4 minutes. These tendencies, most likely, are due to the initial state of the powdered *Spirulina*, and the influence of plasma treatment on the state of the cells(Chaiklahan et al., 2012).

The high C-PC concentration at 0 minutes, specific for the untreated sample, is due to damaged cells in the dried *Spirulina* powder. Cells probably ruptured during preservation, discharging their content within C-PC as well as in the matrix of the powder. That is to mean in the untreated sample, there is a measurable amount of extractable C-PC.(Seo et al., 2013)

The concentration of C-PC decreases right after plasma treatment for1 to 3 minutes. The lower C-PC levels might be due to the degradation of the lysed cells' initially released C-PC and additional C-PC formed due to the plasma exposure. At these initial phases of treatment, exposed C-PC molecules may be degraded by reactive species generated by plasma(Guo et al., 2015).

This explanation emphasizes how plasma treatment has two functions: It can also penetrate cell walls to release bioactive substances and if exposed the pressure can degrade sensitive components. As control from the experiment show that the optimized time to harvest the C-PC from spirulina with least deterioration should be between 4 to 6 minutes(Surowsky et al., 2015).

Most of the plasma treatment periods keep the protein concentration stable while a slight increase can be recognised at 5 minutes. This uniformity suggests that plasma therapy could permeabilize the cell membrane efficiently without causing massive protein degradation (Li et al., 2024). This demonstrates the robustness of proteins in comparison to substances that are more sensitive, such as C-PC (Zhu et al., 2019). Plasma may have a different effect on pigments than it does on cell wall proteins. The selective disruption of pigments may explain why proteins are consistently released, even at shorter intervals, but the release of C-PC is more dependent on the amount of time that they are exposed to plasma.

In addition, the relatively steady protein content over a variety of time points may be a reflection of the non-specific character of plasma extraction for protein molecules. Protein

molecules are often less sensitive to oxidative stress in comparison to pigments such as C-PC. This shows the potential of plasma as a versatile extraction method that may be employed for multi-functional applications, such as the co-extraction of C-PC and proteins for synergistic therapeutic effects in functional foods or nutraceuticals.(Seyedalangi et al., 2024)

As for the therapeutic and cosmeceutical products which demand high purity extracts for better results, it becomes crucial to keep up with the purity of C-PC for further use. Based on the analysis of the results, it could be seen that plasma treatment is capable of preserving the quality of C- PC and at the same time, not compromise the degree of purity of the material as the purity remained constant even through treatment beyond the 5 minutes period although the increase in treatment time from 5 to 7 minutes did not show much difference(Dranseikienė et al., 2022).

Since the purity levels of Plasma extracted C-PC are relatively stable, the drug can be incorporated into long term therapy in the treatment of chronic ailments. This is particularly helpful where it is necessary to sustain bioactivity with a view of providing positive outcomes for patients. For such diseases as neurodegenerative diseases and autoimmune disorders where oxidative stress is the main culprit, then due to C-PC's remarkable ability to reduce oxidative stress in the long run, it becomes an even better treatment option.(Jiang et al., 2017)

4.4 Antioxidant activity of the plasma treated Spirulina maxima

The reduction in antioxidant activity that has been observed over time is attributed to the progressive oxidation of C-PC that occurs when the Spirulina is exposed to plasma for long. (Li et al., 2023) It is established that plasma introduces structural phase transformation in the cell matrix of *Spirulina maxima* and proportionality with respect to the release dynamics of C-PC by plasma. When plasma permeabilizing cell membrane it has effects on the cellular matrix that is surrounding the cell. These alterations may contribute to the possibility of the increased susceptibility of C-PC to undergo oxidative stress during the course of long-term treatments. This decline in antioxidant capacity is attributable to both matrix reorganization and accumulation of reactive species.(Dong & Wang, 2023). This shows why it is important to fine tune the plasma parameters to reduce degradation whilst at the same time enhancing extraction for bio-medical use.

4.5 Cytotoxicity study of plasma treated Spirulina maxima

The results of cell viability of the plasma-treated extract of *Spirulina maxima* exhibited some of toxic effects toward THP-1 macrophage and no toxic effect towards HaCaT human keratinocyte cells. At concentrations of 40% and 60%, both cell lines had viabilities of approximately 55% and 100%, showing that plasma treatment may alter the characteristics of *Spirulina maxima* extract, including biocompatibility and non-toxicity. Following the UNI EN ISO 10993-5 principles of cell toxicity where cell viability above 70% is grouped as non-cytotoxic, the results suggests that there's a slight reduction in cell viability of THP-1 macrophage, but in other hand it does not represent a toxic effect against the HaCaT cell line, the findings endorse the application of the extract in specific contexts; nevertheless, additional research on biocompatibility is necessary to more accurately evaluate safety(Leslie et al., 2017).

The cytotoxicity impact of the extract was investigated on THP-1 cells and the results showed a significant decrease in cell viability to about 55% when compared to TCP or a reduction of about 45% when compared with untreated cells. This suggests that the THP-1 *Spirulina* extract treated using plasma may possess toxicity to THP-1 cells as it is. One possible reason could be the presence of bioactive metabolites in the extract like C-phycocyanin which may have toxic effects at these concentrations. One possible reason may be that nutrient dilution that occurs due to higher concentration of distilled water in the extract reduces nutrient solubility in growth media(Álvarez-Gómez et al., 2019). Similarly, under comparable parameters, HaCaT cells remained almost 100% viable eliminating changes in osmotic pressure as a contributing factor. The results thus prove that the extract induces toxic effects only within THP-1 cells and it was revealed that further tuning with THP-1 immune cell lines is required.(Bechelli et al., 2011)

The results help support prior studies showing that *Spirulina maxima* has limited toxic effects on human cells and its active compound is helpful in giving antioxidant and anti-inflammatory effects.(Dranseikienė et al., 2022) The plasma treatment has advanced in the extraction process without compromising safety aspects, referring plasma treated extract of *Spirulina* for anti-inflammatory therapy, drug carriers, and nutraceuticals where biocompatibility is crucial for application of therapies (Pham et al., 2023). Regarding normal human cell lines, the uniform cell vitality observed HaCaT cell lines points clearly towards the fact that plasma treated extract

of *Spirulina* has numerous applications in human health care right from skin treatments to anti-chronic inflammation. (Fernandes et al., 2023)

4.6 Antibacterial activity of the plasma treated Spirulina maxima

The Figure 3.6 illustrates the logarithmic reduction in colony-forming units (CFUs) of Staphylococcus aureus after exposure to extracts of *Spirulina maxima*, both with and without plasma treatment, at two-time intervals: 0 min (non-plasma group) and 5 min (plasma group). The samples of the no-plasma group and 5-minute plasma-treated group has approximately one log reduction in the number of CFU as compared to the untreated S. aureus culture control, indicating that both extracts are equally effective in killing bacteria.

The log reduction obtained from the present investigation shows that the bioactive compounds in S. maxima, namely C-PC may possess bactericidal or bacteriostatic effects against S. aureus(Venugopal et al., 2020). This is in concordance with previous findings implicating that C-PC exhibits antibacterial properties. This conclusion is supported by the results presented in Figure 3.3, particularly the concentration measurements of C-PC, which are nearly the same for the no-plasma and the 5-minute plasma-treated samples. Since C-PC is known to possess antibacterial properties the observed reduction in the colony forming unit means that the compound could be playing a major role in the reduction observed in both treatment groups.

The antibacterial activity resulted from the no-plasma group was because of the untreated Spirulina powder containing C-PC. During the process of drying, some of the Spirulina cells may have rupture and thus, releasing the cellular components; C-PC inclusive, which was observed in the powder. Thus, the extract from the no-plasma group was enriched with bioactive compounds that prevent bacterial growth and confirms the antibacterial effect of the extract(Chaiklahan et al., 2012).

The observed about 1-log reduction in CFU in both the no-plasma and 5-minute plasma-treated groups indicates that plasma treatment did not enhance antibacterial property of the Spirulina extract to significant level under these conditions. The antibacterial action may be attributed to the inherent bioactive compounds in S. maxima especially C-PC present in both extracts(Safari et al., 2020). Future studies may also explore the various parameters of plasma treatment or enhance the levels of bioactive components present in Spirulina to establish if such changes enhance the antibacterial efficiency of the extracts.

4.7 Morphology study of Spirulina maxima

The Figure 4.7 images suggests that cell wall degradation of *Spirulina maxima* might be achieved through atmospheric plasma treatment, which results in more cell disruption when the treatment time is increased. This correlates well with earlier studies that pointed out that the exposure of bacteria to atmospheric plasma affects negatively their cell wall and particularly the peptidoglycan layer to the degree that depends on the treatment time(Yusupov et al., 2013)This must be through the formation of reactive species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are known to react with cell wall constituents.

The results of the present study indicate that plasma treatment can alter regularity of packing and structure of *Spirulina maxima* cell wall due to breaking of intermolecular forces. The high-energy species from the plasma seem to catalyse chemical reactions that lead to changes in structural conformation, which may alter the content and organization of cell wall components. This disruption may allow for the release of intracellular compounds and could explain why cell disruption is higher with plasma exposure beyond half an hour (Weltmann et al., 2010)

Further, plasma-induced reactive species are expected to affect surface charge, hydrophobicity and permeability of the cell wall. These modifications may additionally also help in the destabilisation of the cell wall. Additional investigations and characterization employing sophisticated instrumental methods are required to explain the structural modifications in detail and to describe the features of the new matrix created after plasma treatment (Cheng et al., 2020)

Table 4.1 Summary of key findings from results.

Aspect of studies	Key findings
Effect of carrier gas treatment on C-PC Concentration, Protein Concentration and Purity of extract of *Spirulina maxima*	No significant difference across Nitrogen, Argon, and Compressed Air treatments on extract.
Effect of plasma time treatment (including extended treatment time) on C-PC Concentration Protein Concentration and Purity of extract of *Spirulina maxima*	For C-PC concentration Significant increase at 5 minutes, decreases after 6,7, 10 and 15 minutes Protein concentration Significant increase at 5 minutes, decreases after 10 and 15 minutes and no significant different between 6 and 7 minutes. Purity highest at 5 minutes, decreases with longer treatments.
Antioxidant Activity of plasma treated extract	No significant change in antioxidant activity across treatments of 0 minute and 5 minute and significant decrease at 10 and 15 minute.
THP-1 Macrophage Cell Viability of plasma treated extract	Reduced cell viability in response to plasma-treated extracts
HaCaT Keratinocyte Cell Viability of plasma treated extract	No significant reduction in cell viability, biocompatible
Bacterial Inhibition (*S. aureus*)	No significant difference between the 0 minute and 5 minute antibacterial activity observed against *Staphylococcus aureus*

Conclusions, Limitations and Future Work

This research has illustrated the efficacy of atmospheric plasma treatment as an innovative, environmentally friendly extraction technique for bioactive compounds from *Spirulina spp*., applicable in pharmaceutical and biological fields. The results indicated that the type of gas (Nitrogen, Argon, and Compressed Air) employed during plasma treatment had no significant impact on extraction efficiency; nevertheless, the time of plasma exposure was critical. A 5-minute plasma treatment was determined to be best, yielding the maximum concentrations of C-PC, protein content, and purity, which are crucial for optimizing the output of bioactive substances. The antioxidant potential of the extracts, quantified by Trolox equivalents, remained statistically insignificant across treatment durations of 0 minutes and 5 minutes, indicating that plasma treatment maintains the antioxidant activity of Spirulina.

However, the study has certain limitations that do not allow having more optimistic results. The concentration on C-PC as the major bioactive compound is a limitation because *Spirulina* contains other bioactive components which include polysaccharides and lipids. This limited focus may have excluded other advantageous chemicals that can perhaps undergo plasma assisted extraction as well. Furthermore, the in vitro cell viability analysis revealed that the viability of THP-1 macrophage cells was reduced following exposure to plasma treated *Spirulina* extracts thereby possibly indicating cytotoxic potentiality. While studying the HaCaT keratinocyte cells, it was observed that they remained viable. As such, how safe PAE *Spirulina* extract is depends on the certain biomedical application and the type of cells the extract is likely to interact with.

Even though *Spirulina* has been known to hold antibacterial properties, the extracts in this study showed antibacterial activity, inferred from the approximate 1-log decrease in CFU of *Staphylococcus aureus* compared to the untreated bacterial culture. This reduction indicates that there are fewer live bacteria in the plasma-treated and no-plasma samples as compared to the control *S. aureus*. However, the present antibacterial effect of PAE was not significantly higher than the extract without plasma treatment. This finding suggests that while the *Spirulina* extract contains active bioactive compounds that should possess antibacterial action such as C-phycocyanin, further studies may need plasma enhancement of these parameters to enhance the microbial activity beyond the observed initial level in the untreated extract. Additionally, while the study established that 5 minutes is the most suitable duration of plasma treatment, other

plasma characteristics such as power density and treatment frequency were not examined. The absence of these characteristics may significantly affect extraction efficiency and bioactivity and thereby limit a proper understanding of how plasma conditions affect the extraction process in the study.

In addition, for the purpose of better description of the plasma-extracted metabolites, it is suggested that other future investigations extend the range of bioactive chemicals to be analyzed from *Spirulina* beyond C-PC. Further studies of the plasma treatment factors such as intensity and frequency of treatment could enhance the extraction process of these complex compounds hence increasing concentrates of diverse bioactive chemicals. Furthermore, next studies should focus on in vivo assays in order to validate the safety and bioactivity of plasma treated *Spirulina extracts* within a live model organism. The observation of moderate toxicity observed in THP-1 cells shows the requirement for additional understanding of such mechanism beginning with applying the Spirulina extracts in the targeted therapy, immune-modulation or anti-cancer healing.

Meanwhile, further studies using other antimicrobial treatments in addition to plasma-treated *Spirulina* extracts may also enhance the observed antibacterial effects because of various synergistic effects. Furthermore, given the plasma treatment as a potential economical and ecologically friendly alternative to conventional solvents used in extraction processes in the future, there is a need to look into the feasibility of the present plasma treatment at industrial level. Alleviating these challenges and improving plasma conditions could pave way to the employment of plasma-treated Spirulina extracts in other therapeutic medications and solutions in the pharmaceutical and biomedical markets as well as functional foods and dietary supplements.

In summary, despite the fact that atmospheric plasma treatment has the potential to be a green method for extracting bioactive compounds from *Spirulina spp*. this study highlights the need to focus future research on overcoming the challenges. Further enlarging the list of analysed compounds, fine-tuning plasmas' characteristics, as well as carrying out more in vivo tests might help to set plasma-assisted extraction as a key technological platform for the generation of active chemicals for various industries and biology.

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APPENDICES

Appendices 1: Total protein content

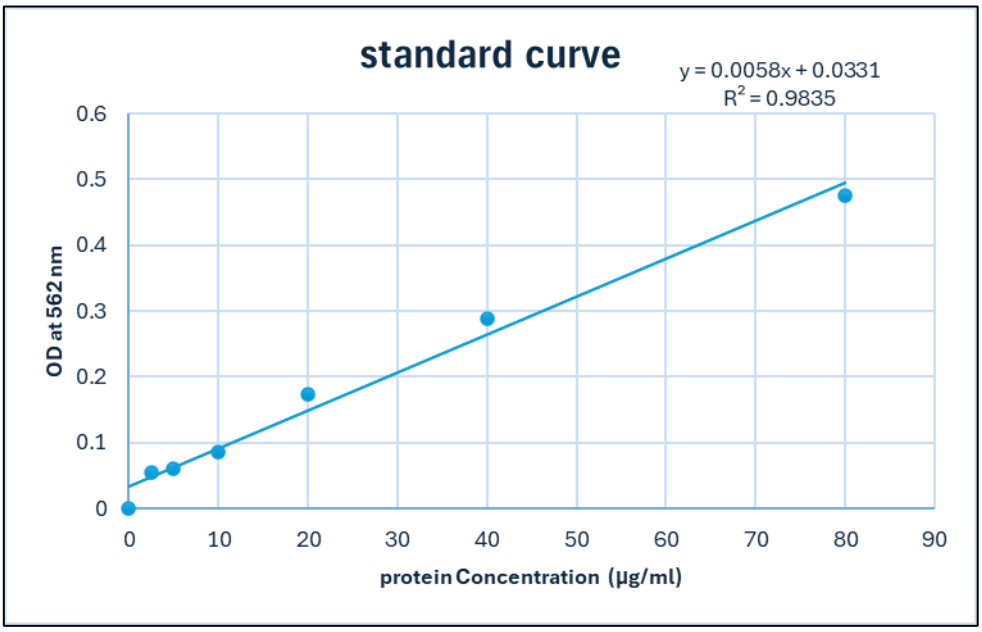

Figure 1: standard curve for total protein content

Appendices 2: antioxidant activity

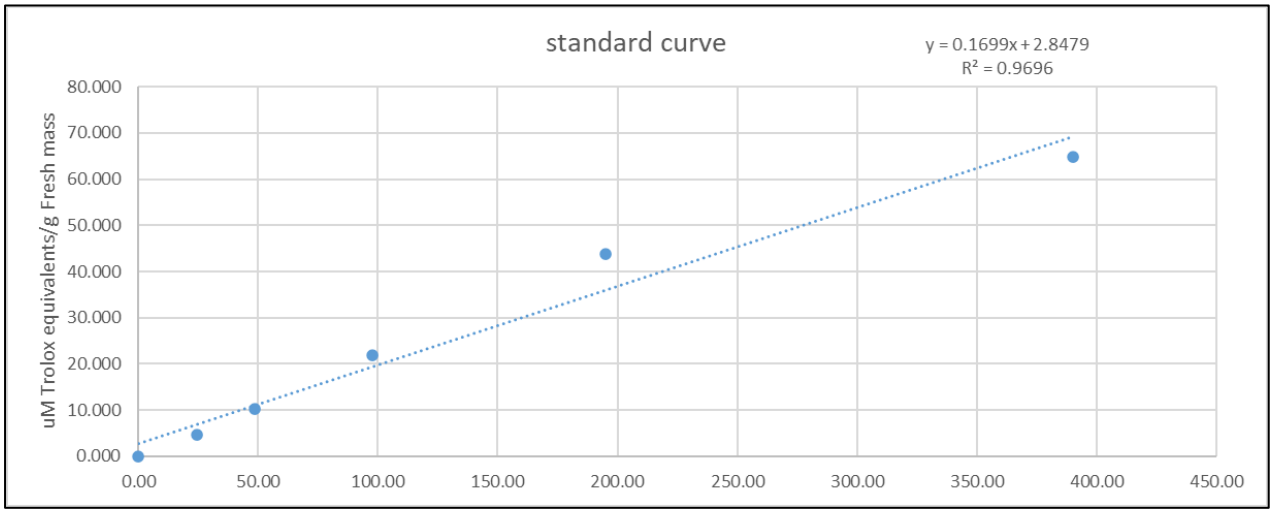

Figure 2: standard curve for antioxidant activity

Appendices 3: Atmospheric plasma treatment to Spirulina with different carrier gas

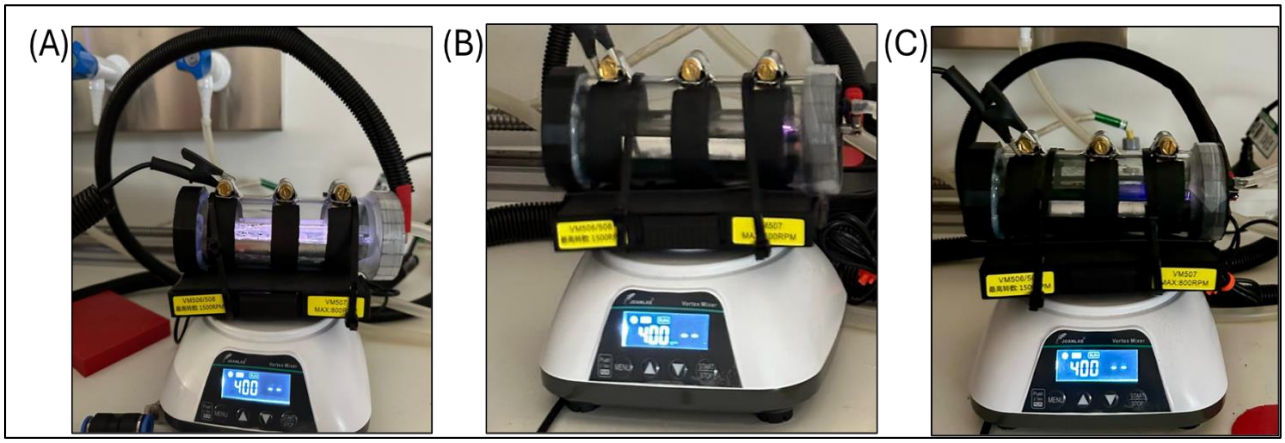

Figure 3: Experimental set-up of Atmospheric plasma treatment for *Spirulina* with different gas

(A) *Spirulina* treated with Argon gas (B) *Spirulina* treated with Compressed Air (C) *Spirulina* treated with Nitrogen Gas

Appendices 4: Colony forming units for antibacterial activity against *S. aureus*

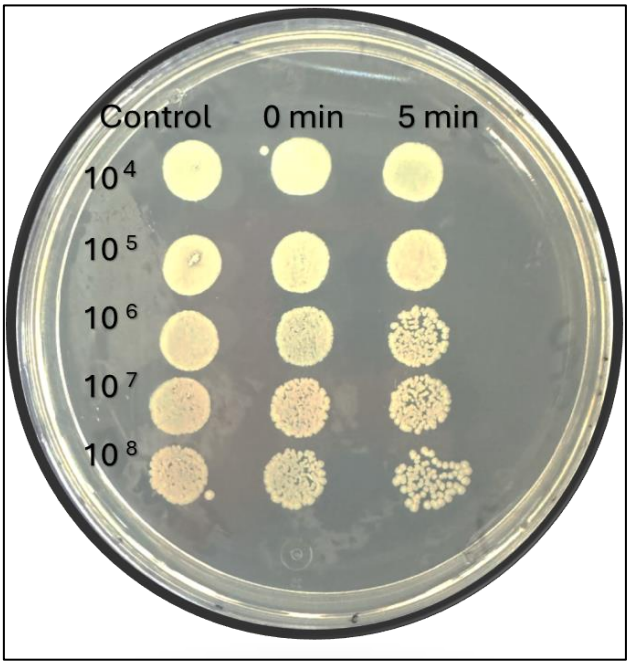

Figure 4: The image presents colony forming units for plasma treated extract of *S.maxima* against *S.aureus*