

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

**OPTIMIZATION OF BATCH BIOREACTOR CONDITIONS FOR
PRODUCTION OF FERMENTED *SPIRULINA* BY MIXED CULTURE
OF *LACTOBACILLUS HELVETICUS* AND *KLUYVEROMYCES*
MARXIANUS AND EFFECT OF BIOREACTOR CONDITIONS ON
SOME BIOACTIVE PROPERTIES**

CANSU YAY
A THESIS SUBMITTED FOR THE DEGREE OF
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**THESIS SUPERVISOR
ASSOC. PROF. DR. MÜGE İŞLETEN HOŞOĞLU**

**GEBZE
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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

***LACTOBACILLUS HELVETICUS* VE
KLUYVEROMYCES MARXIANUS KARIŞIK
KÜLTÜRÜ İLE FERMENTE *SPIRULINA*
ÜRETİMİ İÇİN KESİKLİ BİYOREAKTÖR
KOŞULLARININ OPTİMİZASYONU VE
BİYOREAKTÖR KOŞULLARININ BAZI
BİYOAKTİF ÖZELLİKLER ÜZERİNE
ETKİSİ**

CANSU YAY
YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

DANIŞMANI
DOÇ. DR. MÜGE İŞLETEN HOŞOĞLU

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İMZA/MÜHÜR

SUMMARY

The aim of this study was to find the optimal batch conditions at the bioreactor level for the growth of a mixed culture of *Lactobacillus helveticus* B-4526 and *Kluyveromyces marxianus* Y329 to diversify and/or improve the bioactive properties (antioxidant activity, antimicrobial activity, ACE inhibitory activity, total phenolic content) of *Spirulina platensis* biomass and to investigate the effect of ultrafiltration and *in vitro* digestion on the promising fermented *Spirulina* (FS) product. The operating conditions studied were aeration rate (0.5 vvm FS and 1 vvm FS), dissolved oxygen level (cascade FS) and pH (controlled pH FS). It was observed that all bioreactor conditions supported the growth of microorganisms. The FAA content was higher in all production conditions compared to unFS. The essential amino acid content also increased in all fermentations, with the cascade FS having the highest essential amino acid content. Although FS products did not show antimicrobial activity, they have enhanced bioactivities in terms of other activities (TEAC, ACE-I, TPC), and cascade FS has the most remarkable bioactive properties. Cascade FS also had different groups of VOCs. Sensory analysis revealed that FS products had less "seaweed", "muddy/earthy" and "grass" flavors compared to unFS. Changes in bioactivities during *in vitro* digestion showed that FS had largely conserved its activity at the end of gastrointestinal digestion compared to unFS. FS fractions of 5-10 kDa and <5 kDa were effective in DPPH scavenging activity and TEAC, respectively. Overall, this study demonstrated the importance of optimizing bioreactor conditions to improve the properties of *Spirulina*.

Key Words: *Spirulina platensis*, Fermentation, Bioactive properties, Bioreactor, *Lactobacillus helveticus*, *Kluyveromyces marxianus*

ÖZET

Bu çalışmanın amacı, *Spirulina platensis* biyokütlesinin biyoaktif özelliklerini (antioksidan aktivite, antimikrobiyal aktivite, ACE inhibitör aktivitesi, toplam fenolik içerik) çeşitlendirmek ve/veya geliştirmek için *Lactobacillus helveticus* B-4526 ve *Kluyveromyces marxianus* Y329 karışık kültürünün büyümesi için biyoreaktör seviyesinde optimum kesikli çalışma koşullarını bulmak ve ultrafiltrasyon ve *in vitro* sindirimin umut verici fermente *Spirulina* (FS) ürünü üzerindeki etkisini araştırmaktır. İncelenen koşullar havalandırma hızı (0,5 vvm FS ve 1 vvm FS), çözünmüş oksijen seviyesi (kaskat FS) ve pH (kontrollü pH FS) olmuştur. Tüm biyoreaktör koşullarının mikroorganizmaların büyümesini desteklediği gözlemlenmiştir. FAA içeriği tüm üretim koşullarında unFS'ye kıyasla daha yüksektir. Esansiyel amino asit içeriği de tüm fermentasyonlarda artmış ve kaskat FS en yüksek esansiyel amino asit içeriğine sahip olmuştur. FS ürünler herhangi bir antimikrobiyal aktivite göstermemesine rağmen diğer aktiviteler bakımından gelişmiş biyoaktivitelere (TEAC, ACE-I, TPC) sahiptir ve kaskat FS en dikkat çekici biyoaktif özelliklere sahiptir. Kaskat FS ayrıca farklı VOC gruplarına sahiptir. Duyusal analiz, FS ürünlerinin unFS'ye kıyasla daha az "deniz yosunu" çamurlu/topraklı" ve "çimen" tatlarına sahip olduğunu ortaya koymuştur. *In vitro* sindirim sırasında biyoaktivitelereki değişiklikler, FS'nin gastrointestinal sindirimin sonunda unFS'ye kıyasla aktivitesini büyük ölçüde koruduğunu göstermiştir. FS 5-10 kDa ve <5 kDa fraksiyonları sırasıyla DPPH süpürme aktivitesinde ve TEAC'da etkili olmuştur. Genel olarak, bu çalışma *Spirulina*'nın özelliklerini iyileştirmek için biyoreaktör koşullarının optimizasyonunun önemini göstermiştir.

Anahtar Kelimeler: *Spirulina platensis*, Fermantasyon, Biyoaktif özellikler, Biyoreaktör, *Lactobacillus helveticus*, *Kluyveromyces marxianus*

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Celsius
%	: Percentage
α	: Alpha
β	: Beta
eV	: Electron volt
γ	: Gamma
μg	: Microgram
μL	: Microliter
μm	: Micrometer
μM	: Micromolar
ω -3	: Omega-3
g	: Gram
h	: Hour
kg	: Kilogram
L	: Liter
log	: Logarithm
m	: Meter
M	: Molar
mg	: Milligram
min	: Minute
mL	: Milliliter
mm	: Millimeter
mM	: Millimolar
N	: Normality
nm	: Nanometer
p	: p value
rpm	: Revolutions per minute
v	: Volume

vvm	:	volume of air sparged per unit volume of growth medium per minute
w	:	Weight
AA	:	Amino acid
AAE	:	Ascorbic acid equivalent
ABTS	:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE	:	Angiotensin I Converting Enzyme
ACE-I	:	Angiotensin I Converting Enzyme Inhibition
ALA	:	α -linolenic acid
ANOVA	:	Analysis of Variance
ATP	:	Adenosine triphosphate
BSA	:	Bovine serum albumin
Cd	:	Cadmium
CEPs	:	Cell-envelope proteinases
CFU	:	Colony-forming units
CO ₂	:	Carbon dioxide
C-PC	:	C-phycoerythrin
CVD	:	Cardiovascular diseases
DHA	:	Docosahexaenoic acid
DMB	:	Dried matter basis
DO ₂	:	Dissolved oxygen
DOH	:	Degree of hydrolysis
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
DW	:	Distilled water
EAA	:	Essential amino acid
EC ₅₀	:	Effector concentration to inhibit 50% of radical
e.g.	:	For example
EPA	:	Eicosapentaenoic acid
EPS	:	Extracellular polysaccharides
Eq.	:	Equation
et. al.	:	And others
<i>etc.</i>	:	Other similar things
EU	:	Europe Union

FAA	:	Free amino acid
FAO	:	Food and Agriculture Organization of the United Nations
FEAA	:	Free essential amino acid
FMOC	:	Fluorenylmethyloxycarbonyl chloride
FRAP	:	Ferric reducing antioxidant power
FS	:	Fermented <i>Spirulina</i>
FSR	:	Fermented soybean residue
GAE	:	Gallic acid equivalent
GC–MS	:	Gas chromatography-mass spectrometry
GCO	:	Gas Chromatography-Olfactometry
GLA	:	Gamma - linoleic acid
GRAS	:	Generally recognized as safe
HA	:	Hippuric acid
HHL	:	Hippuryl-His-Leu
HPLC	:	High-performance liquid chromatography
HSC	:	Hepatic stellate cells
HS-SPME	:	Headspace solid phase microextraction
IS	:	Internal standard
IVD	:	<i>In vitro</i> digestion
kDa	:	Kilo Daltons
LAB	:	Lactic acid bacteria
LC-PUFA	:	Long Chain Polyunsaturated Fatty Acids
MRS	:	de Man, Rogosa and Sharpe
MSD	:	Mass selective detector
MW	:	Molecular weight
O ₂	:	Oxygen
OPA	:	o-phthaldialdehyde
ORAC	:	Oxygen radical absorbance capacity
PBPs	:	Phycobiliproteins
PBR	:	Photobioreactor
PBS	:	Phosphate buffered saline
PCA	:	Principal component analysis
PUFAs	:	Polyunsaturated fatty acids

PTFE	:	Polytetrafluoroethylene
QPS	:	Qualified Presumption of Safety
SCP	:	Single-cell protein
SD	:	Standard deviation
SDS-PAGE	:	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE	:	Standard error
SMF	:	Submerged fermentation
sp.	:	Species
SPME	:	Solid phase microextraction
SSF	:	Solid-state fermentation
TAA	:	Total amino acid
TEAA	:	Total essential amino acid
TEAC	:	Trolox Equivalent Antioxidant Capacity
TEMED	:	N, N, N', N'-tetramethyl ethylene diamine
TFAA	:	Total free amino acid
TMP	:	Tetramethyl pyrazine
TPC	:	Total phenolic content
TSPC	:	Total Soluble Protein Concentration
unFS	:	Unfermented <i>Spirulina</i>
VOCs	:	Volatile Organic Compounds
YM	:	Yeast Mold

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1. INTRODUCTION

As we are exceeding the planetary boundaries, it is necessary to consider sustainability in every step we take. In this case, food is an important area where we should create sustainable systems that are sufficient to feed the entire population as well as of good-quality that meets dietary needs and provide health benefits. This has led researchers to investigate alternative protein sources over traditional sources that have a high environmental impact. Edible insects, microalgae, plant proteins, single-cell proteins, lab-grown meat and the use of agro-industrial by-products are among the alternative protein sources that have been investigated. Each of these sources has some advantages and drawbacks due to the current state of technology [Liu et al., 2022].

Microalgae are attracting various industries due to their diverse potential and the food is one of these industries trying utilize the nutritious and bioactive rich content of microalgae in the new food formulations [C. Chen et al., 2022]. *Spirulina platensis* is a microalga with a very long history of consumption. It has nutritious content in terms of proteins, essential amino acids, phycobiliproteins [Habib et al., 2008], [Lupatini et al., 2017]. Despite the fact these good nutritional characteristics, some properties such as odor, color, taste and lack of knowledge about the effect of digestion on the bioactive compounds hinder its use [Hosseinkhani et al., 2022]. Fermentation is a cost-effective technique that can help to overcome these obstacles by changing the sensory characteristics, enhancing bioactive properties and paving the way for microalgae-based food products. Fermentation of *Spirulina* has been investigated by many researchers and it has been shown that *Spirulina* is a suitable medium for a variety of lactic acid bacteria (LAB), *Bacillus* species and yeast species. The selected microorganism and fermentation conditions (e.g. preparation of *Spirulina* medium, fermentation time and temperature) can greatly affect the bioactive properties and the taste and flavor of the final product [Bao et al., 2018], [de Marco Castro et al., 2019], [Jamnik et al., 2022], [Kurt et al., 2023], [Niccolai et al., 2019], [Özyurt et al., 2023], [Sahin et al., 2022], [Tolpeznikaite et al., 2023], [Verni et al., 2021].

1.1. Aim and Contribution of Thesis

In our previous studies, the optimization of the fermentation of *Spirulina* by mixed culture of *Lactobacillus helveticus* and *Kluyveromyces marxianus* was completed in terms of the *Spirulina* concentration and carbon source, fermentation time fermentation medium at the flask level (unpublished data). In this study, the aim was to optimize the co-fermentation of these two microorganisms by optimizing the environmental conditions (such as mixing speed, dissolved O₂ concentration, aeration rate) at the bioreactor level in a bioactivity guided approach and to investigate the effect of *in vitro* digestion and determining the effective fraction in terms of bioactive properties.

Fermentations were carried out under different operating conditions and investigated in terms of microbial growth, degree of hydrolysis, total soluble protein, change in the SDS-PAGE protein profile, total and free amino acid composition, volatile organic compounds, total phenolic content and three different bioactivities (antioxidant, antimicrobial and ACE inhibitory activity) and descriptive sensory evaluation. The bioactive properties of the fermented *Spirulina* (FS) product were considered in order to determine the optimum operating conditions for the process. To find the effective fractionation, the optimum FS was ultrafiltrated and fractionated to different MW fractions. Finally, the effect of *in vitro* digestion on the bioactivity of FS was evaluated. Thus, to the best of my knowledge, this study is the first to optimize the fermentation of *Spirulina* at the bioreactor level with a mixed culture of a yeast with a lactic acid bacteria (LAB) species. Also, since this study involves different stages such as optimization of bioreactor conditions, evaluation of bioactivity before and after *in vitro* digestion and ultrafiltration of the most promising product, it can guide to develop strategies to produce new fermented *Spirulina* products with enhanced bioactive properties and desired sensory characteristics on a large scale.

2. LITERATURE REVIEW

2.1. The Growing Challenges of Today's World, Particularly in the Field of Food and Alternative Proteins as a Solution

There are, and will continue to be, many challenges facing Earthlings in the near future. Climate change is at the center of these challenges, and the others are interdependent issues such as food, energy, pollution and water that cannot be considered in isolation [Chapman et al., 2022], [Finley and Seiber, 2014], [Liu et al., 2022]. It is estimated that the world's population will exceed 9 billion by 2050 [Web 1]. Thus, the number of people affected by these problems will also increase. In this case, food is one of the key issues to be addressed.

Agriculture and food production processes are significant contributors to greenhouse gas emissions. As demand for food production increases in the near future, water and land use will also increase. If not properly addressed, this will lead to deforestation and animal exploitation will eventually increase, but not enough to meet demand [Finley and Seiber, 2014], [Liu et al., 2022]. The main objective should therefore be to produce good quality food in sufficient quantities, taking into account sustainability [Liu et al., 2022].

Proteins are an important part of a healthy diet. Essential amino acid content, digestibility and bioavailability specifies the nutritional quality of a protein source [WHO/FAO/UNU Expert Consultation, 2007]. The recommended daily intake of protein is 0.8-1.0 g by body weight (kg) per day, and increasing to 1.1 g/kg/d for pregnant women and 1.3 g/kg/d for lactating women and 1.2-1.52 g/kg/d for infants (Institute of Medicine, 2005). Today's diet mainly relies on animal proteins (meat, egg, milk etc.). Animal-based proteins have a high environmental and economic impact, making it a major challenge to provide sufficient protein from animal sources sustainably. This leads researchers to find alternative sustainable protein sources (Figure 2.1). Edible insects, microalgae, plant proteins (legumes, cereals, etc.), single-cell proteins, lab-grown meat and agro-industrial by-products are the main sources focused on to obtain alternative proteins [Liu et al., 2022].

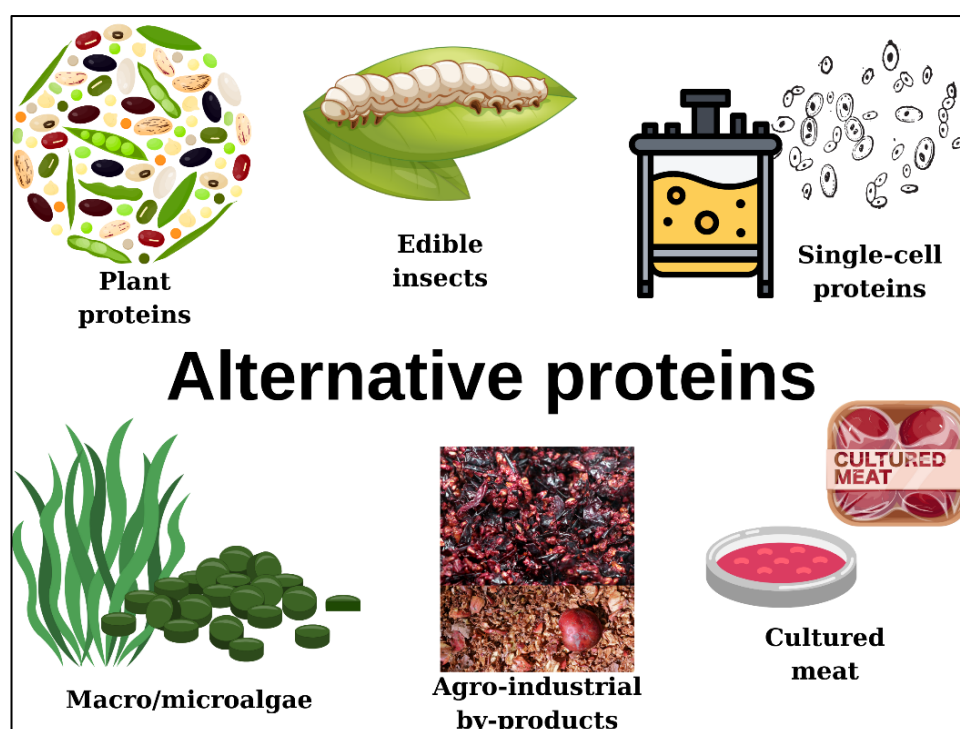


Figure 2.1: Main alternative protein sources (plant, edible insects, single-cell proteins, macro/microalgae, agro-industrial by-products, and lab-grown meat) considered to develop sustainable food systems.

Edible insects are an abundant and sustainable source of protein, requiring less water and land than rearing traditional livestock [Ordoñez-Araque and Egas-Montenegro, 2021]. There are approximately 2000 insects that are suitable for consumption, but this number probably will increase due to the ongoing research in the area. In addition, edible insects have high reproduction rates, high feed conversion efficiency and they are a good source of essential amino acids [Imathiu, 2020], [Ordoñez-Araque and Egas-Montenegro, 2021]. Lipids, minerals and vitamins and chitin are the other nutritional components of the insects (depending on the insect species and insect's diet) [Ordoñez-Araque and Egas-Montenegro, 2021]. Despite the nutritious characteristics of edible insects, safety problems are the main issue hindering consumption. These concerns are related to chemicals (e.g., pesticide residues), biological (e.g., pathogenic microorganisms), allergens and antinutrients and these are affected by the production method, species, harvesting stage and substrate used. To promote the use of insects as whole foods or food ingredients, knowledge of the risks must be well-known. Acceptance of edible insects is another hurdle that needs to be overcome to extend consumption [Imathiu, 2020], [Ordoñez-Araque and Egas-Montenegro, 2021]. Consumer preconceptions can be changed by

finding appropriate processing technique and desirable textures, flavors in foods containing edible insects as ingredients [Mishyna et al., 2020].

Plant proteins are another sustainable and abundant alternative to animal proteins. Legumes, cereals, pseudo-cereals, seeds, leaves and shoots, almond and nuts are some of the sources commonly used for the extraction of plant proteins. Depending on the source, climatic and soil diversity, and agricultural practices some essential amino acids may be deficient in plant proteins. Legumes such as peas, chickpeas, peanuts, soybeans and kidney beans are an important source of plant proteins that rich with protein, carbohydrate, fibers and vitamins [Liu et al., 2022], [Sá et al., 2020]. Except for soybeans, sulfur amino acid (methionine and cysteine) content is limited in most legumes. On the other hand, cereals are often deficient in lysine, thus, combination of legumes with cereals can compensate the deficient amino acids in both source and provide a meal with a more balanced essential amino acid content [Semba et al., 2021]. Although the high amounts of macronutrient and micronutrient content of legumes and cereals, they also contain some anti-nutritional factors such as tannins, saponins, protease inhibitors and amylase inhibitors. These anti-nutritional factors can reduce the bioavailability of and digestibility of the macro- and micronutrients. Several processing methods can be applied to reduce the content of anti-nutritional factors in foods including fermentation, germination, cooking and autoclaving [Samtiya et al., 2020]. For instance, in a study performed by Coda et al. (2015) air classification and fermentation of faba bean flour by *Lactobacillus plantarum* VTT E-133328 decreased the anti-nutritional factors (vicine and convicine) and significantly reduced trypsin inhibitor activity. In the same study, fermentation also increased the amount of free amino acids and improved the *in vitro* protein digestibility. Pseudo-cereals such as amaranth, buckwheat and quinoa are rich in protein and nutrient profile, and they do not contain gluten. People with celiac disease must follow a gluten free diet for a lifetime. Pseudo-cereals are therefore becoming increasingly important in the development of gluten-free products. [Alvarez-Jubete et al., 2010]. Overall, plant proteins have great potential to fulfill the requirements of a nutritious and healthy diet as knowledge of their nutritional profile and their combination in food products increases and as long as the right processing techniques are chosen.

Single-cell proteins (SCPs) refer to dried biomass of protein-rich single celled organisms such as algae, yeast, fungi and bacteria that can be used in human or animal diet. In addition to high protein content (60-82% on dry matter basis), SCPs also have carbohydrate, nucleic acids, lipids and minerals [Sharif et al., 2021]. “Microbial protein” and “petroprotein” were the terms previously used in the field and were replaced by “single cell protein”, which was thought to be more aesthetic than the former two, at a meeting at the Massachusetts Institute of Technology (MIT) in 1968 [Mateles and Tannenbaum, 1968]. The production of SCPs do not require large areas of land, large amounts of water and their production is independent of seasonal changes (depending on the cultivation system algae can be affected) compared to plant protein sources [Sharif et al., 2021]. Due to short doubling times, bacterial biomass increases rapidly. Bacterial proteins are mostly used as feed for the animals in farms or aquaculture. Various substrates such as waste products from industries (potato processing waste, waste of fruit processing and methane broth) can be used to cultivate them [Hadi and Brightwell, 2021], [Sharif et al., 2021]. Fungal proteins, mycoproteins, are another important source of SCPs. Growing interest in alternative proteins resulted in the discovery of a filamentous fungus *Fusarium graminearum* A3/5 at the end of 1960s. The Quorn brand was launched in 1985 to produce mycoprotein from this filamentous fungus to offer alternatives to meat, and later this fungus was reclassified as *Fusarium venenatum*. Although *Fusarium venenatum* is not known to produce mycotoxins, regular monitoring is carried out to guarantee mycotoxins-free end products. In addition, amino acid composition of fungal SCPs meet the Food and Agriculture Organization of the United Nations (FAO)’s recommendations at a great extent but allergenicity of the mycoproteins must be considered as adverse reactions to mycoproteins have been reported previously [Amara and El-Baky, 2023], [Hadi and Brightwell, 2021]. Yeasts such as *Saccharomyces cerevisiae*, *Candida utilis*, *Kluyveromyces marxianus*, *Yarrowia lipolytica* and *Pichia pastoris* have important biotechnological applications. They have high protein content (45-55%), dry weight and ability to grow in various substrates, and as they have been used for fermentation for a long time, they are also consumer acceptable [Bajić et al., 2023]. There are several commercial yeast products such as Vegemite, an Australian spread made from spent brewer's yeast flavored with vegetables and spices, Marmite, a British spread made from beer

brewing, and the German spread VITAM-R, which contains yeast extract and is rich in B vitamins [Amara and El-Baky, 2023].

Lab-grown meat or cultured meat means that animal cells have been produced *in vitro* conditions using stem cells [Liu et al., 2022]. The combination of skeletal muscle tissue engineering, stem cells and tissue culture methods to cultivate meat *in vitro* seems promising to prevent the problems associated with the livestock system such as slaughter of animals, animal diseases, epidemics and misuse antibiotics [Zhang et al., 2020]. Mimicking real meat, being free of allergens and anti-nutritional factors, and having a lower environmental impact are some of the advantages of lab-grown meat [Liu et al., 2022]. On the other hand, there are also challenges, such as selecting a suitable cell source, obtaining a sufficiently large number of homogeneous starter cells, ensuring effective proliferation and differentiation, scaling-up the technology and reducing the cost of media. In addition to these technical challenges, mimicry of conventional meat in terms of its physical sensations (e.g., texture, smell and visual aspect), taste and acceptance are the other obstacles [Post, 2012], [Zhang et al., 2020].

The use of by-products from agriculture and agriculture-related industries, agro-industrial by-products, is a potential strategy to create value-added products while reducing by-products that might otherwise be treated as "waste" or low-value feed. Meals obtained from oil extraction from such as rapeseed and sunflower can be used for protein extraction [Liu et al., 2022]. Depending on the composition of the by-products, they can be used in microbial protein production processes. Rice bran, orange peel, whey, potato starch processing waste and carrot pomaces are some of the by-products utilized in the bioprocesses as substrate for various microorganisms (fungi, bacteria and yeast). The general workflow consists of obtaining residues or by-products and then applying pre-treatment or hydrolysis, which is necessary in most cases, fermenting the by-product with suitable microorganism(s) and then applying the necessary downstream processing to obtain the final product for the food & feed industry [Bajić et al., 2023]. It can be a good strategy to fortify the frequently consumed food products to promote a healthier lifestyle. Bianchi et al. (2021) reviewed the fortification of different types of pasta (noodles, macaroni, spaghetti, fettuccini and etc.) with agro-industrial by-products such as okara, olive pomace, and onion skins. The technological properties (hardness, cohesiveness and

extensibility) of fortified pasta were changed. In addition, the use of by-products affected the nutritional and bioactive compounds, in some cases increasing the total phenolic and flavonoid content or the dietary fiber content.

Algae can be included in the SCP, but as they can contain other valuable food ingredients such as carotenoids, fatty acids, proteins and phenolic compounds they can also be classified as a separate subcategory [Plaza et al., 2009]. Microalgae have several advantages over terrestrial plants, such as high growth rate, rich protein content and ability to grow in non-arable lands and ability to grow in non-potable water [Hosseinkhani et al., 2022]. One of the major bottlenecks in the microalgal food industry is the cost and development of efficient technologies for the production cycle [Y. Chen et al., 2022]. This environmentally friendly food source is discussed in more detail in the following section.

In a study where Smetana et al. (2015) performed an analysis to compare the environmental impact of different alternative proteins (dairy based, lab-grown, insect based, gluten based, soy meal based and mycoprotein based) and chicken as a conventional meat. They concluded that lab-grown meat had the highest environmental impact indicating the recent technology has to be improved for this meat-substitute to be sustainable, followed by mycoproteins and chicken. The study also showed that insect-based and soymeal-based alternatives had the lowest impact. On the other hand, insect proteins require larger area of land compared to mycoprotein based and lab-grown meat. And, since the production of microalgal proteins required more energy, their production causes higher carbon emissions compared to other protein sources [Hadi and Brightwell, 2021]. In conclusion, although all alternative protein sources seem promising, the drawbacks of these sources can be overcome in the near future, depending on advances in the technologies associated with these sources.

2.2. Microalgae, Specifically *Spirulina platensis*, as a Sustainable Source to Develop Functional Food Products and Ingredients

2.2.1. General Properties of Microalgae

The evolution of oxygenic photosynthetic metabolism enabled the rise of atmospheric oxygen and the evolution of complex life forms on Earth [Sánchez-Baracaldo and Cardona, 2020]. Cyanobacteria, also known as blue-green algae, are the first known oxygenic photosynthetic microorganisms and play a crucial role in the oxygenation of the Earth [Zahra et al., 2020]. Algae can occupy different habitats on the earth, as some resemble plants and are attached to a substrate, some are mobile, some live in aquatic environments and some grow on the ground [Richmond and Hu, 2013]. Depending on their size, they can range from micrometers (microalgae) to meters (macroalgae). Based on their pigmentation they can be classified as “green algae” “red algae” and “brown algae”. Chlorophyta (green algae) have several classes commonly found in fresh water. Red algae (Rhodophyta) have phycoerythrin pigment. Such classification is not sufficient to represent the evolutionary course, but it provides practical information for the valorization of the algae from both the producer and the consumer point of view. Blue-green algae (Cyanophyta) are an ancient group of prokaryotic algae. They resemble bacteria biochemically and cytologically, but they have the ability to photosynthesize like eukaryotic algae. Algae have several taxonomic groups: Chlorophyta (green algae), Charophyta, Glaucophyta, Chlorarachniophyta, Euglenozoa, Diatoms, Dinoflagellate, Eustigmatophyceae and Cryptophyta. [Fleurence, 2021], [Richmond and Hu, 2013].

Microalgae are photosynthetic microorganisms that resemble the plant metabolism in their ability to grow and produce biomass using carbon dioxide (CO₂), light and nutrients (e.g., carbon, nitrogen, phosphorus and potassium). In addition to autotrophic growth, some microalgal species can grow heterotrophically using organic carbon sources. They can be also cultivated mixotrophically where heterotrophic and autotrophic growth is used simultaneously [Enamala et al., 2018], [Hosseinkhani et al., 2022].

Microalgae can be grown in open pond systems or in photobioreactor (PBR) systems. Open systems can be natural ponds or artificial raceways. PBRs are closed culture environments where there is better control over growth conditions and the process [Mutale-Joan et al., 2023]. A suitable culture system can be chosen based on the climate of the region, geographical conditions and budget.

Microalgae are a valuable green source with several advantages. Microalgae can grow faster than plants, do not require arable land and can even grow in wastewater. They are environmentally friendly as they use CO₂ and have high rates of photosynthesis. They can also be a solution to the food versus fuel dilemma, where the diversion of arable land or crops for fuel purposes is a concern [Enamala et al., 2018]. Depending on the cultivation strategies, there can be some drawbacks. For example, open ponds are prone to contamination which decreases efficiency, have high evaporation rates, poor light penetration, high downstream costs and they are dependent on weather changes. On the other hand, the ability of PBR to provide tight control brings high construction and operating costs and difficulty in scaling up [Alishah Aratboni et al., 2019], [Mutale-Joan et al., 2023].

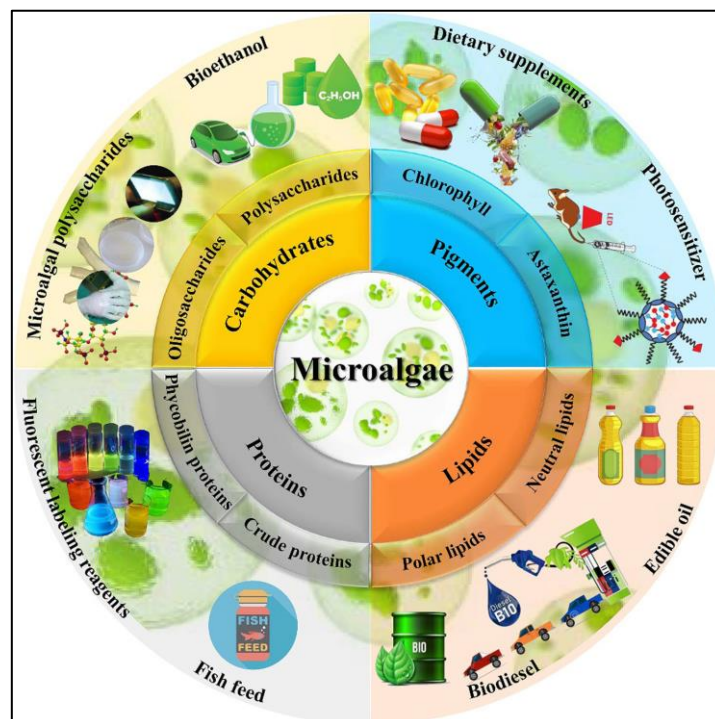


Figure 2.2: Some microalgal compounds and their application areas [Hoang et al., 2022].

Microalgae have several compounds that attract different industries (Figure 2.2). Some of these compounds are polysaccharides to produce bioethanol or biodegradable materials, lipids to produce biodiesel [Hoang et al., 2022]. Microalgae also have several bioactive compounds that make them valuable for human consumption, such as proteins, pigments, bioactive peptides, polyunsaturated fatty acids (PUFAs), vitamins, polyphenols and minerals. Protein is one of the most important compounds found in microalgae, making it comparable to meat or eggs. In the 1950s, microalgae began to be considered as a source of protein for humans. This was followed by the commercialization of *Chlorella* as a single-cell protein in the 1960s and the commercialization of *Spirulina* in the 1970s. Pigment production from *Dunaliella* and *Haematococcus* were the next events that the industry started to commercialize in the 1980s. PUFAs from microalgae were then considered in the 1990s [Hosseinkhani et al., 2022]. Bioactive compounds from microalgae have attracted increasing interest as they have been linked to the prevention or beneficial effects of several health problems, including cardiovascular disease (CVD) (hypertension, oxidative stress), diabetes, inflammation and cancer [Ejike et al., 2017], [Martínez Andrade et al., 2018].

2.2.2. Utilization of Microalgae as a Green and Sustainable Source for Food Industry

Microalgae are a promising sustainable source for the development of novel foods as they contain various bioactive compounds. As there are several microalgae that are generally recognized as safe (GRAS) status, they can be safely used in the diet. *Arthrospira platensis*, *Arthrospira maxima*, *Chlorella protothecoides*, *Haematococcus pluvialis*, *Schizochytrium* sp. and *Ulkenia* sp. are some of the microalgae that have GRAS status [C. Chen et al., 2022].

Microalgae can be incorporated into food formulations in two ways: dried microalgal biomass or specific microalgal extracts. Food formulations containing microalgae can have improved nutritional properties such as phenolic and protein content, carotenoids and fiber. Sensory characteristics such as taste, color, odor and texture can vary depending on the microalgae used and processing conditions. These properties are summarized in Figure 2.3. As whole dried biomass, the incorporation of microalgae has been studied in various foods such as cereals, biscuits, fish

burgers, snacks, beverages (kefir, apple juice, etc.) and dairy products such as yogurt and cheese [C. Chen et al., 2022]. One aspect of the use of microalgae can be the enrichment of commonly used food products such as bread. Graça et al. (2018) investigated the addition of *Chlorella vulgaris* on wheat flour dough rheology and its effect on bread texture. They observed that the addition of 3.0 g of microalgal biomass, between 1.0 and 5 g per 100 g of wheat flour, strengthened the gluten network and improved the rheology and viscoelastic properties of the dough. In another study, Batista et al. (2017) studied the addition of different microalgal biomass, *Arthrospira platensis* F & M-C256, *Chlorella vulgaris* Allma, *Tetraselmis suecica* F & M-M33 and *Phaeodactylum tricornutum* F & M-M40 at low (2% w/w) and high (6% w/w) levels on the functional properties of cookies. They found that cookies prepared with *A. platensis* and *C. vulgaris* had higher protein content than control cookies containing no microalgal biomass. According to the sensory analysis, the cookies containing *A. platensis* were more acceptable. Microalgae containing cookies had higher total phenolic content and antioxidant capacity compared to the control, while in terms of, there were no significant differences in *in vitro* digestibility between microalgae-containing cookies and the control. Beverages can be another application are for microalgae. Ścieszka et al. (2021) fermented soya drink containing *Chlorella vulgaris* with lactic acid bacteria (*Levilactobacillus brevis* LOCK 0944). They found that the survival rate of lactic acid bacteria increased in drinks containing *C. vulgaris* compared to drinks without microalgae during 30 days of storage at 4°C. The storage experiment was followed by the gastrointestinal tract simulator, bacteria showed a good survival rate, which may indicate that the use of *C. vulgaris* and LAB in a soya drink has potential to be a probiotic product.

Although microalgae contain bioactive compounds, these compounds have to go through several steps such as release, absorption, distribution, metabolism and elimination to exert their beneficial effects. And this digestive fate affects the bioefficacy of the compounds [Rein et al., 2013]. Therefore, formulating food with the microalgae extracts can improve bioaccessibility of the compounds and help them to show their effects, as the cell walls of the algae can act as a physical barrier for these compounds [C. Chen et al., 2022]. Bernaerts et al. (2020) investigated the effect of cell disruption of *Nannochloropsis* sp. to evaluate the *in vitro* lipid digestibility and bioaccessibility of omega-3 long chain polyunsaturated fatty acids

(ω 3-LC PUFA) compared to untreated biomass. And they saw that, as a result of high-pressure homogenization, improved lipid digestibility increased bioaccessibility of carotenoids (from 1-6% to 8-16%) and ω 3-LC PUFA (from nearly 13% to 29%) were observed. Hence, cell disruption based on microalgal species can be considered when incorporating them into foods to increase effectiveness of the bioactive compounds. Mofasser Hossain et al. (2017) studied the substitution of whole meal flours (wheat, barley and oat) with astaxanthin and investigated the changes in some bioactive properties (antioxidant capacity and glycemic glucose equivalents) and physical properties (e.g., texture, color). They found that cookies with increasing astaxanthin content (5%, 10%, 15%) had a lower free glucose release compared to the control, which was the corresponding flour's cookie without astaxanthin. Total phenolic content, DPPH radical scavenging and Oxygen Radical Absorbance Capacity (ORAC) value also increased with increasing astaxanthin content. Formulation of the cookies with astaxanthin decreased the lightness and increased the redness of the cookies due to red nature of astaxanthin.

Some microalgal products and compounds are recognized as novel food in the European Union Regulation (EU) 2015/2283 of the European Parliament and of the Council and specified by some food categories and maximum levels [Official Journal of the European Union, 2017]. Authorized as novel food includes algal oil from *Ulkenia* sp. that is specified in various food categories bakery products (breads, rolls and sweet biscuits) (200 mg/100 g maximum levels of DHA), cereal bars (500 mg/100 g maximum levels of DHA) and non-alcoholic beverages (including milk-based beverages) (60 mg/100 ml maximum levels of DHA), astaxanthin-rich oleoresin from *H. pluvialis* (40-80 mg/day of oleoresin, with a maximum of 8 mg astaxanthin/day) and *Schizochytrium* sp. oil rich in DHA and EPA as food supplements for pregnant and lactating women as defined in Directive 2002/46/EC 450 mg/day as maximum levels of DHA and EPA combined and for adult population excluding pregnant and lactating women 3000 mg/day as maximum levels of DHA and EPA combined.

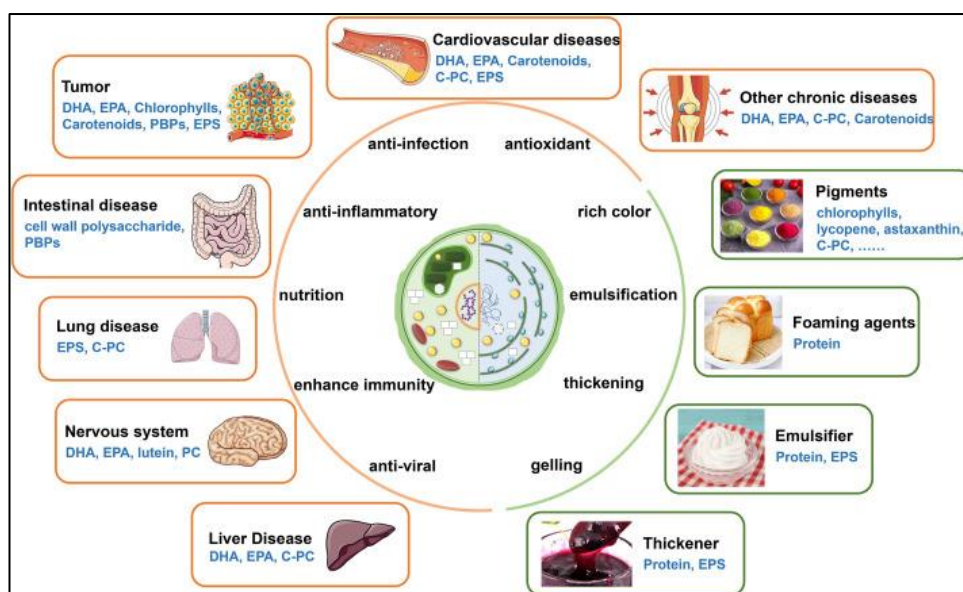


Figure 2.3: Some techno-functional properties and physiological activities of bioactive compounds of microalgae. DHA: Docosahexaenoic acid, EPA: Eicosatetraenoic Acid, EPS: extracellular polysaccharides, PBPs: phycobiliproteins, C-PC: cphycocyanin [C. Chen et al., 2022].

2.2.3. Potential of *Spirulina platensis* in the Development of Functional Foods

Spirulina is a filamentous, multicellular microalga that has a long history of use as a food. During the Spanish conquest of Mexico in the 16th century, the Spaniards discovered that the Aztecs collected 'slime' from lakes and dried it in the sun to make loaves that tasted like cheese [Farrar, 1966]. They named this substance "Techuitlatl". After the end of the 16th century, this name was not mentioned by Spanish chronicles, possibly due to smallpox outbreak and the draining of the lake due for urban activities [Farrar, 1966], [Habib et al., 2008]. Today, Lake Texcoco still harbors the living *Spirulina* population. The tradition of harvesting the biomass from the lake and using it for food continues: the Kanembu people, who live on the shores of Lake Chad, still harvest wet spirulina from the lake, dry it in the sun and sell algae cakes called "dihé" on the market (Figure 2.4) [Habib et al., 2008].



Figure 2.4: People harvesting *Spirulina* from the shores of Lake Chad and drying it. (Photos by Marzio Marzot from the FAO Report the Future is an Ancient Lake, 2004).

Spirulina is a trade name and describe two species: *Spirulina platensis* and *Spirulina maxima* [Lafarga et al., 2020]. *Spirulina* is an obligate photoautotroph that has a helical shape with length of 200-300 μm and 5-10 μm in width (Figure 2.5) and grows in alkaline waters (a pH value of 8.5-11) that rich in minerals and the optimum growth temperature ranges from 35 to 40 $^{\circ}\text{C}$, although under laboratory conditions the optimum temperature range is between 35 to 37 $^{\circ}\text{C}$. Therefore, alkaline, saline water in the tropics favor the growth of *Spirulina* in the lakes of Africa and Mexico [Fais et al., 2022], [Habib et al., 2008], [Lupatini et al., 2017].

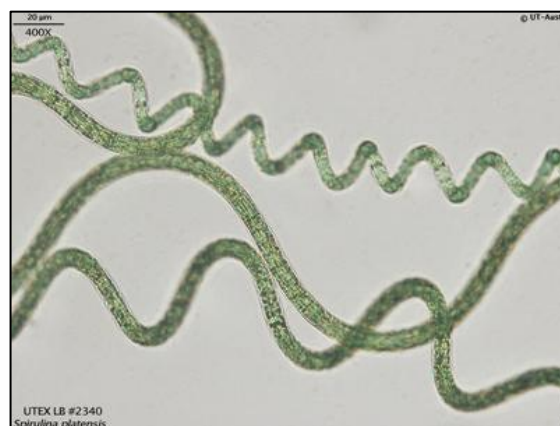


Figure 2.5: Microscopic image of *Spirulina platensis* UTEX LB 2340 (UTEX Culture Collection of Algae at The University of Texas).

The investigation of the nutritional profile of *S. platensis* and due to its high content of macro and micronutrients, it has been considered as a rich food source since the 1970s [Habib et al., 2008]. The composition of some macro- and micronutrients of *Spirulina* is listed in Table 2.1 [Web 2]. However, such compositions needs to taking into consideration carefully as the composition of microalgal biomass is affected by many factors such as cultivation conditions (temperature, light-dark cycle, medium, cultivation system and the light intensity) and cultivation system [Lafarga, 2019], [Lupatini et al., 2017]. One of the most outstanding macronutrients available in *Spirulina* is its high protein content. Its protein content (460-630 g kg⁻¹ dried matter basis (DMB)) is comparable to that of meat (710-760 g kg⁻¹ DMB) and soybeans (around 400 g kg⁻¹ DMB). In addition, it has a balanced amino acid composition and it contains all essential amino acids [Lupatini et al., 2017]. Besides the high protein content, *Spirulina* has phycobiliproteins, light harvesting proteins that play role in photosynthesis, C-phycocyanin and allophycocyanin. Phycocyanin can be used as a healthy natural coloring ingredient for its characteristic blue color and for its health benefits such as anti-inflammatory, antioxidant and anti-cancer properties [Habib et al., 2008], [Lupatini et al., 2017].

Table 2.1: Nutritional profile of dried *Spirulina*.

Composition Macronutrients	Per 100 g	Composition Vitamins	Per 100 g
Calories	290 kcal	Vitamin C	10.1 mg
Water	4.7 g	Vitamin E	5 mg
Lipids	7.7 g	Vitamin K	25.5 µg
Protein	57.5 g	Vitamin B1	2.4 mg
Carbohydrates (by difference)	23.9 g	Vitamin B2	3.7 mg
Ash (g)	6.2 g	Vitamin B3	12.8 mg
		Vitamin B5	3.5 mg
		Vitamin B6	0.4 mg
		Vitamin A	570 IU
Minerals		Amino acids	
Calcium	120 mg	Tryptophan	0.93 g
Iron	28.5 mg	Threonine	2.97 g
Magnesium	195 mg	Isoleucine	3.21 g
Phosphorus	118 mg	Leucine	4.95 g

Table 2.1. continued.

Potassium	1360 mg	Lysine	3.02 g
Sodium	1050 mg	Methionine	1.15 g
Zinc	2 mg	Cysteine	0.66 g
Copper	6.1 mg	Phenylalanine	2.78 g
Manganese	1.9 mg	Tyrosine	2.58 g
Selenium	7.2 µg	Valine	3.51 g
		Arginine	4.15 g
		Histidine	1.08 g
		Alanine	4.52 g
		Aspartic acid	5.79 g
		Glutamic acid	8.39 g
		Glycine	3.10 g
		Proline	2.38 g
		Serine	3.00 g

Bioactive peptides are specific amino acid sequences embedded in the proteins that show their biological activity when the proteins are hydrolyzed. *Spirulina* is also a potential source of bioactive peptides. These peptides can be obtained by disrupting the cells, extracting the proteins and applying a hydrolysis method (e.g., enzymatic hydrolysis), screening for bioactivity and purification. Their health benefits may include antimicrobial, antihypertensive, antitumor and immunomodulatory properties. Thus, bioactive peptides from *Spirulina* can be utilized as functional ingredients in development of nutraceuticals [Ovando et al., 2018].

Spirulina is also important for fatty acids: palmitic acid, oleic acid, linoleic acid, alpha-linoleic acid (ALA) and gamma-linoleic acid (GLA) are the major fatty acids found [Habib et al., 2008], [Mühling et al., 2005], [Tokuşoglu and Unal, 2003]. GLA is an essential polyunsaturated fatty acid and is of interest to the industry as it is associated with various health benefits on body such as selective killing tumor cells, treatment of diabetes and rheumatoid arthritis. Therefore, the extraction of GLA from *Spirulina* has been studied previously [Mendes et al., 2006], [Sajilata et al., 2008].

Enrichment of frequently consumed foods with *Spirulina* is increasing. As consumer acceptance is important, it is important to find the amount that provides the most palatability as well as bioactive properties in such studies. For instance, in a study performed by Barkallah et al. (2017), they investigated the addition of *Spirulina* to yogurt at different concentrations (0.25, 0.5, 0.75 and 1%) and its impact on the textural properties, antioxidant activity and sensory acceptability. They found

that the addition of 0.25% provided overall acceptability as increasing *Spirulina* concentration decreased the color, flavor, taste and texture of *Spirulina* and eventually the overall acceptability. And the addition of 0.25% *Spirulina* improved antioxidant activity in terms of DPPH scavenging activity (%) and reducing power. Pasta is another popular food that can be fortified to promote a healthy lifestyle. Rodríguez De Marco et al. (2014) substituted wheat flour in the bread wheat pasta by *Spirulina* biomass at three levels: 5, 10 and 20 g/100 g. Increasing the *Spirulina* content in the pasta increased the total phenolic compounds and antioxidant activity (in terms of ferric reducing power, FRAP, and Trolox equivalent antioxidant capacity, TEAC methods) and the protein content. However, pasta containing *Spirulina* had lower *in vitro* digestibility compared to control pasta. Cookie, smoothie, broccoli soup, snacks and sausage are another examples of *Spirulina* enriched products [Hosseinkhani et al., 2022]. Due to the potential of *Spirulina*, space agencies, such as The National Aeronautics and Space Administration (NASA), have been considering *Spirulina* as food supplement to ensure the well-being of astronauts on space missions. In conclusion all these properties have made *Spirulina* attractive to the food industry to develop sustainable and healthier foods on Earth and beyond [Fais et al., 2022].

2.2.4. Factors Limiting the Use of Microalgae in Food

There are a number of challenges that need to be overcome in order to expand the commercial use of microalgae in food products. These include sensory acceptance, legislative and regulatory issues, and bioaccessibility and digestibility [Hosseinkhani et al., 2022].

When developing a new food product, sensory properties are one of the most critical factors in determining consumer acceptance. If the dried biomass of microalgae is incorporated directly into food, the characteristic aroma of microalgae and fishy taste of the end product may be unpleasant to consumers [Hosseinkhani et al., 2022]. Isleten Hosoglu (2018) investigated the aroma compounds of five industrially important microalgae species, three marine microalgae species (*Cryptocodinium cohnii*, *Schizochytrium limacinum* and *Tetraselmis chuii*) and two freshwater microalgae species, *Chlorella vulgaris*, *Chlorella protothecoides* using

some instrumental analysis (HS-SPME/GC-MS and GCO) and sensory evaluations. The results showed that *C. cohnii* had high levels of sulfur, ester and alcohol compounds that characterized with “sulfur-cabbage”, “fruity”, “rosy” and “boiled potato” aromas. On the other hand, aldehydes and alcohols were more dominant in *S. limnancium* which was characterized with “mushroom”, “cucumber” and “fatty-grassy” aromas. The other three microalgae species had higher levels of ketone and terpene compounds, resulting in “woody” and “cereal-like” aromas. In another study Durme et al. (2013) investigated the volatile composition and sensory properties of four species of marine microalgae *Botryococcus braunii*, *Rhodomonas*, *Tetraselmis* species and *Nannochloropsis oculata*) and one freshwater microalga, *Chlorella vulgaris* through instrumental (HS-SPME GC-MS) and descriptive sensory analysis. The principal component analysis (PCA) showed that freshwater microalga *C. vulgaris* were different from the marine microalgae according to the HS-SPME-GC-MS results. *Botryococcus* were represented by mostly alkenals, alkenals and alkatrienals whereas *Rhodomonas* and *Tetraselmis* were represented by a variety of sulfuric compounds. *C. vulgaris* had “grassy, vegetable, cucumber” aromas whereas *Tetraselmis* and *Rhodomonas* had “fresh marine, fishy” and “cooked shrimp/cooked seafood” aroma profiles. Such studies are important because knowing the odor characteristics of microalgae species can guide their use in food formulations and promote the development of fortified healthy foods. A flavor may be unpleasant for one type of food formulation, but highly desirable for another food category. For instance, “seafood” aroma might not be very desirable for dairy products but can be used in fish oil products. Thus, knowing the flavor and taste that microalgae impart to foods can increase the use of microalgae in foods.

Despite the fact that there are several studies related to the health benefits of microalgae, the number of studies investigating the absorption of bioactive compounds (carotenoids, PUFAs, vitamins, proteins, etc.) and the effect of *in vivo* and *in vitro* digestion is limited. It is therefore important to investigate whether the bioactive compound is released from the food matrix and to demonstrate its effect [Zhou et al., 2022]. The cell wall of microalgae is one of the barriers that hinders the use of microalgae as a protein source due to digestibility problems. Several cell disruption methods such as enzymatic treatment, high pressure homogenization, bead milling, ultrasound and microwave treatment have been used to overcome this

problem [Barros de Medeiros et al., 2022], [Demarco et al., 2022]. Matrix interferents such as dietary fibers, minerals and lipids can be other factors affecting the absorption and distribution of compounds from microalgae such as carotenoids [Barros de Medeiros et al., 2022]. Thus, knowledge of digestibility, bioaccessibility and bioactivity are important concepts for utilizing microalgae in food [Barros de Medeiros et al., 2022].

2.3. Fermentation as a Strategy to Overcome the Limitations of the Use of Microalgae

2.3.1. Fermentation as an Ancient Way of Developing Novel Food Products

Fermentation is as old as the history of mankind or maybe even older. Alongside drying, it is the oldest method of preserving food. As civilizations developed, fermentation was used not only as a method of preserving food, but also to produce foods with different flavors and tastes and make them more palatable [Bourdichon et al., 2012], [Farnworth, 2008], [Marco et al., 2021]. Bread, cheese, wine, beer, traditional fermented milk from different geographical locations such as kumis (Central Asia), dahi (India) and Långfil (Sweden) are old products obtained from fermentation [Farnworth, 2008]. Louis Pasteur's definition of fermentation: "La vie sans l'air", meaning "life without air" [Bourdichon et al., 2012]. Traditionally, fermentation is defined as the metabolic process of generating ATP from organic compounds that are both electron acceptors and donors. This definition is narrow as it only emphasizes the absence of oxygen and therefore only provides information for anaerobic fermentations such as ethanol and lactic acid fermentation. However, from a broader perspective, it can be defined as the breakdown of organic compounds by microorganisms in the presence (aerobic) or in the absence (anaerobic) oxygen to produce ATP and employing metabolism of microorganisms to produce value-added products that can be used in the food industry as well as other industries [Marco et al., 2021], [McNeil and Harvey, 2008].

There is an increasing interest in fermented foods as the research associated with their health benefits increases. Also, perception of fermentation as a natural process increases the acceptability of fermented foods by consumers. Depending on the metabolism of selected microorganisms, fermentation provides a unique transformation of raw materials and imparts sensorial, physical and chemical changes [Shiferaw Terefe and Augustin, 2020]. The taste related metabolites during the fermentation can bring sweetness in the case of mannitol and some amino acids, bitterness in the case of oligopeptides and hydrophobic peptides and sourness from the organic acids such as lactic acid [Smid and Kleerebezem, 2014]. Fermentation also enhances the nutritional value of food, as microorganisms can synthesize proteins and other essential nutrients. Microbial metabolism can also improve the bioavailability and digestibility of macromolecules in the raw material [Shiferaw Terefe and Augustin, 2020].

2.3.2. Fermentation of *Spirulina* and its Effect on Bioactive and Sensory Properties

Due to all the aforementioned properties of *Spirulina* and the importance of fermentation as a conventional and cost-effective strategy, fermentation of *Spirulina* has recently attracted the interest of many researchers [Bao et al., 2018], [de Marco Castro et al., 2019], [Jamnik et al., 2022], [Kurt et al., 2023], [Niccolai et al., 2019], [Özyurt et al., 2023], [Sahin et al., 2022], [Tolpeznikaite et al., 2023], [Verni et al., 2021]. The common strategy in these studies was to use *Spirulina* as a substrate rich in bioactive compounds and to ferment it by a variety of microorganisms to take advantage of their metabolism and to investigate the effect of fermentation on microbial growth and some bioactive properties such as total phenolic content (TPC), antioxidant activity, antimicrobial activity and sensory properties. Lactic acid bacteria (LAB), especially *Lactobacillus plantarum*, were the most commonly used microorganisms in these studies [Bao et al., 2018], [de Marco Castro et al., 2019], [Jamnik et al., 2022], [Kurt et al., 2023], [Niccolai et al., 2019], [Özyurt et al., 2023], [Sahin et al., 2022], [Tolpeznikaite et al., 2023], [Verni et al., 2021]; while some studies used *Bacillus* strains [Bao et al., 2018], [Kurt et al., 2023] and only one study used yeast species (*S. cerevisiae*, *D. hansenii* and *K. marxianus*) [Sahin et al., 2022].

Overall, these studies showed that *Spirulina* is a suitable medium to support the growth of a variety of microorganisms.

Several studies showed that fermentation of *Spirulina* by *L. plantarum* increased the TPC, antioxidant activity DPPH radical scavenging capacity, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) [de Marco Castro et al., 2019]; [Jamnik et al., 2022], [Niccolai et al., 2019]. Tolpeznikaite et al. (2023) used submerged (SMF) and solid-state fermentation (SSF) of *Spirulina* with different Lactobacilli strains. Their results showed that most of the fermented *Spirulina* (FS) products exhibited antimicrobial activity against *Staphylococcus aureus*. In addition, fermentation of *Spirulina* can be used to remove off-flavors and make it more palatable for the consumers. Kurt et al. (2023) found that the most abundant group of volatile organic compounds (VOCs), the pyrazines, were significantly reduced by using LAB strains for the fermentation. They also found that FS by LAB species had a higher level of sourness due to the low pH value, while bitterness was a notable taste in the FS by *B. licheniformis* according to the sensory analysis. Among four different LAB species, *L. helveticus* B-4526 achieved the highest performance among them by 24 h of fermentations in terms of growth, sensory properties, total amino acid and VOCs compositions Kurt et al. (2023). Sahin et al. (2022) found that FS by yeast species also affected the amino acid composition and VOCs content. They found that glutamic acid, methionine, lysine, isoleucine and valine were dominant in FS products compared to unfermented *Spirulina* (unFS). Fermentation caused a decrease in the pyrazine, ketone and aldehyde contents in FS products compared to unFS. In addition, sensory analysis revealed that FS by *K. marxianus* had the lowest “seaweed” and “umami” flavor attributes and higher “rose” and “fermented” flavor attributes. In conclusion, fermentation is a suitable technique to improve the bioactivity of *Spirulina*, bringing new flavors as well as changing the nutritional profile (e.g., essential amino acids) depending on the characteristic of the selected microorganisms under the designated environmental conditions.

2.4. Microorganisms Used in Fermentation: *Lactobacillus helveticus* and *Kluyveromyces marxianus*

The choice of microorganisms is as important as the choice of raw material, as they will be the “microbial cell factories and their metabolism will determine the changes in the properties of the raw material, a working in the medium.

2.4.1. *Lactobacillus helveticus*

Lactobacillus helveticus is a rod-shaped thermophilic bacterium that belongs to the group of lactic acid producing microorganisms, the Lactic Acid Bacteria (LAB) (Figure 2.6). Earlier, at the beginning of the 20th century, LAB was identified as “milk-souring organisms”. LAB play important role in food fermentations and even some strains have health-promoting activities referred as probiotics [Lahtinen et al., 2012]. The Emmental cheese isolate was where *Lactobacillus helveticus* was identified by Orla-Jensen in 1919, and characterized by ability to grow at high temperature (~55°C) and proteolytic activity [Slattery et al., 2010]. The efficiency of the proteolytic system of *L. helveticus* makes it suitable for processes such as cheese ripening and dairy fermentation. The proteolytic system includes cell-envelope proteinases (CEPs), transport system and intracellular peptidases. Proteinases, CEPs, carry through the first stage of protein hydrolysis and degrade protein into oligo-peptides. In the second stage, peptide and amino acid transport systems take place and transport di-, tri-, and oligo-peptides into the cell by various peptide transport systems. The final step that is performed by peptidases is very important for cells as it involves the hydrolysis of peptides and release of essential amino acids. *L. helveticus* has specific CEPs as sites of cleavage changes among different strains and depending on the type of casein [Griffiths and Tellez, 2013]. *L. helveticus* strains are auxotrophic for several amino acids, meaning that they must have efficient proteolytic activity to obtain essential amino acids from the medium [Griffiths and Tellez, 2013], [Sadat-Mekmene et al., 2011]. Hébert et al. (2002) investigated the effect of the peptide content of the medium on the cell surface proteinase of different lactobacilli strains (five strains of *L. helveticus* and two strains of *L. delbrueckii* subsp. *lactis*). Their results showed that proteinase activities were significantly

reduced when lactobacilli were grown in peptide-rich medium. This implicates the importance of the composition of the growing medium on *L. helveticus*.

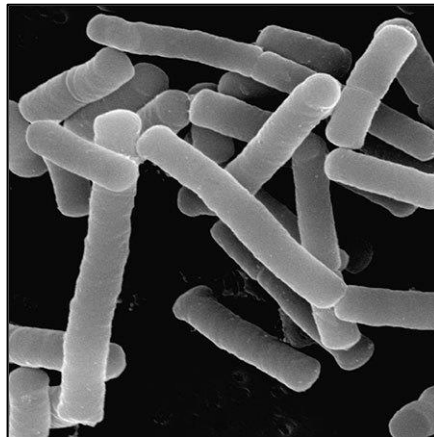


Figure 2.6: Microscopic image of *Lactobacillus helveticus*.

Due to the good proteolytic system of *L. helveticus*, it is widely used to produce bioactive peptides from milk with various bioactivities such as antihypertensive, immunomodulatory, antibacterial and calcium binding. Among these peptides, antihypertensive peptides which have Angiotensin converting enzyme (ACE) inhibition activity have been studied more than the others [Griffiths and Tellez, 2013], [Sadat-Mekmene et al., 2011], [Taverniti and Guglielmetti, 2012]. Fermentation with *L. helveticus* can also improve the content of bioactive compounds, antioxidant properties and digestibility of the raw material. Wang et al. (2022) investigated the effect of fermentation of kiwifruit juice from two cultures by three LAB, *Lactobacillus acidophilus* 85 (La85), *Lactobacillus helveticus* 76 (Lh76) and *Lactobacillus plantarum* 90 (Lp90) on the phenolic compounds, antioxidant activity and volatile compounds. Their results showed that kiwifruit juices were suitable food matrices for the LAB growth. And Lh76 significantly increased the total phenolics and flavonoids. LAB fermentation significantly increased the total contents of alcohols, and this increase was the highest in the Lh76 fermentation. In a recent study, Dong et al. (2023) investigated the effect of fermentation by LAB, *L. helveticus* 76 (Lh) and *Bifidobacterium lactis* 80 (Bla) on the *in vitro* digestion and bioactive compounds of an important Chinese herb, Chinese wolfberry (*Lycium barbarum*) pulp. Lactic acid fermentation significantly increased the total phenolic content (TPC), and this increase was higher in the Lh-fermented pulp. As a result of

simulated gastric digestion, the TPC of unfermented pulp increased, but after stimulated intestinal digestion, the TPC decreased due to instability and degradation of phenolic compounds. Stimulated digestion did not cause a significant change in the TPC of fermented pulp, and fermented pulp still contained higher TPC after digestion compared to unfermented, indicating that fermentation promoted the passage of phenolic compounds to the intestine and increases the possibility of them exerting their functional activity.

2.4.2. *Kluyveromyces marxianus*

Saccharomyces cerevisiae is a commonly used yeast in biotechnology due to its availability, well-known genome and physiology, and ease of use. Although, the characterization of non-conventional yeasts is limited compared to *S. cerevisiae*, the use of non-conventional yeasts such as *Y. lipolytica*, *P. pastoris*, *K. lactis* and *H. polymorpha* is accelerating due to their potential applications in various fields. *K. lactis* was the first species to be granted GRAS status after *S. cerevisiae*. *Kluyveromyces marxianus*, the sister species of *K. lactis*, has gained importance due to its attractive properties. These include the ability to utilize a wide range of sugars such as lactose and inulin, thermotolerance, high growth rate and the synthesis of lytic enzymes. In addition, *K. marxianus* can be isolated from different habitats resulting in a high metabolic diversity leading its use in different biotechnological areas such as the production of enzymes (β -glucosidase, inulinase), single-cell protein, aroma compounds and of ethanol and use as a host for heterologous protein production. It also has Qualified Presumption of Safety (QPS) status in the European Union and GRAS status in the United States, making it suitable for use in the production of pharmaceuticals and food [Baptista and Domingues, 2022], [Fonseca et al., 2008], [Karim et al., 2020].

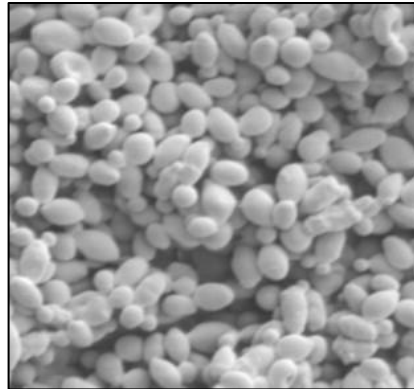


Figure 2.7: *Kluyveromyces marxianus* observed by scanning electron microscopy.

Kluyveromyces marxianus was first described by Emil Christian Hansen in 1888 and named as *Saccharomyces marxianus*. Later, due to differences in spore, morphology, fermentation and utilization of different sugars compared to *S. cerevisiae*, *S. marxianus* was reclassified into the genus of *Kluyveromyces* [Fonseca et al., 2008]. The morphology of *K. marxianus* is shown in Figure 2.7. Most of the *K. marxianus* strains are Crabtree negative, meaning they do not produce alcohol in the presence of oxygen. In addition, *K. marxianus* can grow in a variety of low-cost media such as molasses and whey, and the production of extracellular enzymes that enable the hydrolysis of these raw materials makes it suitable for the utilization of industrial by-products. [Karim et al., 2020]. Hu et al. (2019) investigated the effect of solid-state fermentation (SSF) by *K. marxianus* on the okara which is a by-product of soy products such as tofu and soymilk. Their results showed that the fermented soybean residue (FSR) had higher soluble dietary fibers, polysaccharides and the trypsin inhibitor activity of the FSR decreased. And the characteristic beany odor of the okara was reduced by fermentation with *K. marxianus*. In another study tomato pomace, which is produced in large quantities in the tomato processing industry, was pretreated and enzymatically hydrolyzed to produce tomato pomace hydrolysate. The tomato pomace hydrolysate was then fermented by *K. marxianus*. Their results showed that isoamyl alcohol, phenyl ethyl alcohol, ethyl acetate and phenyl ethyl acetate were abundant as volatile organic compounds (VOCs). Rose and sweet floral aromas were also found by the sensory analysis [Kılmanoğlu et al., 2021]. Such studies show the potential of valorization of industrial by-products by *K. marxianus*. As different aroma volatiles can be produced by *K. marxianus*, Plessas et al. (2008) used the two starter cultures *Lactobacillus delbrueckii* ssp. *bulgaricus* or

Lactobacillus helveticus mixed with *K. marxianus*. They investigated the bread flavor consumer preference, aroma volatiles and shelf-life. They observed that breads made with *K. marxianus* and *L. bulgaricus* had longer shelf-life, the highest scores in the sensory evaluation and more VOCs. The daily change in the aroma composition showed that bread containing *K. marxianus* and *L. bulgaricus* contained higher levels of alcohols and carbonyls, which are important for the sensory preference of bread. Fermentation with *K. marxianus* can also affect the bioactive properties of the food product. In a study in which 20 dairy yeasts strains belonging to the species *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* were investigated for the production of milk protein-derived antihypertensive hydrolysates. The strains were grown in medium supplemented with casein or lactoferrin as the only nitrogen source. The promising casein and lactoferrin hydrolysates were selected and fractionated into permeates with molecular masses lower than 3 kDa. The *K. marxianus* Km2 strain grown on either casein or lactoferrin produced permeates with the strongest ACE-inhibitory activity [García-Tejedor et al., 2013].

2.5. Importance of Evaluating the Effects of *In Vitro* Digestion of Newly Developed Foods

A newly developed food product may have various beneficial properties due to their high protein, high total phenolic contents and good bioactive properties such as antioxidant activity, antimicrobial activity and ACE inhibitory activity, but it is also important how these properties are affected by digestion and what will be the fate of the bioactive food compounds after digestion. It is therefore important to mimic the physiological conditions of digestion and understand the changes and bioactivity of nutrients in order to process foods in such a way that they continue to show benefits after digestion. In this case, simulated gastrointestinal digestion is a commonly used method to assess the bioaccessibility of nutrients and the digestibility of macronutrients. Although *in vivo* studies are the 'gold standard' for answering nutritional questions and provide more accurate results, *in vitro* digestion models are advantageous because they are cost effective, less labor intensive and do not raise ethical issues compared to *in vivo* studies [Lucas-González et al., 2018], [Minekus et al., 2014].

There are several terms used to assess the effect of digestion on foods, including “bioavailability”, “bioaccessibility” and “bioactivity”. Bioavailability is a general term that includes bioaccessibility, absorption, metabolism and bioactivity. It can be defined as the proportion of ingested components that enter the bloodstream where they are distributed to different tissues and organs and are “available” for use and to exert their effect. On the other hand, bioaccessibility is the proportion of ingested components that become “accessible” for absorption through the gastrointestinal tract as a result of a series of events such as release from the food matrix and solubilization and biotransformation. Bioactivity is the result of the overall digestion process; by which the bioactive compound exerts its biological effect at the target site [Dima et al., 2020], [Fernández-García et al., 2009], [Lucas-González et al., 2018].

There are various methods for the investigation of *in vitro* digestion (IVD). These methods differ in complexity, automation and experimental conditions. To harmonize the conditions and facilitate comparisons of results in the literature, a standardized protocol has been proposed for static and semi-dynamic stimulated digestion by INFOGEST (an international network of excellence on the fate of food in the gastrointestinal tract) [Brodkorb et al., 2019], [Minekus et al., 2014], [Rodrigues et al., 2022]. The physiological conditions of the oral, gastric and small intestinal phases are set by the experimenters, and they are constant in the static IVD system. However, the gastric phase is dynamic and gradual pH change is performed by using a pump and automatic titrator in the semi-dynamic IVD. There are also dynamic IVD systems such as the SHIME (Simulator of the Human Intestinal Microbial Ecosystem from Ghent University, Belgium) which includes a colonic fermentation stage [Rodrigues et al., 2022]. The use of these kinds of systems is quite important as it can guide researchers to determine suitable food design and food processing techniques in a way that guarantees the effect of the bioactive compounds [Fernández-García et al., 2009]. For instance, Chen et al. (2014) investigated the effect of *in vitro* digestion on the total phenolic content and antioxidant activities of 33 fruits. Their results showed that the total phenolic content (TPC) of 8 fruits increased after the gastric phase and the TPC of 25 fruits increased after the duodenal phase of digestion. They also found that the antioxidant properties determined by DPPH, ABTS and FRAP assays, decreased for some fruits but increased for other

fruits after the gastric phase of digestion. They also found that before the gastric and duodenal phases of *in vitro* digestion there was a high correlation between the DPPH, FRAP, ABTS and the TPC which was poor after the digestion. This shows that there is no generalized behavior of foods products during the *in vitro* digestion.

2.6. Bioreactors and Operating Conditions: The Hub of Bioprocesses

Bioreactors are an imperious part of bioprocessing, providing suitable conditions and control over the bioprocess to obtain the desired product. There are many types of bioreactors designed for different applications, such as airlift bioreactors, hollow fiber bioreactors and stirred tank bioreactors. Among them, stirred tank bioreactors are the most commonly used in bioprocessing (Figure 2.8). The probes such as pH, dissolved oxygen (DO₂) and temperature provide continuous monitoring of the bioprocess and the tubing pumps allow the necessary components such as microbial culture, acid/base or fresh media to be transferred to the vessel tank via the ports and tubing system. The stirrer provides mechanical agitation and also, the sparger provides the introduction of air bubbles into the system. Stirrer and sparger are key parts of the oxygen transfer in the tank as the stirrer reduces the size of the air bubbles entering through the sparger and thus, giving the cells more time to use the oxygen [McNeil and Harvey, 2008].

In a study investigating the symbiotic relationship between *Lactococcus lactis* subsp. *lactis* and *K. marxianus* for nisin production, the researchers constructed a cascade control system in which the pH of the environment was controlled by linking the lactate consumption rate of *K. marxianus* to the amount of DO₂; in this system, changing the amount of DO₂ changed the lactic acid consumption rate, which in turn changed the pH of the environment. When DO₂ increased, the lactate consumption rate of *K. marxianus* increased, which raised the pH of the medium and affected nisin production. When lactic acid accumulated in the medium, the growth of *L. lactis* stopped and nisin production decreased [Wardani et al., 2006]. In another study, the effect of oxygen percentage in the inlet gas (1.75%, 11% and 20.95%) and temperature (30 °C and 41 °C) on the ethanol fermentation by *K. marxianus* CBS712 strain using glucose and xylose, was investigated. While the glucose consumption rate varied with temperature rather than oxygen content, both the oxygen content in

the inlet gas and temperature were effective in the change in xylose consumption rate that occurred after the glucose exhaustion [Signori et al., 2014]. Overall, such studies demonstrate the importance of the operating conditions (pH, temperature and DO₂) for the metabolism of the microorganisms and the fate of the bioprocesses and the need to optimize them.



Figure 2.8: A lab-scale stirred bioreactor production set up used in this study.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Materials and Microorganisms

Spirulina platensis biomass was obtained from a local manufacturer based in Çanakkale, Turkey. Proximate composition (w/w) of the biomass was characterized by Sahin et al. (2022) as: 49.3% of protein, 19.4% % of carbohydrate, 13.5% of lipid, 7.8% of ash, and 7.5% of moisture (in dry basis).

Based on the previous studies performed in our laboratory, *Lactobacillus helveticus* (B-4526) and *Kluyveromyces marxianus* (Y-329) were chosen for the fermentation and bought from NRRL Culture Collection, USDA Agricultural Research Service (IL, USA). The test pathogens (*Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*) were obtained from the Department of Food Engineering, Uşak University.

3.1.2. Chemicals Used

All chemicals used in this study were of analytical grade, including Fluorenylmethyloxycarbonyl chloride (Fmoc), Disodium hydrogen phosphate (Na_2HPO_4), Acetonitrile, Methyl nonanoate, 2-methyl valeric acid, Boric Acid (Fisher Scientific), L-leucine (Fisher Scientific), Sodium Tetraborate Decahydrate (Fisher Scientific), Sodium Dodecyl Sulfate (Fisher Scientific), Acrylamide (Fisher Scientific), Bis-Acrylamide (Fisher Scientific), Ammonium Persulfate Crystals (Fisher Scientific), Glycerol (Fisher Scientific), Brilliant Blue G-250 (Fisher Scientific), TEMED (N, N, N', N'-tetramethyl ethylene diamine) (Fisher Scientific), n-alkane series (C5-C30) (Aldrich), Silver Nitrate Acs Reagent, ($\geq 99.0\%$, Sigma-Aldrich), Amino acid mixture (Sigma-Aldrich), Methanol (Merck), Trichloroacetic Acid (Sigma-Aldrich), Ninhydrin Reagent (Sigma-Aldrich), Formaldehyde (Sigma-Aldrich), Sodium Thiosulfate (Sigma-Aldrich), Sodium Carbonate (Sigma-Aldrich), Sulfuric Acid (H_2SO_4) (Acs Reagent, 95.0-98.0 %, Sigma-Aldrich), Albumin from

Bovine Serum (Sigma), Phthalaldehyde (Acros), Acetone (Isolab), Sodium Carbonate Anhydrous (Isolab), Hydrochloric Acid Fuming (Isolab), Cadmium Chloride, (Acros Organics), Ethanol (Isolab), Acetic Acid (Glacial, 100 %, Isolab), Sodium Chloride (Isolab), Yeast Extract (Biolife), Peptone Bacteriological (Biolife), Glucose (Biolife), Lactose (Biolife), Agar Technical (Biolife), β -Mercaptoethanol (Alfa Aesar), Kjeldahl Tablet (Merck), Tris (Merck), Glycine (Merck), Folin-Ciocalteu's Phenol Reagent (Merck), Gallic Acid (Anhydrous) (Merck). ABTS (Sigma-Aldrich), DPPH (Sigma-Aldrich), Trolox (Acros Organics), OPA-reagent (Agilent Technologies), Muller Hinton (Biolife), Nutrient Broth (Biolife) Antimicrobial Susceptibility Test Discs (Bioanalyse).

3.1.3. Equipment Used

Table 3.1: Laboratory devices used in this study.

LABORATORY DEVICES	BRANDS
Micropipettes	Rainin Pipet-Lite XLS
Vortex stirrer	VELP Scientifica
Autoclave	N-Biotek; Nuve NC 90M
Bioreactor	Bundle 3L Microbial EZ2, Applikon Biotechnology
Magnetic stirrer	Witeg MSH-20D
SDS electrophoresis	Bio-Rad Laboratories Ltd., Watford, UK
DEN-1 McFarland	
Densitometer	Biosan
Deep freezers -80°C	Nuve DF 490
Centrifuges	Nuve, NF 1200R; Sigma 1- 16K
Ultrasonic homogenizer	Bandelin Sonopuls

Table 3.1. continued

Water-bath	Stuart
pH meter	Mettler Toledo
Shaking incubator	N-Biotek
Biological Safety Cabinet (Class 2)	SafeFast Classic
Microscope	XSZ-107BN of Biological Microscope
Spectrophotometer	EPOCH2 Microplate Reader, BioTek; Shimadzu UV-1280
Burning Unit	Gerhardt, Germany
Rotary evaporator	Wiggens Strike 280
Gas chromatography-mass spectrometry (GC-MS) (HP 6890 GC and 7895C mass selective detector)	Agilent Technologies, Wilmington, DE, USA
Steam distillation	Gerhardt, Germany
HLPC sysyem (Infinity 1260 Autosampler, 1260 Multiple Wavelength UV detector)	Agilent Technologies Inc., Folsom, CA
Ultrasonic homogenizator (sonicator)	Bandelin SONOPULS

3.2. Methods

3.2.1. Preparation of Microbial Inoculum and *Spirulina* for Fermentations

Spirulina solution (60 g/L) was prepared by dissolving dried *Spirulina* biomass in distilled water and freeze-thawing process was applied 3-4 times followed by homogenization in a sonicator. The degradation of *Spirulina* was visually checked using a microscope and the total soluble protein concentration was measured to start

each batch with similar protein content. Lactose stock solution was prepared at a concentration of 10 g/80 mL.

The pH probe was calibrated using a 2-point calibration (pH 4.01 and 7.0 standard solutions). Then, 1.2 L of *Spirulina* was transferred to the bioreactor tank. The rest of the *Spirulina* solution was separated into a glass bottle as an unfermented *Spirulina* control. The necessary connections were made, and the bioreactor and the control *Spirulina* were autoclaved at 121 °C for 20 minutes. After sterilization, the bioreactor was connected to its control unit and kept stirring overnight for cooling. The following day, the pH was checked to ensure that there was no contamination in the system (a lower pH compared to the initial value of pH can indicate bacterial contamination). The dissolved oxygen was calibrated using 1-point calibration. The bioreactor was saturated with air and this point was assigned as 100% for the dissolved oxygen. 1M sodium bicarbonate was added to the system via tubing to start each batch with an initial pH of around 6.1-6.3. The required volume (57.6 mL) of lactose stock solution was transferred to the system to provide 6 g/L lactose in the medium.

K. marxianus was activated in YM (Yeast Mold) broth medium and *L. helveticus* was activated in MRS (de Man, Rogosa and Sharpe) broth medium and incubated at 37°C 100 rpm for 18-20 h. The active cultures of *K. marxianus* and *L. helveticus* were centrifuged at 5000 rpm for 10 minutes. The supernatants were removed, and the pellets were solubilized using the sterile saline solution (0.85% (w/v) NaCl). The McFarland turbidity of the cultures was adjusted to 5 and equal amounts of each of their solutions were taken and inoculated into the bioreactor, with a total inoculation of 2.5% (v/v) in the bioreactor and the fermentation was started. For all bioreactor productions, the constant stirring speed was 150 rpm, and the production temperature was set at 37°C. The effect of aeration was studied separately with two productions with constant "0.5 vvm" and "1 vvm" aeration. The dissolved oxygen level parameter was investigated by adjusting the "cascade" control to maintain the dissolved oxygen at 30%; in this system, when the dissolved oxygen fell below the desired value (30%), the mixing speed was first adjusted to increase to a maximum of 500 rpm, and if this was not sufficient, the system was adjusted to introduce 1 vvm of air into the bioreactor in addition to mixing. To examine the effect of pH, when the pH dropped to 5.75, pH control was activated and sterile 1M

sodium bicarbonate and 1M phosphoric acid were used to keep the pH at 5.75 throughout the production and this production mode was referred as “controlled pH”. In all figures and tables, the productions are coded as "**0.5 vvm**" and "**1 vvm**" for constant aeration productions, "**cascade**" for the production where dissolved oxygen was kept at 30% and "**controlled pH**" for the production where pH was kept constant when pH dropped to 5.75 at constant 1 vvm aeration. On the first day of production, if there was excessive foam, small amounts of an antifoam agent (4-5 drops) were added to the bioreactor.

3.2.2. Microbiological analysis

Microbial growth was monitored by sampling the bioreactor at specified time intervals (0, 24, 48 and 72 h) aseptically and performing viable cell counts. The sample was serially diluted with sterile saline (0.85% (w/v) NaCl) and the pour plate technique was used. MRS agar medium and YM agar medium were used for the enumeration of viable cells of *L. helveticus* and *K. marxianus*, respectively. Plates were incubated at 37°C for 48 hours and colony forming units were counted as (log CFU.mL⁻¹). The morphological differences between yeast and bacterial cells helped to differentiate these two microorganisms during counting.

3.2.3. Chemical Analysis

Prior to chemical analysis (total soluble protein content, degree of hydrolysis, SDS-PAGE, free amino acid content, volatile organic compounds), the samples were centrifuged at 10,000 rpm for 40 minutes at 4°C to remove cells and remaining *Spirulina*, and if solid particles remained in the supernatant, the centrifugation process was repeated. The supernatants were stored at -20°C and used for chemical analysis.

3.2.3.1. Determination of Total Soluble Protein Concentration

Total soluble protein concentration (TSPC) was measured according to Lowry et al. (1951). Bovine serum albumin (BSA, Sigma-Aldrich) at concentrations ranging

from 0-0.2 mg/mL was used to prepare standard calibration curve for TSPC measurements (Appendix A). Measurements were performed at a wavelength of 750 nm using an Epoch2 microplate reader.

3.2.3.2. Determination of Degree of Hydrolysis

The colorimetric cadmium ninhydrin method of Doi et al. (1981) was used to assess the degree of hydrolysis, as this method uses peptidase activities on peptides containing free amino acid groups. Briefly, the Cd-ninhydrin reagent was prepared by dissolving 0.4 g ninhydrin and 1 g CdCl₂ in a mixture of 80 mL 95% ethanol, 10 mL acetic acid and 20 mL water. 1 mL of Cd-ninhydrin was added to 0.5 mL of sample and vortexed. The tubes were heated in a water bath at 80°C for 10 minutes. The tubes were cooled, and the absorbance was read at 508 nm using an Epoch2 microplate reader. Leucine concentrations in the range 0-120 mg/L were used to prepare a standard calibration curve (Appendix B).

3.2.3.3. Investigating the Protein Profile Using SDS-PAGE

SDS-PAGE analysis was performed using 4% stacking and 12% running polyacrylamide gel using the Mini-Protean BioRad electrophoresis system. Prior to the analysis, a Lowry protein assay was performed to make sure each well contained the same protein concentration. The samples were mixed with 4X Laemmli buffer (3:1, v.v⁻¹) and were heated at 100°C for 5 min. At the end of electrophoresis, gels were stained with Coomassie Blue G-250. Protein Ladder in range of 11-245 kDa (GangNam-STAIN™) was used as protein marker.

3.2.3.4. Determination of Total and Free Amino Acid Content

The total and free amino acid content of unFS and FS products was performed using an Agilent amino acid HPLC method as used by Sahin et al. (2022) and Kurt et al. (2023). The sample preparation step was different for total amino acids (TAA) and free amino acids (FA), while the measurements with HPLC were the same. To prepare samples for TAA, 10 mL of supernatants were added to the

digestion tubes and 5 mL of 6N HCl was added. The samples were kept in a drying oven at 110 °C for 24h. Then, the samples were cooled to room temperature and 5 mL of ultrapure water was added and the pH was adjusted with HCl and NaOH to have a value around neutral pH (6.7-7.3). The total volume was then completed to 25 mL by the addition of ultrapure water. Following centrifugation at 5000 rpm for 5 min, the samples were filtered through a 0.45 µm pore size PTFE filter and transferred to a 1 mL HPLC vial. To determine the FA content, 20 mL of supernatants were transferred to a volumetric flask and 1 mL of salicylic acid was added. The total volume was adjusted to 25 mL with ultrapure water. After centrifugation at 5000 rpm for 15 min, the samples were filtered through a 0.45 µm pore size PTFE filter and transferred to a 1 mL HPLC vial. The Agilent 1260 infinity HPLC system (Agilent Technologies Inc., Folsom, CA) consisting of an Infinity 1260 Autosampler, 1260 Multiple Wavelength UV detector, and ZORBAX Eclipse-AAA (4,6 x 150 mm, 3.5µ PN 963400-902) was used for chromatographic separation. Amino acid derivatization was performed using OPA (o-phthaldialdehyde, 10mg/mL) and FMOC (fluorenylmethyloxycarbonyl chloride, 2.5mg/mL). The mobile phases were solvent A (40 mM Na₂HPO₄ at pH 7.8) and solvent B (acetonitrile: methanol: water 45:45:10). Elution conditions were: 0-1.9 min, solvent A 100 % and 1.9-18 min, solvent B from 0 to 57%; 18–18.6 min, solvent B increased to 100%; 18.6–22.3 min, solvent B was kept at 100 %; 22.3-26 min, solvent B decreased from 100% to 0 %. The flow rate was 2 mL.min⁻¹. The mixture containing 17 amino acids (Sigma-Aldrich, No 79248) was used as an external standard, and the quantification of amino acids in the samples was performed based on this standard mixture and expressed as µM.

3.2.3.5. Determination of Volatile Organic Compounds (VOCs)

Volatile organic compounds (VOCs) were extracted by solid-phase microextraction (SPME) technique. Briefly, 2 g of supernatant was transferred to SPME vial (Supelco, Bellafonte, ABD) and 1 g of NaCl and 10 µL of internal standard were added to the vial. The vials were vortexed for 1 min. The vials containing the samples were kept at 40°C water bath for 20 min to accumulate volatiles in the headspace of the vials. At the end of this period, SPME fiber (2 cm-

50/30 μm DVB/Carboxen/PDMS stable flex, Bellafonte, ABD) was inserted in the headspace of the vial and kept there for 20 min at 40°C in a water bath. Then, SPME fiber was removed and injected into gas chromatography-mass spectrometry (GC-MS). A highly polar column, HP-INNOWAX Polyethylene Glycol Column (60m $\times$ 0.25mm i.d. $\times$ 0.25 μm film thickness, J&W Scientific, Column number 19091N-136, Folsom, CA) was used to separate VOCs in GC-MS. GC oven program was adjusted to gradually increase from 40°C to 250°C at a rate of 5 °C min⁻¹ with initial and final hold times of 10 min. And as a carrier gas helium with a flow rate of 1.0 mL min⁻¹ was used. Mass selective detector (MSD) conditions were as follows: capillary direct interface temperature, 280°C; ionization energy, 70 eV; mass range 35–350 amu; scan rate, 4.45 scans. s⁻¹. A stock solution of the internal standard (IS) containing 8.75 $\mu\text{g/mL}$ methyl nonanoate for basic and neutral aroma and 186 $\mu\text{g/mL}$ of 2-methyl valeric acid for the acidic aroma compounds prepared in high-performance liquid chromatography (HPLC) grade diethyl ester was prepared. 10 μL of IS solution was added to the samples. National Institute of Standards and Technology (NIST, 2008) and Wiley Registry of Mass Spectral Data (Wiley, 2005) libraries were used to identify the VOCs. To quantify the VOCs Eq. (3.1) was used:

$$\text{Mean relative abundance } (\mu\text{g kg}^{-1}) = \frac{\text{Concentration of IS} \times \text{Peak area of compound}}{\text{Peak area of IS}} \quad (3.1)$$

3.2.4. Bioactivity Assays

Prior to the total phenolic content and bioactivity assays, the supernatants of the unfermented *Spirulina* products and fermented *Spirulina* products at different operating conditions were concentrated using a rotary evaporator at 45°C under vacuum. The concentrated products were freeze-dried. The dried form of the products was prepared at a concentration of 50 mg/mL with distilled water for the total phenolic content, DPPH inhibition and TEAC assays. For the antimicrobial assays, a higher concentration of 150 mg/mL was used as 50 mg/mL did not give an antimicrobial effect.

3.2.4.1. Determination of Total Phenolic Content

Total phenolic content (TPC) was measured according to Singleton et al. (1999), which measures the phenolic compounds by means of the Folin-Ciocalteu reagent. Gallic acid at concentrations between 0-1.5 mg/mL and 0-1.25 mg/mL was used to prepare standard calibration curve for TPC measurements on the well-plate reader and spectrophotometer, respectively (Appendix C). Briefly, 900 μ L of distilled water was added to 100 μ L of the sample. Then, 5 mL of 2N Folin-Ciocalteu reagent was added and vortexed. Finally, 4 mL of sodium carbonate (75 g/L) was added and vortexed. The tubes were incubated for 2 hours in the dark and at room temperature. Measurements were performed at a wavelength of 765 nm using either an Epoch2 microplate reader or a spectrophotometer (Shimadzu UV-1280), depending on the equipment availability.

3.2.4.2. Determination of Antioxidant Activity Measuring DPPH Radical Scavenging Activity and Trolox Equivalent Antioxidant Capacity

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity measurements were done by according to the methods described by Benvenuti et al. (2004) and Cemeroğlu (2010). 1 mM DPPH radical solution was prepared using methanol as solvent freshly on the day of measurements and kept at 4°C and dark. Different volumes of samples, selected based on color change and linearity of radical inhibition, were transferred to the tubes. Then, 600 μ L of 1 mM DPPH radical was added to the samples. Finally, the total volumes of each tube were adjusted to 6 mL by adding methanol. The tubes were incubated at dark and room temperature for 30 minutes. The absorbances were measured at 517 nm. The analysis was performed in duplicate and if necessary, in triplicate. The inhibition percentage was calculated using the Eq. (3.2):

$$\% \text{ DPPH inhibition} = \left(\frac{Abs_{DPPH} - Abs_{sample}}{Abs_{DPPH}} \right) \times 100 \quad (3.2)$$

Abs_{DPPH} is the absorbance of the blank containing only the radical solution and methanol without the sample, Abs_{sample} is the absorbance in the presence of sample, radical solution and methanol. The percentage inhibition was plotted against sample volume. According to the linear regression, EC₅₀ (half maximum effective concentration) values, the amount of sample required to provide 50% inhibition of the radical, were calculated and expressed in μL and converted to mg using the stock concentration of the sample (50 mg/mL). The graphs for DPPH inhibition of samples are shown at Appendix D.

Trolox Equivalent Antioxidant Capacity (TEAC) is another commonly used assay based on the decolorization of an ABTS (2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)) radical. In this method, Trolox, a compound with antioxidant activity, is used as a standard to determine the antioxidant activity of samples as equivalent to that of Trolox. The method proposed by Re et al. (1999) and Cemeroğlu (2010) was used. First, 7 mM ABTS radical solution was prepared by dissolving 0.0384 g ABTS in some distilled water followed by the addition of 2 mL of 12.25 mM potassium persulfate. The volume of the radical solution was completed to 10 mL with distilled water in the volumetric flask. This solution was then stored in the dark at room temperature for 12-16 h prior to the use. As the stability of this solution is maximum 3 days, the fresh solution was prepared when necessary. Before the analysis, the radical solution was diluted with 0.1 M PBS buffer (phosphate-buffered saline, pH 7.7) to get an absorbance of 0.700 ± 0.020 at a wavelength of 734 nm and this value (absorbance of the radical solution) was recorded. Different sample volumes were added to the absorbance adjusted radical solution in a way to reach 1 mL in the cuvette (e.g., 10 μL sample - 990 μL radical solution). The cuvette was then mixed and the change in the absorbance values was recorded every 1 minute for 6 minutes at 734 nm. For each sample, at least 4-5 different sample volumes and for each sample volume 2 replicates, when necessary 3 replicates were done. For the calibration of standard, 10 μL stock solutions were added to 990 μL of the absorbance adjusted radical solution and thus, the final Trolox concentrations were 5 μM , 10 μM , 15 μM and 20 μM , respectively. For each concentration 3 replicates were done. The standard calibration curve for TEAC is shown at Appendix E.

Percentage inhibition was calculated using the Eq. 3.3:

$$\% \text{ ABTS inhibition} = \frac{Abs_{ABTS} - Abs_{Sample}}{Abs_{ABTS}} \quad (3.3)$$

Abs_{ABTS} is the absorbance of the ABTS radical solution that was adjusted between 0.680±0.720. Abs_{Sample} is the absorbance read at the end of 6 minutes in the presence of sample. Percentage inhibition versus sample volume was plotted. The slope of the equation for the samples was divided by the slope of the standard curve. The result was expressed in “mM Trolox/mL sample” and then, converted to “mM Trolox/g sample” by using the sample concentration (50 mg/mL). Appendix F shows the “Inhibition versus sample volume” graphs for some samples.

3.2.4.3. Evaluation of Antimicrobial Activity

The antimicrobial activity of the unfermented and fermented *Spirulina* products was evaluated against pathogens, a gram-negative bacterium *Escherichia coli*, a gram-positive bacterium *Staphylococcus aureus* and a spore-forming bacterium *Bacillus cereus* by means of agar well and disc diffusion methods. The microorganisms were grown in nutrient broth at 37°C for *E. coli* and *S. aureus* and 25 °C for *B. cereus* for 18-24 h. For the agar disc diffusion a method similar to that used by Jain and Anal (2017) was applied. Active bacterial cultures of *E. coli*, *S. aureus* and *B. cereus* were adjusted to the McFarland standard turbidity of 0.5 with the help of sterile saline (0.85% NaCl) and 100 µL of each were spread on the surface of Mueller-Hinton agar medium, separately. 15 µL of the supernatants of fermented and unfermented *Spirulina* products (150 mg/mL) were absorbed onto sterile blank discs (Antimicrobial Susceptibility Test Discs, Bioanalyse) with a diameter of 6 mm and placed on the bacteria-spread surface of agar plates. Sterile distilled water absorbed discs were used as a negative control and standard discs containing chloramphenicol (0.3 mg/mL) (Antimicrobial Susceptibility Test Discs, Bioanalyse) were used as a positive control. For the agar well diffusion method, 0.1 mL of the active cultures with the McFarland standard turbidity of 0.5 were added to 20 mL of Brain Heart Infusion containing 0.75% (w/v) agar, mixed and poured into

sterile petri dishes. Plates were placed in the refrigerator (4 °C) for 2 hours to solidify. Wells were opened in the solidified agar medium using sterile 1 mL pipette tips. 100 µL of supernatants were loaded to the wells and filter sterilized distilled water and 0.3 mg/mL of chloramphenicol solution were used as negative and positive controls, respectively. *E. coli* and *S. aureus* plates were incubated at 37°C and *B. cereus* plates were incubated at 25°C for 24 h. At the end of the incubation periods, the diameters of the inhibition zones were measured.

3.2.4.4. Angiotensin Converting Enzyme Inhibitory (ACE-I) Activity

ACE-I was determined by using the method of Martínez-Alvarez et al. (2016), which is based on the release of hippuric acid (HA) as a result of the reaction of hippuryl-His-Leu (HHL) as a substrate with the ACE enzyme. 25 milliUnit.mL⁻¹ concentration of the enzyme solution was prepared in ultrapure water. 5 mM of HHL solution was prepared in the 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl. Then, 40 µL of samples were mixed with 200 µL HHL solution followed by addition of the 40 µL of the enzyme solution. The test tubes were vortexed and incubated at 37°C for 60 minutes at a water bath. The reaction was stopped using 100 µL of 1M HCl. The HA released from the reaction was quantitated using HPLC in Gemini C18 column (Phenomenex, 110A°, 150x 46 mm, particle size of 5 µm, Phenomenex Co., Torrance, CA, USA). The mobile phases that were used were: solvent A, 0.1% (v/v) distilled water containing trifluoroacetic acid and solvent B 0.1% (v/v) acetonitrile containing trifluoroacetic acid. The elution conditions were solvent B at 20% between 0-5 min, solvent B from 20% to 60% between 5-15 min, and solvent B 60% between 15-19 minutes and then, it was returned to initial elution conditions (solvent B 20%) between 19-21 minutes. The flow rate was adjusted 0.8 mL.min⁻¹ and the column temperature was adjusted to 25 °C. The peaks for HA and HHL were monitored at a wavelength of 228 nm. ACE-I was calculated from the Equation 3.4:

$$ACE - I (\%) = \left[1 - \frac{A_{inhibitor}}{A_{control}} \right] \times 100 \quad (3.4)$$

$A_{\text{inhibitor}}$ and A_{control} indicate the relative peak areas of the HA with samples and without samples, respectively.

3.2.5. Descriptive Sensory Analysis

For the descriptive sensory analysis, a sensory analysis panel consisting of a group of 7 people experienced in sensory analysis was established. Spectrum™ method was applied to describe the taste and flavor attributes of the unfermented and fermented *Spirulina* products. 20 mL of the samples were transferred to a small jar (40 mL) with cap and the jars were kept in water bath and at 40°C for 20 min to provide the accumulation of volatiles in the headspace. The jars were labelled with 3-digit numbers and 3 jars were prepared for each sample. The terminology developed by Sahin et al. (2022) and Kurt et al. (2023) was used as a basis. The panelists quantified the attributes on a 15-point scale (0=no aroma intensity, 15=very high aroma intensity).

3.2.6. Ultrafiltration of Unfermented *Spirulina* and Cascade Fermented *Spirulina* Products

The supernatants of the cascade fermented *Spirulina* products and unfermented *Spirulina* products were fractionated to molecular weights above 10 kDa, between 5-10 kDa and below 5 kDa molecular weight using tangential flow filtration and 10 kDa (VivaFlow 50R, Sartorius Stedim Biotech GmgH, Germany) and 5 kDa (Vivaflow 200, Sartorius Stedim Biotech GmgH, Germany) cut-off ultrafiltration cassettes. Prior to fractionation, the supernatants were vacuum filtered through Glass-Microfibre Discs (Sartorius, Goettingen) with a pore size of 1.2 µm and then through a pore size of 0.45 µm Cellulose Nitrate Membrane Disc Filter (mdi, India) to prevent the clogging of the cassettes. Samples were collected by diluting with 0.1 M PBS (pH 7.7) gradually to maximize the separation of compounds in the circulation of the system. The samples that could not pass through the 10 kDa cut-off cassette and remained in the reservoir were the “>10 kDa” fraction. The portion of samples that passed through the 10 kDa cut-off membrane but did not pass the 5 kDa cut-off membrane and remained in the reservoir was the “5-10 kDa” fraction. The part of the samples passed the 5 kDa cut-off membrane was the “<5kDa” fraction.

The ultrafiltration system was demonstrated in Fig. 3.1. As dilution with PBS resulted in large volumes of diluted samples at this stage, the fractions were evaporated in a rotary evaporator at 45°C under vacuum and the concentrated fractions were freeze-dried.



Figure 3.1: Fractionation of unFS and cascade FS products by tangential flow filtration using ultrafiltration units (Vivaflow 200, Sartorius). The sample that could not pass through the cassette and collected in the reservoir (on the left); the sample that passed through the cassette (on the right). (The sample in the reservoir and the sample collected in the filtrate section were kept in an ice box to prevent heating of the samples and cables).

3.2.7. Effect of *In Vitro* Gastrointestinal Digestion on the Bioactivity of Unfermented *Spirulina* and Cascade Fermented *Spirulina* Products

To evaluate the effect of gastrointestinal digestion, a static gastrointestinal digestion model proposed by Minekus et al. (2014) was performed by Prof. Dr. Sibel Karakaya's laboratory team (Department of Food Engineering, Ege University). Samples collected at the end of gastric and gastrointestinal digestion were first passed through 10 kDa ultrafiltration tubes (Vivaspin 15R, Sartorius) by dilution with 0.1 M PBS (pH 7.7). The samples were then centrifuged at 4000 rpm for 10 min. Dilution and centrifugation were repeated for several times to remove digestive enzymes with a molecular weight higher than 10 kDa, and the filtrate was collected and analyzed for total phenolic content, DPPH, TEAC and ACE-I activities as described previously. The procedure is summarized in Figure 3.2.

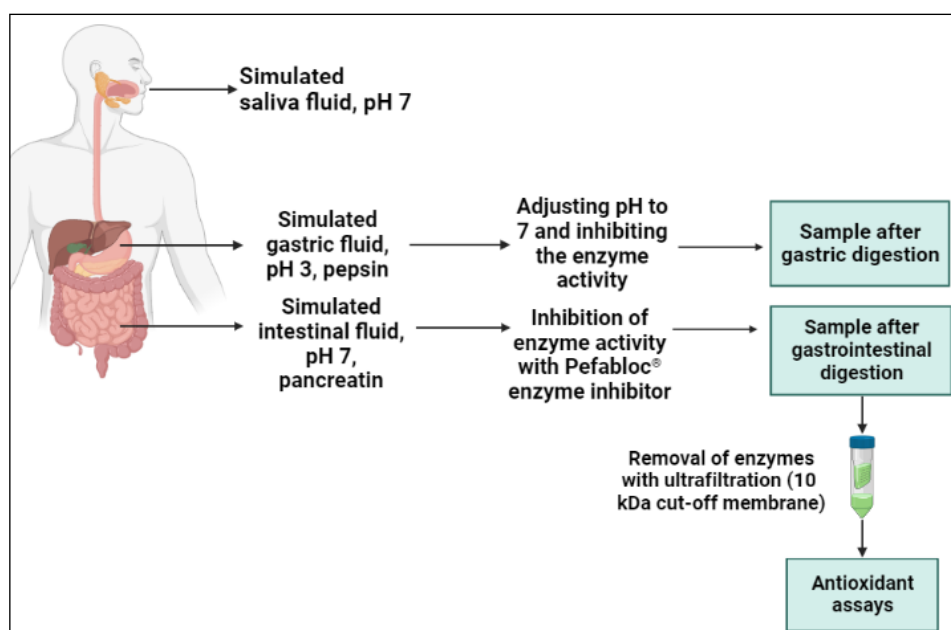


Figure 3.2: Summary of workflow to investigate the effect of gastrointestinal digestion on the bioactive properties of unFS and cascade FS products.

3.3. Statistical Analysis

Experimental results were averages of duplicate analyses. Data was analyzed using Minitab 17 (Minitab Inc., State College, PA). One way analysis of variance (ANOVA) was followed by the TUKEY HSD test for multiple comparisons with a confidence level of 95% (differences were considered significant at $p \leq 0.05$). To perform principal component analysis (PCA) a free trial version of XLSTAT statistical program was used.

4. RESULTS and DISCUSSION

According to our previous experiments performed in shake flasks level, 60 g/L *Spirulina* and 6 g/L lactose concentrations were suitable for the fermentation by mixed culture of *L. helveticus* and *K. marxianus* (unpublished results). Therefore, the same media composition was used in the bioreactor conditions, but optimization of some environmental parameters (such as stirring speed, dissolved O₂ concentration, aeration rate, and pH), which couldn't be optimized at the flask level, were performed in bioreactor scales in the scope of the current study. The effect of these conditions on the growth of the mixed culture of *L. helveticus* and *K. marxianus* and on biochemical properties of FS products in terms of their protein content, degree of hydrolysis, protein fragmentation, and some bioactive properties (total phenolic content (TPC), antioxidant activity, ACE inhibitory and antimicrobial activity) were investigated. Taken together all these properties among all conducted bioreactor conditions, the optimum bioreactor operating condition was determined. Additionally, for the chosen bioreactor production the effect of *in vitro* digestion and its different UF fractionations (>10 kDa, 5-10 kDa and <5 kDa) were investigated in terms of some bioactive properties (TPC, antioxidant activity and ACE inhibitory activity).

4.1. Microbial Growth of *L. helveticus* and *K. marxianus* in Different Bioreactor Conditions

During the fermentation period (72 h), samples were taken at regular intervals and diluted with sterile saline (0.85% NaCl) and plated on YM agar medium and MRS agar medium by pour plate method to determine the number of viable yeast cells and bacteria, respectively. As can be seen in Figure 4.1, similar cell numbers for both *L. helveticus* and *K. marxianus* were observed at different production parameters.

Many studies have investigated the suitability of *Spirulina* for the growth of different microorganisms, especially lactic acid bacteria (LAB) [Bao et al., 2018], [de Marco Castro et al., 2019], [Kurt et al., 2023], [Niccolai et al., 2020]. At the beginning of the fermentations, the number of viable *L. helveticus* cell counts in the

medium started with 6.5-6.8 log CFU/mL and exceeded 9 log CFU/mL as a result of 24 h fermentations in 4 different batch bioreactor operation conditions, and the highest number of viable cells was reached with 9.6 log CFU/mL in a bioreactor performed with “controlled pH” condition (Figure 4.1a). In a recent study that investigating the effect of fermentation of lactic acid bacteria (LAB) and different *Bacillus* strains, *L. helveticus* also reached maximum viable cell number (around 9 log CFU/mL) by 24 h of fermentation; however, a significant decrease occurred in the viable cell number by 48 h of fermentation in the same study [Kurt et al., 2023]. In contrast, in this study, cell numbers were maintained in all productions after 48 hours of fermentation. And all productions maintained their viable cell numbers, except for the “controlled pH” mode at the end of the fermentation, which had a slightly less viable cell number compared to its viable cell number at 48 h.

The inoculum required for yeast fermentations were prepared with *K. marxianus* of similar activity. The turbidimetry of the culture was adjusted to McFarland 5 using a McFarland densitometer to keep the initial yeast cell counts in a similar range across all productions. However, it was found that there were differences in the initial yeast cell counts of bioreactors performed under different operation conditions. Under constant “0.5 vvm” and “1 vvm” aeration conditions, the productions started with cell counts of 5.5 and 5.7 log CFU/mL, respectively, while the “controlled pH” production started with cell counts of 6.4 log CFU/mL and the “cascade” production started with cell counts of 5.1 log CFU/mL (Figure 4.1b). However, at the end of 24 hours, when these four different bioreactor productions were compared, the largest increase in yeast cells was found in the “cascade” mode with approximately 3.1 log. At the end of fermentation, the cell numbers were similar in “0.5 vvm”, “1 vvm” and “cascade”, whereas it was lower in the “controlled pH” production. In a recent study performed by Sahin et al. (2022) to investigate the effects of fermentation of *Spirulina* with three different yeast species (*Debaryomyces hansenii*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*), *Spirulina* was a suitable medium for these yeast species to grow without the need for additional carbon source and as result of 48 h of fermentation, viable cell number was 8.26 log CFU/mL for *K. marxianus*. However, in the present study, *K. marxianus* reached similar cell numbers in 24 h fermentation and maintained this number in general. This suggests that performing a similar process in the bioreactor level where aeration

and mechanical stirring took place, might accelerate the growth of the yeast cells compared to the flask level productions.

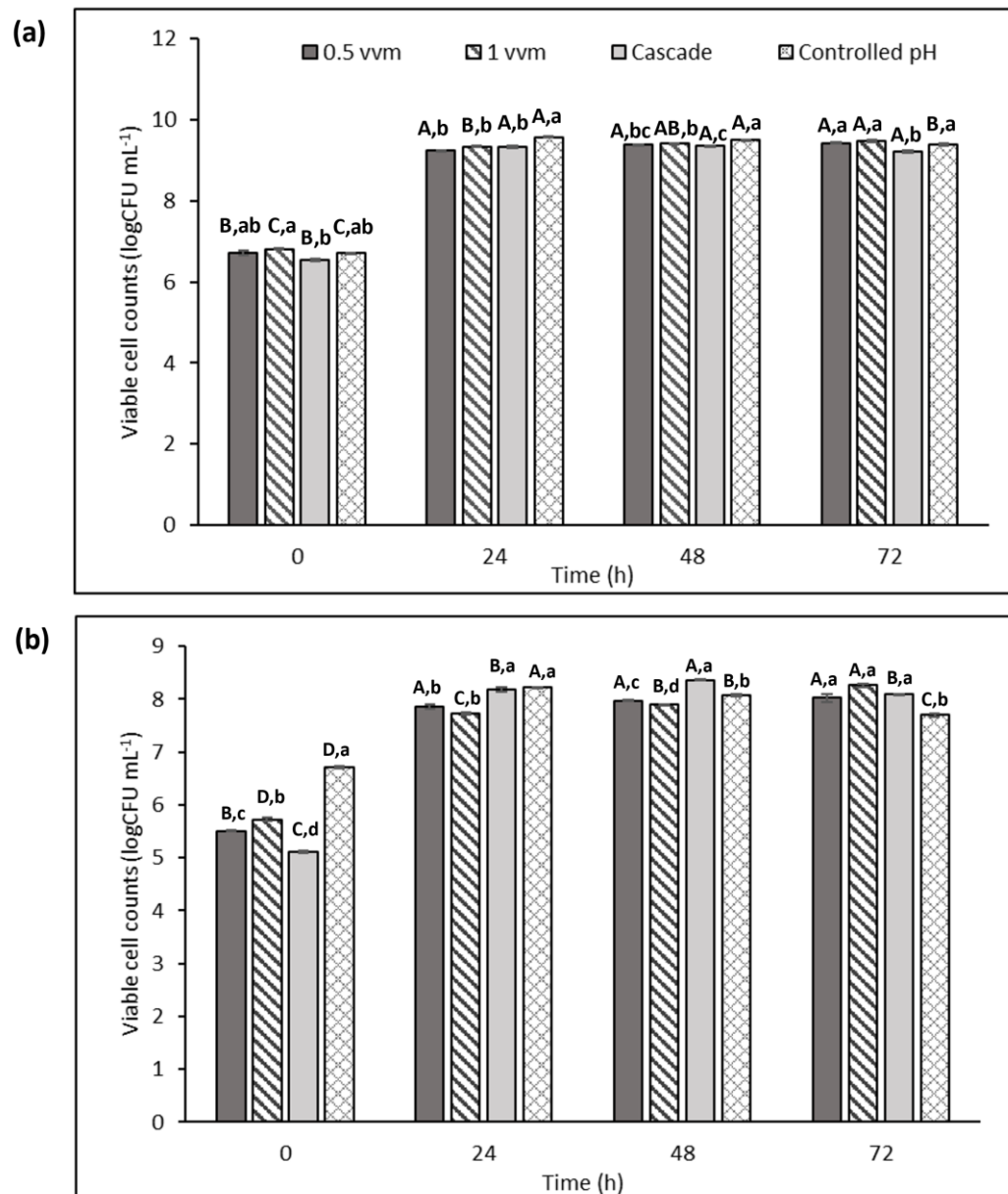


Figure 4.1: Effects of different parameters (0.5 vvm and 1 vvm constant air, cascade mode and controlled pH) at 3-L bioreactor level on the growth of (a) *L. helveticus*, (b) *K. marxianus* during 72 h of fermentation with mixed culture of *L. helveticus* and *K. marxianus* for the production of fermented *Spirulina*. ^{A-D} Means in the same production condition followed by different capital letters indicate significant changes during fermentation. ^{a-d} Means in the same fermentation time followed by different lowercase letters indicate significant changes between different production conditions (p<0.05).

Changes in pH and dissolved oxygen values were two other important parameters showing the progress of the process, and these changes are shown in Figure 4.2. For all productions, the initial pH was around 6.1-6.3. In the "0.5 vvm" and "1 vvm" operating conditions, a decrease in pH value was observed during the first 24 hours, while an increase in pH was observed in the "cascade" production during this period. Thereafter, an increase in pH was observed in all these three operating conditions. Sahin et al. (2022) observed a decrease in pH after 24 h of fermentation, reaching 5.5-5.9 followed by an increase after 48 h of fermentation, in which FS by *K. marxianus* showed the highest increase compared to other yeast species *S. cerevisiae* and *D. hansenii*, reaching a pH value of 7.5. On the other hand, fermentation of *Spirulina* by different LAB species caused a decrease in pH to the range of 2.9-3.1 after 24 h of fermentation due to the production of organic acids (Kurt et al. 2023). Thus, in the current study a slight decrease in the pH at the beginning of the process and then an increasing trend in pH may also be associated with the consumption of organic acids [Hamme et al., 2009]. Also, as a result of the proteolytic activity of the microorganisms, an increase in the amino acid content, hydrolyzed proteins and other metabolites, may have increased the pH. In the case of "controlled pH" production, the pH reached 5.70 after about 12 h of fermentation and the "constant pH" control was initiated.

During the first 24 h of fermentation, the dissolved oxygen level remained low in all productions; it was found that the cascade production was insufficient to maintain the dissolved oxygen at 30% between 10 and 20 h. Since this period coincides with the hours when the microorganisms were in the logarithmic phase, the rapid increase in the number of cells can cause a rapid decrease in dissolved oxygen.

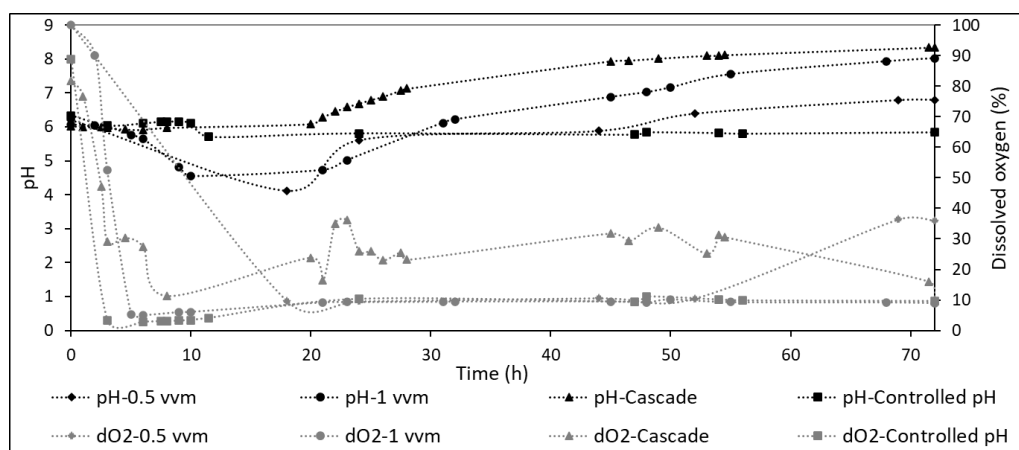


Figure 4.2: Changes in pH and dissolved oxygen (DO2) levels during 72 hours of fermentation with mixed culture of *L. helveticus* and *K. marxianus* at 3-L bioreactor level with different parameters (0.5 vvm and 1 vvm constant air, cascade mode and controlled pH) for fermented *Spirulina* production.

4.2. Total Soluble Protein Concentration, Degree of Hydrolysis and Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) Protein Profiles of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions

The differences in total soluble protein concentration (TSPC) and degree of hydrolysis (DOH) for different bioreactor productions were investigated and presented in Figure 4.3. The initial TSPC for the productions carried out under different bioreactor conditions were determined as 3.66 g/L for "cascade", 4.67 and 4.93 g/L for "0.5 vvm" and "1 vvm", and 5.74 g/L for "constant pH" conditions, respectively. There was no significant change in TSPC during the "constant pH" condition, while an increase was observed in the other conditions (Appendix G with statistical analysis). The highest increase in TSPC was observed in "cascade" production, and TSPC reached 9.10 g/L at the end of the production. In "1 vvm" conditions, a value close to the "cascade" production of 8.65 g/L was determined, in contrast to the production carried out under "0.5 vvm" conditions, of which TSPC was determined to be 5.75 g/L. Alkaline conditions have been reported to increase the extraction of soluble proteins from *S. platensis* as by aiding in the solubilization of the labile cell wall compounds and thus, production under "controlled pH" conditions may have a negative effect on the TSPC [Safi et al., 2014]. A similar trend with TSPC was observed in the results of the degree of hydrolysis. While there

was no significant increase in the degree of hydrolysis at 48 and 72 h in the "controlled pH" and "0.5 vvm" production conditions, there was a significant increase in the degree of hydrolysis at the end of fermentation in the "1 vvm" and "cascade" production conditions, reaching 4.14 and 5.71 g/L for "1 vvm" and "cascade", respectively. Although the increasing trend of the degree of hydrolysis in the "controlled pH" condition was similar to that of the "0.5 vvm" production and higher at all hours, no significant change in the concentration of TSPC was observed in the "controlled pH" production, while an increase in the TSPC was observed in the "0.5 vvm" product.

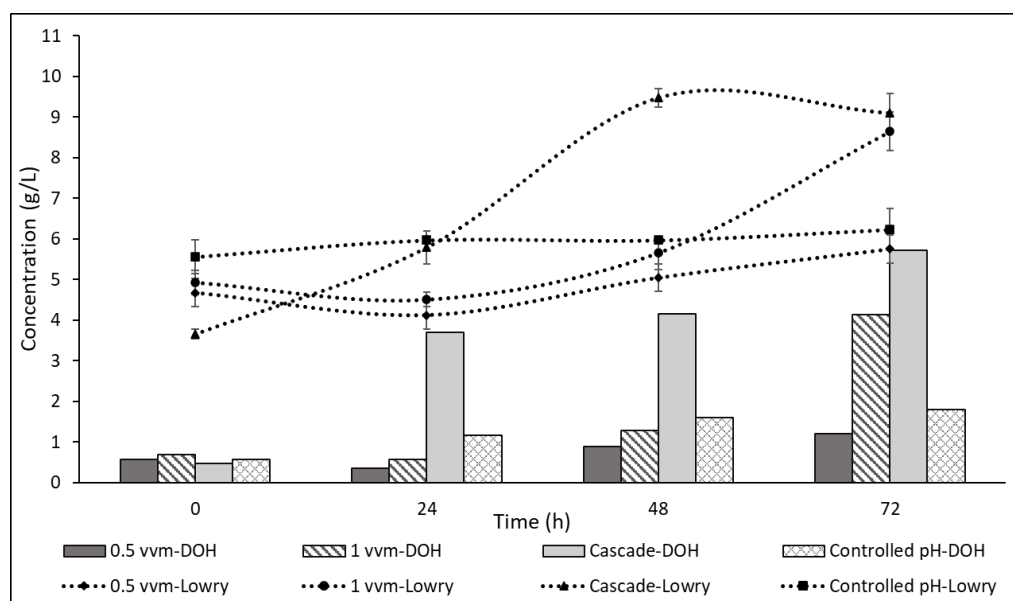


Figure 4.3: Changes in total soluble protein concentration and degree of hydrolysis (DOH) determined by Lowry and cadmium-ninhydrin method, respectively during 72 hours of fermentation with mixed culture of *L. helveticus* and *K. marxianus* at 3-L bioreactor level with different parameters (0.5 vvm and 1 vvm constant air, cascade mode and controlled pH) for fermented *Spirulina* production.

The protein profiles obtained by SDS-PAGE gel electrophoresis also supported the results of the degree of hydrolysis. As seen in Figure 4.4, there was a thick band between 75 kDa-100 kDa and a band around 135 kDa in unfermented *Spirulina* (unFS) samples. There were also faint bands between 48 kDa-63 kDa and 35 kDa-48 kDa and a thick band between 17-20 kDa in unFS, which could be the subunits of phycocyanin as the molecular weight of α and β subunits are around 18.4 kDa and 21.3 kDa [Chaiklahan et al., 2011]. This band remained unfragmented in the fermentation of *Spirulina* by *L. plantarum* in the study of De Marco Castro et al.

(2019), whereas it was fragmented in all bioreactor productions in the current study. This might be due to the differences in the fermentation conditions as well as the difference in microorganism species. UnFS also contained weak medium molecular weight bands (35-63 kDa) compared to high molecular weight bands (75-135 kDa), which may be a result of freeze-thawing the *Spirulina* solution prior to fermentation and additional disruption using a sonicator. In all productions, the high molecular weight protein bands found at the unFS were replaced by various lower molecular weight proteins as a result of fermentation. Although low-molecular-weight bands were present in all bioreactor productions, there were some differences in their profile and intensity. For example, although the products obtained from "0.5 vvm" and "1 vvm" production were similar in some aspects, such as the decrease in intensity of the band between 75-100 kDa and the presence of a dark band around 48 kDa, some differences were also observed. The bands between 63-75 kDa in "0.5 vvm" became weaker in "1 vvm", the number of bands between 35-48 kDa in "1 vvm" was less than in "0.5 vvm", and intensity of the bands were high compared to the ones that formed in the "0.5 vvm". On the other hand, the number of bands decreased in "cascade" production, and the bands seen in "0.5 vvm" and "1 vvm" production were found to be weaker in "cascade" production. This not only supported the high degree of hydrolysis in the "cascade" production, but also indicates that the small molecular weight protein structures formed during the fermentation can be degraded or used by *L. helveticus* & *K. marxianus*. In the "constant pH" production, the number of bands formed was higher than in the other productions. Different bands between 25-35 kDa and 35-48 kDa were formed. It has been reported that high pH during fermentations could positively affect the activities of microbial proteases [Jain and Anal, 2017]. In this study, the low number of bands in the protein profile of the "cascade" product, in which the pH increased throughout the fermentation, could support this phenomenon.

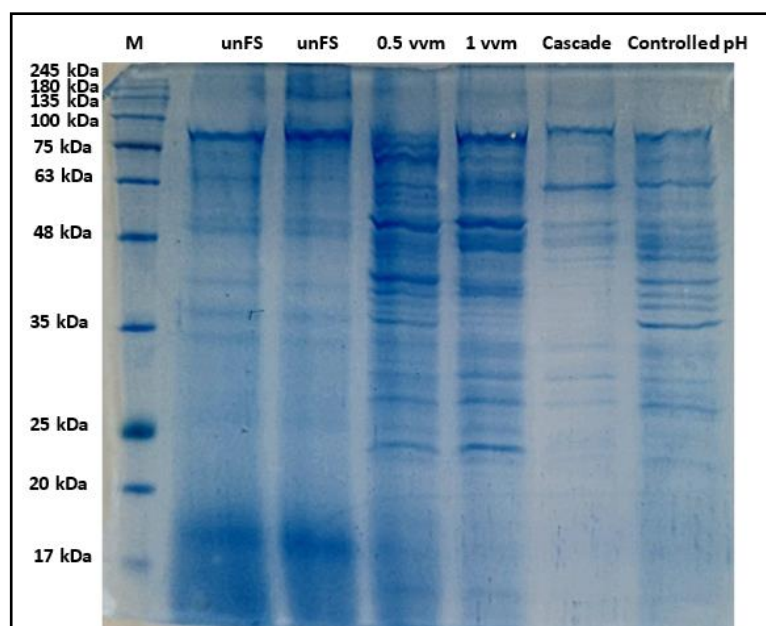


Figure 4.4: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of unfermented control products and fermented products obtained at the end of 72 h fermentation with mixed culture of *L. helveticus* and *K. marxianus* at 3-L bioreactor level with different parameters (0.5 vvm and 1 vvm constant air, cascade mode and controlled pH) to produce fermented *Spirulina*. M indicates the standard protein marker between 10.5 kDa-175 kDa.

4.3. Total and Free Amino Acid Composition of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions

The amino acid profile of a given food protein is an important indicator of its nutritional value. The changes in total amino acid (TAA) and free amino acid (FAA) concentrations of unfermented *Spirulina* (unFS) and fermented *Spirulina* (FS) products obtained under different bioreactor conditions (0.5 vvm, 1 vvm, cascade and constant pH) are presented in Table 4.1 and Table 4.2, respectively. In order to see the changes clearly, the heat map plots for the TAA and the FAA are also shown in Figure 4.5 and 4.6, respectively. When *Spirulina* fermented under different conditions was analyzed in terms of TAA composition, high contents of TAA were found in "constant pH" and "0.5 vvm" products and were $43953.9 \pm 577.2 \mu\text{M}$ and $43514.98 \pm 583.18 \mu\text{M}$, respectively. These were followed by the product produced under "1 vvm" condition ($32436 \pm 1798.79 \mu\text{M}$). The lowest TAA concentration was found in the "cascade" production with $27015.48 \pm 347.04 \mu\text{M}$, which may be due to cell growth and high catabolism rate of amino acids such as, decarboxylation,

transamination and deamination [Ardö, 2006], [Yvon and Rijnen, 2001]. In contrast to the results obtained for FS by *K. marxianus* and *L. helveticus* in monoculture fermentation at flask level, the TAA composition of the fermented products obtained under "constant pH" and "0.5 vvm" bioreactor conditions was found to be higher than in the unFS groups ($34322.35 \pm 4304.62 \mu\text{M}$) [Kurt et al., 2023], [Sahin et al., 2022]. On the other hand, the total essential amino acid (TEAA) content was higher in all FS products compared to the unFS. FS products obtained under different bioreactor conditions were found to have differences in TAA profiles. Glutamic acid was the most abundant amino acid with $7344.86 \pm 741.41 \mu\text{M}$ in the unFS and increased to 8879.41 ± 15.4 and $8242.57 \pm 16.44 \mu\text{M}$ in "0.5 vvm" and "constant pH" productions, respectively; it decreased to $5781.17 \pm 334 \mu\text{M}$ in "1 vvm" production and decreased significantly to $3475.13 \pm 24.32 \mu\text{M}$ in the "cascade" production. A similar situation was found for the amino acids alanine and aspartic acid; the concentrations, which were similar to the unFS in productions other than "cascade" conditions, decreased in "cascade" production. Cysteine was the less abundant amino acid in both unFS and FS groups similar to the study by Verni et al. (2021).

Mohammadi et al. (2022) investigated the effect of hydrolysis of *Spirulina* by pancreatin and pepsin to obtain *Spirulina* hydrolysates, pancreatin hydrolysate and pepsin hydrolysate respectively. They reported that hydrolysis had no significant effect on the percentage content of amino acids of pepsin hydrolysate. Both hydrolysates were found to be rich in acidic amino acids (aspartic acid and glutamic acid), arginine, valine, lysine, alanine, glycine, threonine and leucine. And, when they compared their results with Food and Agriculture Organization (FAO) recommendation for adults, they found that *Spirulina* hydrolysates contained all essential amino acids at concentrations higher than the FAO recommendation. Only sulfur-containing amino acids were limited before and after hydrolysis.

Table 4.1: Total amino acid content of unfermented *Spirulina* and fermented *Spirulina* products obtained at the end of 72 hours of fermentation with different parameters (“0.5 vvm” and “1 vvm” constant air, “cascade” mode and “controlled pH”) at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus*.

Amino acid	Amino acid concentration ($\mu\text{M}\pm\text{SE}$)				
	unFS	0.5 vvm	1 vvm	Cascade	Controlled pH
Aspartic acid	3495.17 $\pm$ 480.09	4657.13 $\pm$ 57.86	3759.28 $\pm$ 214.82	2648.28 $\pm$ 4.28	5113.66 $\pm$ 0.56
Glutamic acid	7344.86 $\pm$ 741.41	8879.41 $\pm$ 15.40	5781.17 $\pm$ 334.00	3475.13 $\pm$ 24.32	8242.57 $\pm$ 16.44
Serine	2064.22 $\pm$ 322.44	2744.48 $\pm$ 10.93	1424.58 $\pm$ 58.08	811.28 $\pm$ 28.43	2405.25 $\pm$ 0.50
Histidine	167.94 $\pm$ 25.77	282.98 $\pm$ 4.67	241.68 $\pm$ 30.91	334.95 $\pm$ 6.51	307.86 $\pm$ 0.14
Glycine	3425.63 $\pm$ 420.52	4274.06 $\pm$ 12.59	3028.34 $\pm$ 162.79	2250.53 $\pm$ 17.80	4451.04 $\pm$ 4.34
Threonine	1487.22 $\pm$ 391.98	2434.41 $\pm$ 113.52	1643.97 $\pm$ 95.17	903.39 $\pm$ 10.62	2431.33 $\pm$ 5.50
Arginine	1191.53 $\pm$ 164.44	1299.68 $\pm$ 21.74	558.74 $\pm$ 30.84	1443.50 $\pm$ 12.28	1576.19 $\pm$ 0.74
Alanine	4173.67 $\pm$ 562.43	5236.05 $\pm$ 24.95	3689.26 $\pm$ 200.19	1705.72 $\pm$ 18.44	4173.13 $\pm$ 9.16
Tyrosine	642.33 $\pm$ 78.14	777.52 $\pm$ 8.34	945.54 $\pm$ 48.50	1470.60 $\pm$ 12.30	1197.70 $\pm$ 0.92
Cysteine	71.82 $\pm$ 7.25	54.54 $\pm$ 0.19	59.59 $\pm$ 9.99	86.20 $\pm$ 2.79	50.17 $\pm$ 0.95
Valine	1650.02 $\pm$ 225.53	2385.86 $\pm$ 4.02	1824.18 $\pm$ 102.37	1718.90 $\pm$ 13.97	2582.28 $\pm$ 4.10
Methionine	345.95 $\pm$ 74.70	387.56 $\pm$ 1.92	490.58 $\pm$ 149.44	865.70 $\pm$ 0.07	851.03 $\pm$ 2.75
Phenylalanine	913.42 $\pm$ 136.25	1868.25 $\pm$ 9.53	1266.32 $\pm$ 80.30	1705.89 $\pm$ 81.25	1474.66 $\pm$ 185.82
Isoleucine	1008.68 $\pm$ 119.99	731.43 $\pm$ 0.93	1235.50 $\pm$ 70.09	917.57 $\pm$ 22.57	1528.84 $\pm$ 32.51
Leucine	2132.08 $\pm$ 329.70	2634.53 $\pm$ 10.28	1919.69 $\pm$ 113.32	1767.17 $\pm$ 20.70	2631.96 $\pm$ 4.00
Lysine	848.82 $\pm$ 163.92	1108.48 $\pm$ 9.11	1085.48 $\pm$ 80.45	1311.60 $\pm$ 14.25	1477.64 $\pm$ 6.98
Proline	3358.99 $\pm$ 60.06	3758.61 $\pm$ 277.20	3482.97 $\pm$ 17.53	3599.07 $\pm$ 56.46	3458.59 $\pm$ 301.79
Total AA	34322.35 $\pm$ 4304.62	43514.98 $\pm$ 583.18	32436.87 $\pm$ 1798.79	27015.48 $\pm$ 347.04	43953.9 $\pm$ 577.20
Total EAA	8554.13 $\pm$ 1467.84	11833.50 $\pm$ 153.98	9707.40 $\pm$ 722.05	9525.17 $\pm$ 169.94	13285.60 $\pm$ 241.80
Total EAA/Total AA (%)	24.92	27.19	29.93	35.26	30.23

AA: Amino acid, EAA: Essential amino acid, SE: Standard error.

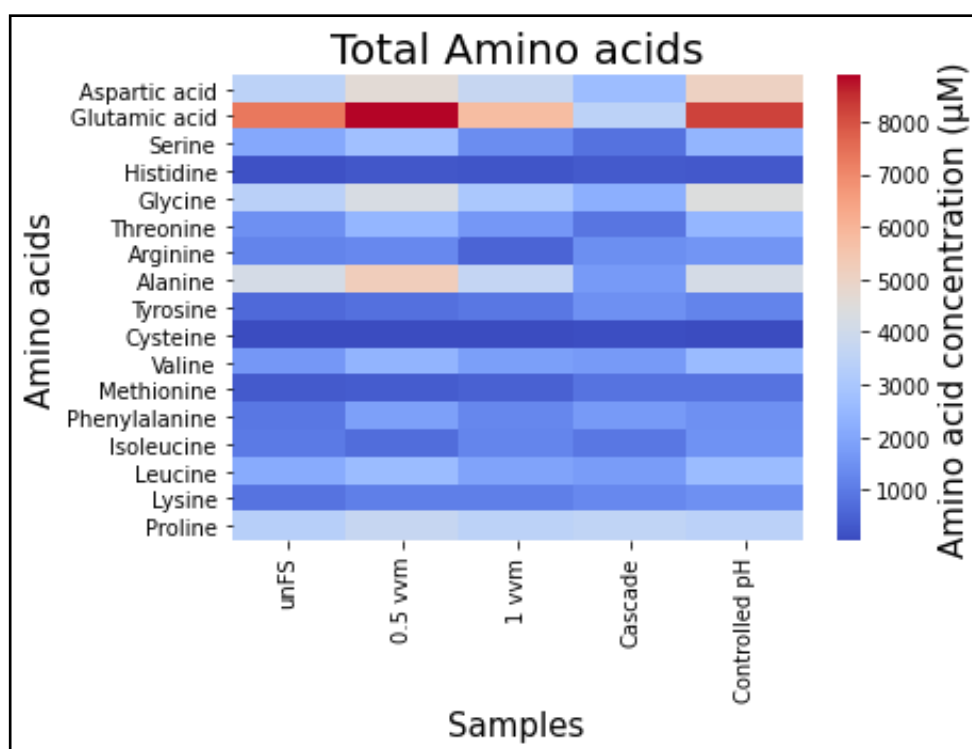


Figure 4.5: Visualization of total amino acid content of unfermented *Spirulina* and fermented *Spirulina* products obtained at the end of 72 hours of fermentation with different parameters (0.5 vvm and 1 vvm constant air, cascade mode and controlled pH) at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus* in heatmap.

Considering the changes in FAA concentrations of the products, it was found that there was an increase in FAA contents in all FS products compared to the unFS group. However, when analyzed on the basis of different conditions, the total FAA concentration was 4495.14 ± 251.45 μM in the unFS product, while it reached 12952.70 ± 157.54 and 14939.45 ± 431.48 μM in the "0.5 vvm" production and "constant pH" production, respectively. This increase was at the highest level in the "1 vvm" and "cascade" conditions and reached 38870.56 ± 216.32 and 32479.12 ± 334.82 μM , respectively. Although the total TAA profile was similar in these two conditions, there were differences in terms of some amino acid concentrations. For example, while the highest concentrations of glutamic acid and alanine were determined as 6260.44 ± 58.89 and 6158.99 ± 34.68 μM , respectively, under "1 vvm" conditions; the concentrations of glutamic acid and alanine were 3135.97 ± 62.66 and 2166.66 ± 20.68 μM , respectively, under "cascade" condition. On

the other hand, amino acids such as arginine, tyrosine, valine, methionine and phenylalanine were more prominent in "cascade" compared to "1 vvm" condition. In particular, it is noteworthy that aromatic amino acids (phenylalanine and tyrosine) were more significantly increased in "cascade" FS. These amino acids are known to contribute to the antioxidant and ACE inhibitory activities of proteins and peptides, and the presence of such amino acids may support the high free radical capture and ACE inhibitory abilities obtained from protein hydrolysates as a result of fermentation of egg shell membranes by LAB [Jain and Anal, 2017]. For example, in a study in which longan fruit was fermented with lactic acid bacteria, fermentation decreased the concentrations of free amino acids such as phenylalanine, tyrosine, and leucine, which can cause bitterness, while increasing cysteine and gamma-aminobutyric acid, which have the potential to show antioxidant effects [Khan et al., 2018]. In another study on egg yolk, it was stated that tryptophan and tyrosine amino acids make a great contribution to the antioxidant properties of egg yolk [Nimalaratne et al., 2011]. Therefore, these differences in total TAA contents contain important findings that can shed light on the evaluation of the bioactive properties of the products. The presence of different amino acids in such products may cause differences in their bioactive properties as well as taste/flavor characteristics as amino acid catabolism can lead to formation of different aromas. For instance, 3-methylbutanal, methional and isovaleric acid are major odorants found in Cheddar, Camembert and Emmental cheeses that formed as a result of the amino acid degradation [Ardö, 2006], [Yvon and Rijnen, 2001]. Also, similar to De Marco Castro et al. (2019), the free methionine content of different bioreactor products increased considerably in all FS products except the "0.5 vvm" condition. The total FAA content was higher at all FS products compared to unFS, with the highest content belonging to "1 vvm" followed by the cascade FS. The FS products in these two operating conditions had a good degree of hydrolysis which supported the higher FAA content. The FEAA content of the "cascade" was the highest followed by "1 vvm" FS, which also had considerably high amounts of the total FEAA. Although the "controlled pH" FS did not contain as much FEAA as the "1 vvm" and "cascade" FS, when the total FEAA content was compared to its total FAA, FEAA was a significant portion with 42%.

Table 4.2: Free amino acid content of unfermented *Spirulina* and fermented *Spirulina* products obtained at the end of 72 hours of fermentation with different parameters (“0.5 vvm” and “1 vvm” constant air, “cascade” mode and “controlled pH”) at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus*.

Amino acid	Amino acid concentration ($\mu\text{M}\pm\text{SE}$)				
	unFS	0.5 vvm	1 vvm	Cascade	Controlled pH
Aspartic acid	67.50 $\pm$ 3.47	234.56 $\pm$ 1.64	2946.29 $\pm$ 15.12	1466.70 $\pm$ 8.93	445.43 $\pm$ 1.45
Glutamic acid	1035.29 $\pm$ 17.56	2221.64 $\pm$ 22.41	6260.44 $\pm$ 28.89	3135.97 $\pm$ 62.66	2004.70 $\pm$ 13.20
Serine	140.09 $\pm$ 8.38	1004.88 $\pm$ 5.71	1682.32 $\pm$ 8.07	571.09 $\pm$ 31.99	788.30 $\pm$ 8.71
Histidine	55.68 $\pm$ 3.39	131.76 $\pm$ 0.28	349.85 $\pm$ 2.41	391.28 $\pm$ 4.68	105.51 $\pm$ 2.35
Glycine	218.15 $\pm$ 6.23	701.70 $\pm$ 10.76	2554.80 $\pm$ 28.94	1876.14 $\pm$ 6.64	548.00 $\pm$ 0.68
Threonine	73.29 $\pm$ 4.29	716.80 $\pm$ 6.31	1662.50 $\pm$ 8.47	403.02 $\pm$ 7.08	569.76 $\pm$ 3.57
Arginine	153.84 $\pm$ 12.63	366.12 $\pm$ 3.65	223.67 $\pm$ 2.62	2556.85 $\pm$ 18.19	571.48 $\pm$ 3.33
Alanine	911.88 $\pm$ 18.70	2213.18 $\pm$ 48.54	6158.99 $\pm$ 34.68	2166.66 $\pm$ 20.68	1311.09 $\pm$ 9.65
Tyrosine	64.18 $\pm$ 6.61	413.11 $\pm$ 6.73	1932.26 $\pm$ 10.64	3055.84 $\pm$ 53.46	958.20 $\pm$ 6.45
Cysteine	148.19 $\pm$ 3.80	728.02 $\pm$ 7.79	1833.81 $\pm$ 6.20	1768.22 $\pm$ 9.66	1045.42 $\pm$ 6.60
Valine	36.77 $\pm$ 5.44	181.44 $\pm$ 1.14	1329.25 $\pm$ 2.90	2104.26 $\pm$ 7.54	441.72 $\pm$ 4.16
Methionine	85.93 $\pm$ 9.14	67.77 $\pm$ 0.48	1341.50 $\pm$ 3.55	2220.70 $\pm$ 9.57	1196.83 $\pm$ 14.10
Phenylalanine	108.43 $\pm$ 15.79	919.02 $\pm$ 7.99	2525.39 $\pm$ 14.38	3026.12 $\pm$ 24.66	737.61 $\pm$ 0.25
Isoleucine	100.51 $\pm$ 3.39	669.96 $\pm$ 4.06	1605.60 $\pm$ 14.40	1348.91 $\pm$ 17.83	848.31 $\pm$ 8.54
Leucine	174.53 $\pm$ 25.74	1135.47 $\pm$ 0.43	2901.09 $\pm$ 21.74	2866.22 $\pm$ 25.08	1652.21 $\pm$ 20.46
Lysine	130.73 $\pm$ 6.41	492.91 $\pm$ 2.68	1845.62 $\pm$ 7.10	2291.66 $\pm$ 22.59	786.89 $\pm$ 153.29
Proline	990.15 $\pm$ 100.48	754.36 $\pm$ 26.94	1717.18 $\pm$ 6.21	1229.48 $\pm$ 3.58	927.99 $\pm$ 174.69
Total FAA	4495.14 $\pm$ 251.45	12952.7 $\pm$ 157.54	38870.56 $\pm$ 216.32	32479.12 $\pm$ 334.82	14939.45 $\pm$ 431.48
Total FEAA	765.87 $\pm$ 73.59	4315.13 $\pm$ 23.37	13560.80 $\pm$ 74.95	14652.17 $\pm$ 119.03	6338.84 $\pm$ 206.72
Total FEAA/Total FAA (%)	17.04	33.32	34.89	45.11	42.43

FAA: Free amino acid, FEAA: Free essential amino acid, SE: Standard error.

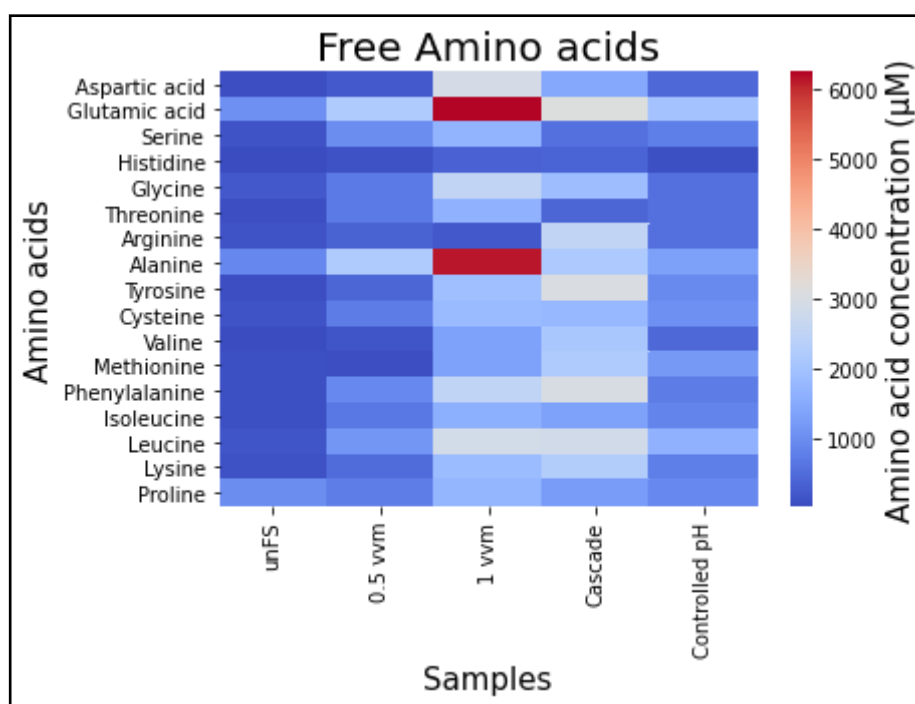


Figure 4.6: Visualization of free amino acid content of unfermented *Spirulina* and fermented *Spirulina* products obtained at the end of 72 hours of fermentation with different parameters (0.5 vvm and 1 vvm constant air, cascade mode and constant pH) at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus* in heatmap.

4.4. Evaluation Bioactive Properties of Unfermented and Fermented *Spirulina* Products

It is important to evaluate the bioactive properties of foods that can provide benefits when consumed. The content of total phenolic compounds are important and can affect the antioxidant activity. Antioxidant activity is one of the most important bioactive properties that can play role in the prevention of oxidative stress, DNA damage and cardiovascular diseases. It can be measured in a variety of ways such as Oxygen Radical Absorbance (ORAC), Ferric Reducing Power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC) and inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) [Cömert and Gökmen, 2018]. Antimicrobial activity is also an important bioactive property for preserving food and extending shelf life.

4.4.1. Determination of Antioxidant Activity of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions According to Total Phenolic Content, DPPH Radical Scavenging Activity and Trolox Equivalent Antioxidant Capacity (TEAC)

The bioreactor products and their unfermented counterparts were also analyzed for total phenolic content (TPC) and antioxidant properties (DPPH radical scavenging and Trolox equivalent antioxidant capacity (TEAC)).

The total phenolic content (TPC) was determined for unFS control samples and FS products under various bioreactor conditions. Accordingly, it was found that the phenolic content of all products obtained under different bioreactor conditions increased significantly compared to the unFS (Table 4.3). This increase was 28% and 46% for “0.5 vvm” and “controlled pH” productions, respectively and 187% and 351% for “1 vvm” and “cascade” productions, respectively. Increasing the constant aeration to “1 vvm” affected the total phenolic content positively. In the “controlled pH” production, the constant aeration was set also at “1 vvm”. However, apparently maintaining the pH at 5.75 value hindered the increase in total phenolic content. On the other hand, it can be clearly seen that the dissolved oxygen (DO₂) level is quite important for this process as the TPC of the “cascade” production was superior compared to the other production modes. TPC and the antioxidant property of *Spirulina* had been gaining interest for a long time. Wu et al. (2005) investigated the antioxidant and antiproliferative activities of *Spirulina* and *Chlorella* water extracts for the inhibition of hepatic stellate cell (HSC) proliferation. They found out that *Spirulina* had a much higher TPC of 6.86 mg tannic acid equivalent/g than *Chlorella* extracts (1.44 mg tannic acid equivalent/g). In addition, both extracts were able to inhibit HSC and human liver cancer cells, HepG2, but the *Spirulina* extracts were more potent than the *Chlorella* extracts. De Marco Castro et al. (2019) and Niccolai et al., (2019) fermented *Spirulina* with a lactic acid bacterium *Lactobacillus plantarum* for 72 h and investigated the effect of fermentation as well. In De Marco Castro et al. (2019)’s study, untreated *Spirulina* had around 8 mg GAE /g and the maximum TPC was reached at the 24 h of fermentation with a value of 17.87 mg GAE /g and then, TPC decreased afterwards; on the other hand, Niccolai et al., (2019)’s study, untreated *Spirulina* had lower TPC of 4.5 mg GAE/g and increased to 18.9 mg GAE/g in 48 h and decreased to 10.9 mg GAE/g after 72 h. Here, unFS had

a higher TPC (10.88 mg GAE/g) compared to these previous studies. The freeze-thawing of the *Spirulina* solution (fermentation medium) for 3-4 cycles followed by ultrasound-assisted cell wall disruption performed in this study prior to the fermentation may have led to the release of phenolics. In addition, the cultivation conditions (light, temperature, production system etc.) and the harvesting and drying methods (lyophilization, drying at low temperature) of *Spirulina* may have affected the final *Spirulina* biomass content [Verni et al., 2021]. Also, the decrease in TPC at some point in those studies could be due to selected microorganisms as well as the conditions of the studies. In this study, the TPC of before and after fermentations were known, the trend of TPC during fermentation was unknown; therefore, it is difficult to speculate whether such a decrease occurred or not.

The DPPH method is one of the most used methods to evaluate the antioxidant activity of a product. The required sample volumes to provide 50% inhibition (EC_{50}) against the DPPH radical are listed in Table 4.3. A lower EC_{50} value indicates higher antioxidant activity, as the product has strong antioxidant activity, and a lower volume of product can provide 50% inhibition. There was no significant difference between different bioreactor productions or between unFS. However, similar to the total phenolic content, “cascade” mode had the lowest EC_{50} value, 4.55 mg, followed by “1 vvm” production, 4.99 mg, and “0.5 vvm”, “controlled pH” and unFS had similar values with 6.54 mg, 7.07 mg, and 6.97 mg, respectively. De Marco Castro et al., (2019) also analyzed the FS by DPPH method and expressed their results as “mg Ascorbic Acid Equivalent/g (mg AAE/g)”, due to the differences between the protocol of their study and this one, direct comparison cannot be made. However, the trend in their DPPH result was similar to the TPC of their work, as the antioxidant property reached a maximum at 24 h of fermentation with a value of 21.00 mg AAE/g and then, started to decrease, ending with 4.73 mg AAE/g at the end of fermentation which was lower than the untreated *Spirulina* (12.58 AAE/g). Niccolai et al. (2019) observed an increase in the DPPH scavenging capacity from 20.5% to 36.6% as a result of fermentation of *Spirulina* with *L. plantarum*.

In another study, Niccolai et al., (2020) investigated the use of *Spirulina* biomass in the development of a plant-based soybean beverage by *Lactiplantibacillus plantarum* ATCC 8014. The DPPH radical scavenging activity increased up to 48 h of the fermentations, and at the end of the fermentation (72 h) a

decrease was observed but the combined use of *Spirulina* and soybean drink and *Spirulina* solution alone in the fermentation as substrate still contained a high DPPH scavenging activity (>75%) at the end of the fermentation. They also reported that the TPC and DPPH radical scavenging capacity was positively correlated ($r=0.85$).

Trolox Equivalent Antioxidant Capacity (TEAC) is another antioxidant assay performed in this study. Trolox is a vitamin E analog and has antioxidant properties. Therefore, a higher TEAC value indicates that the product has a higher antioxidant property. The TEAC values of unFS and FS at different bioreactor conditions are listed in Table 4.3. The highest TEAC value was observed in the cascade FS with 317.03 mM Trolox/g, followed by 1 vvm FS with 145.53 mM Trolox/g and 0.5 vvm FS with 86.09 mM Trolox/g. On the other hand, controlled pH FS, and unFS had similar TEAC values with 60.89 and 62.99 mM Trolox/g, respectively. Verni et al. (2021) also found that processed *Spirulina* had higher ABTS scavenging activity compared to the unprocessed *Spirulina*, with combined Alcalase and fermentation with *L. plantarum* having the highest value 9.24 mmol Trolox equivalent/kg.

Table 4.3: Total phenolic content, EC₅₀ and TEAC values of unfermented *Spirulina* and fermented *Spirulina* obtained at the end of 72 hours of fermentation with mixed culture of *L. helveticus* and *K. marxianus* at 3-L bioreactor level with different parameters (“0.5 vvm” and “1 vvm” constant air, “cascade” mode and “controlled pH”).

Sample	Total Phenolic Content (mg GAE /g sample)	EC ₅₀ (mg sample)	TEAC (mM Trolox/g sample)
unFS	10.88±0.82 ^E	6.97±0.65 ^A	62.99±2.40 ^C
0.5 vvm	13.94±0.29 ^D	6.54±1.74 ^A	86.09±1.67 ^C
1 vvm	31.26±0.21 ^B	4.99±0.082 ^A	145.53±12.44 ^B
Cascade	49.02±0.11 ^A	4.55±0.002 ^A	317.03±3.10 ^A
Controlled pH	15.93±0.31 ^C	7.07±0.37 ^A	60.89±1.26 ^C

Mean± SD. GAE: gallic acid equivalent, SD: standard deviation.

^{A-E} Means followed by different capital letters indicate significant changes between different production conditions ($p<0.05$).

UnFS shows the mean of unfermented control counterparts of the bioreactor productions.

4.4.2. Determination of Angiotensin I Converting Enzyme Inhibitory (ACE-I) Activity of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions

Angiotensin I converting enzyme inhibitor (ACE-I) activity is an important functional property that lowers the blood pressure. Therefore, in order to see the ACE-I activity of *Spirulina* (unFS) and the effect of fermentation on this activity, ACE-I activity was assessed with an HPLC method and expressed as percentage inhibition. The results showed that *Spirulina* was already a good source to inhibit ACE-I as it provided 66.88% inhibition. 0.5 vvm FS and 1 vvm FS had similar ACE-I activity with 69.83% and 72.71%, respectively. Cascade FS showed the highest ACE-I activity with 86.53% followed by controlled pH FS with 82.01% (Table 4.4).

Differences in the degree of hydrolysis between productions may be one of the factors affecting ACE-I activity. In a study in which reconstituted skim milk was fortified with various molecular weight milk peptide fractions (<3 kDa, 3-10 kDa and >10 kDa) and fermented with *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Streptococcus thermophilus* monocultures, proteolytic and ACE-I activity were monitored [Gandhi and Shah, 2014]. All bacterial cultures increased soluble peptide concentrations in milk supplemented with high molecular weight peptides compared to fermentations supplemented with low molecular weight fractions, and the highest soluble peptide concentration was achieved with *L. helveticus* fermentation. In fermentations with *L. helveticus* and *Lactobacillus acidophilus*, supplementation of <3 kDa and 3-10 kDa fractions caused a decrease in ACE-I activity. The researchers reported that this may be due to the possibility that the small peptides already present in the medium may meet the essential needs of the microorganisms, causing a decrease in proteolytic activity and reducing the ability of the microorganisms to constitute ACE-I peptides. This supported the highest ACE-I activity in "cascade" production with high proteolytic activity compared to other productions. On the other hand, it is known that proteolytic activity is not the only factor affecting ACE-I activity, ACE-I activity is affected by many factors such as the microorganism used and process parameters (such as fermentation time) [Xue et al., 2021]. In a study conducted by Begunova et al. (2021) in which the antioxidant, proteolytic and ACE-I activities of the products as a result of 72 hours of fermentation of cow's milk with *Lactobacillus helveticus*,

Lactobacillus rhamnosus and *Lactobacillus reuteri* monocultures were investigated. *L. helveticus* showed the highest proteolytic activity and the antioxidant activity increased with the increasing proteolytic activity. On the other hand, this trend was not observed in ACE-I activity and *L. rhamnsosus*, which had lower proteolytic activity than *L. helveticus*, gave a similar activity to *L. helveticus* in terms of ACE-I. The researchers attributed this to the fact that the antioxidant activity of peptides depends on conditions such as being composed of fewer amino acids and containing hydrophobic amino acids, and therefore, if the proteolytic activity is high, the number of peptides increases and eventually the probability of peptides with antioxidant activity increases; on the other hand, they thought that for peptides with ACE-I activity, microorganisms need to degrade proteins and peptides more specifically. This can also be the case for controlled pH FS, whose proteolytic activity was lower than the 1 vvm FS, but its ACE-I activity tended to be higher than the 1 vvm FS. Another study attempted to obtain ACE inhibitory peptides from whey fermentation by screening microflora from ripened cheeses and dairy products [Hamme et al., 2009]. The researchers focused on the microflora that showed the highest ACE-I activity, which was accompanied by high peptide production, and they identified the microorganisms involved in the microflora as *Kluyveromyces marxianus* and *Lactobacillus rhamnosus*. In conclusion, the microorganisms used in this study, *L. helveticus* and *K. marxianus*, had previously been associated with the production of ACE inhibitory peptides and products with ACE-I activity from the fermentation of dairy products. Although the substrate in this study was not a dairy product, fermentation of *Spirulina* also resulted in improved ACE-I activity.

Table 4.4: Angiotensin I Converting Enzyme Inhibitory activity (ACE-I) of unfermented *Spirulina* and fermented *Spirulina* obtained at the end of 72 hours of fermentation at different parameters ("0.5 vvm" and "1 vvm" constant air, "cascade" mode and "controlled pH") at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus*.

Sample	ACE-I (%)
unFS	66.88±3.50 ^C
0.5 vvm	69.83±2.71 ^C
1 vvm	72.71±2.69 ^{BC}
Cascade	86.53±0.69 ^A
Controlled pH	82.01±0.82 ^{AB}

Mean± SD. ^{A-C} Means followed by different capital letters indicate significant changes between different production conditions (p<0.05).

UnFS shows the mean of unfermented control counterparts of the bioreactor productions.

4.4.3. Determination of Antimicrobial Activity of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions

An important factor in preserving the shelf life of food is the antimicrobial activity contained in the product; therefore, if a newly developed food product has antimicrobial activity, spoilage and nutritional loss can be prevented or retarded by the nature of the product itself. Antimicrobial activity was evaluated using two approaches, agar disc and well diffusion, against gram-negative bacteria (*E. coli*), Gram-positive bacteria (*S. aureus*), and spore-forming bacteria (*B. cereus*). First, the analysis was performed with 50 mg/mL of products and there was no antimicrobial activity (data not shown). Therefore, the 150 mg/mL concentration was used for this analysis. While FS products showed no antimicrobial activity (Figures 4.7, 4.9, 4.10, 4.11 and 4.12), only unFS showed a static effect around 2.1-2.5 cm on *E. coli* in the agar well diffusion method (Figure 4.8). On the other hand, this activity by unFS was not observed in the agar disc diffusion method. The observation of a static effect against *E. coli* in the agar well diffusion method but not in the disc diffusion method was most likely due to the sample volume involved in the methods; 100 µL of

samples were used in the well diffusion method, whereas 15 μ L of samples were used in the disc diffusion method.

Antimicrobial activity is one of the properties that has been studied for *Spirulina*. In a recent study the rich phenolic content of *Spirulina* has attracted the attention of Alshuniaber et al. (2021). They extracted polyphenolic compounds from *Spirulina* and investigated their antibacterial potential against food-borne bacterial pathogens (*E. coli* and *S. aureus*). They showed that the bioactive compounds in fraction B were effective against both *E. coli* and *S. aureus* in terms of minimum inhibitory concentration and minimum bactericidal concentration. In addition, these findings were supported by scanning electron microscopy and transmission electron microscopy images that the bacteria treated with these bioactive compounds had different cell morphologies with lack of cellular integrity and malformed cells compared to untreated bacteria. Although many studies have investigated the antimicrobial activity of unfermented or fermented *Spirulina*, it is difficult to draw conclusions from these studies because many factors differ in these studies, such as the *Spirulina* used in the study, differences in extractions (solvents used) or processing methods (fermentation, enzymatic hydrolysis), and the microorganisms tested [Ozdemir et al., 2004], [Tolpeznikaite et al., 2023], [Verni et al., 2021]. Ozdemir et al. (2004) evaluated the antibacterial activity of different extracts (methanol, dichloromethane, petroleum ether, ethyl acetate) and volatile components of *Spirulina*. Methanol extracts were more potent than the other extracts and for some microorganisms tested such as *B. subtilis* ATCC 6643, *S. aureus* 6538/P and *S. epidermidis* ATCC12228, and increasing the methanol extract concentration from 1 to 8 mg/mL did not affect the diameter of the inhibition zone that much for those microorganisms while increasing the methanol extract concentration provided the observation of inhibition zone around 7 mm at 4 and 8 mg/mL against *E.coli* ATCC11230. In a recent study performed by Tolpeznikaite et al. (2023), effect of submerged and solid-state fermentation by different LAB (*Lacticaseibacillus paracasei* No. 244; *Levilactobacillus brevis* No. 173; *Leuconostoc mesenteroides* No. 225; *Liquorilactobacillus uvarum* No. 245) on the antimicrobial activity against various pathogenic and opportunistic strains were investigated. Unfermented *Spirulina* showed no antimicrobial activity against the microorganisms tested, while most of the FS products showed antimicrobial activity only against *S. aureus*, and in

some cases the fermentation time significantly affected the diameter of the inhibition zone. Verni et al. (2021) investigated the effect of fermentation (with *Lactiplantibacillus plantarum* T0A10) and enzymatic (Alcalase) treatment on the wet, dried at low temperature and freeze-dried *Spirulina* in terms of the proteolytic activity and some functional properties (antioxidant and antimicrobial). Similar to the present study, they also observed inhibition of *E. coli* growth in wet and lyophilized *Spirulina* biomasses (unfermented *Spirulina*) with an inhibition diameter of 3-4 mm, and 1-2 mm, respectively. Fermentation and Alcalase treatment of these biomasses increased the diameter of the inhibition zone up to 9 mm. They also investigated the antifungal effect against *Penicillium roqueforti*. In general, fermentation, treatment with Alcalase and their combination of these processes caused inhibition of *Penicillium roqueforti* growth, however, the potency of antifungal activity was higher when lyophilized *Spirulina* was used for fermentation or enzyme treatment. The reason for not observing antibacterial activity in fermented *Spirulina* for the current study, could be related to the microorganisms used for the fermentation as well as the fermentation conditions such as fermentation time, *Spirulina* biomass and the pre-treatments applied and the scale of the process.

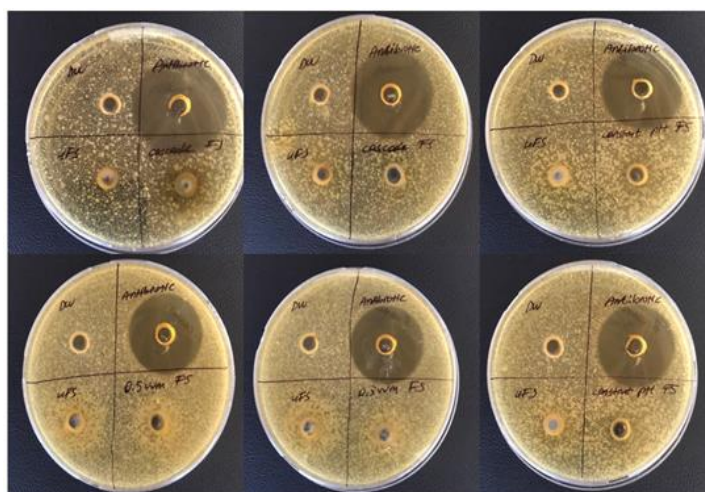


Figure 4.7: Investigation of antimicrobial activity of bioreactor products (150 mg/ml) and their unfermented counterparts (150 mg/ml) on *B. cereus* by agar well method.

(uFS: unfermented *Spirulina*, DW: distilled water, antibiotic: 0.3 mg/ml chloramphenicol, FS: fermented *Spirulina*, 0.5 vvm FS: fermented *Spirulina* at 0.5 vvm, 1 vvm FS: fermented *Spirulina* at 1 vvm, cascade FS: fermented *Spirulina* at cascade mode, const. pH FS: fermented *Spirulina* at controlled pH).

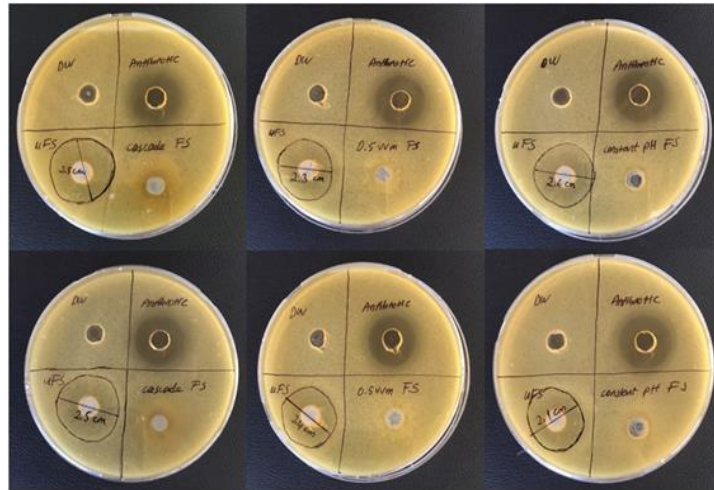


Figure 4.8: Investigation of antimicrobial activity of bioreactor products (150 mg/ml) and their unfermented counterparts (150 mg/ml) on *E. coli* by agar well method.

(uFS: unfermented *Spirulina*, DW: distilled water, antibiotic: 0.3 mg/ml chloramphenicol, FS: fermented *Spirulina*, 0.5 vvm FS: fermented *Spirulina* at 0.5 vvm, 1 vvm FS: fermented *Spirulina* at 1 vvm, cascade FS: fermented *Spirulina* at cascade mode, const. pH FS: fermented *Spirulina* at controlled pH).

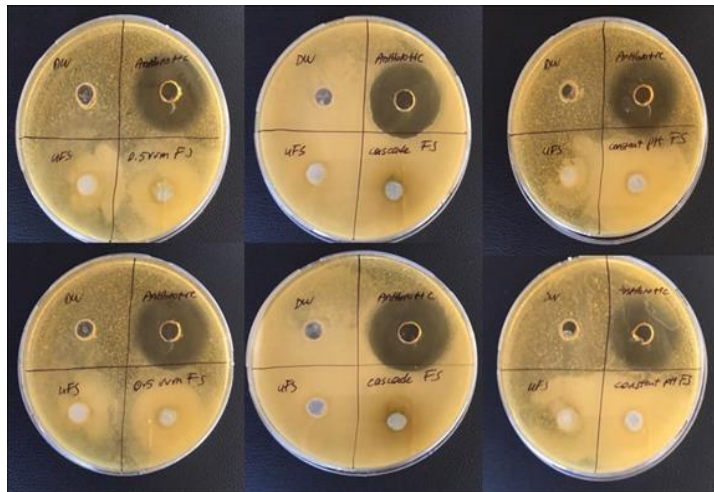


Figure 4.9: Investigation of antimicrobial activity of bioreactor products (150 mg/ml) and their unfermented counterparts (150 mg/ml) on *S. aureus* by agar well method.

(uFS: unfermented *Spirulina*, DW: distilled water, antibiotic: 0.3 mg/ml chloramphenicol, FS: fermented *Spirulina*, 0.5 vvm FS: fermented *Spirulina* at 0.5 vvm, 1 vvm FS: fermented *Spirulina* at 1 vvm, cascade FS: fermented *Spirulina* at cascade mode, const. pH FS: fermented *Spirulina* at controlled pH).

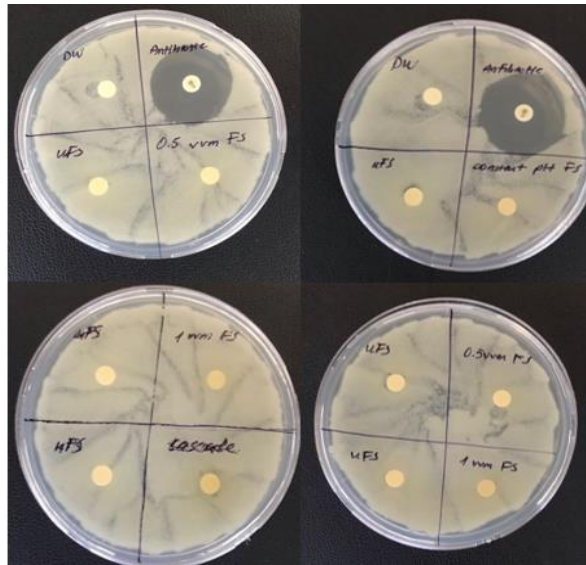


Figure 4.10: Investigation of antimicrobial activity of bioreactor products (150 mg/ml) and their unfermented counterparts (150 mg/ml) on *B. cereus* by disc diffusion method. (uFS: unfermented *Spirulina*, DW: distilled water, antibiotic: standard chloramphenicol disc, FS: fermented *Spirulina*, 0.5 vvm FS: fermented *Spirulina* at 0.5 vvm, 1 vvm FS: fermented *Spirulina* at 1 vvm, cascade FS: fermented *Spirulina* at cascade mode, const. pH FS: fermented *Spirulina* at controlled pH).

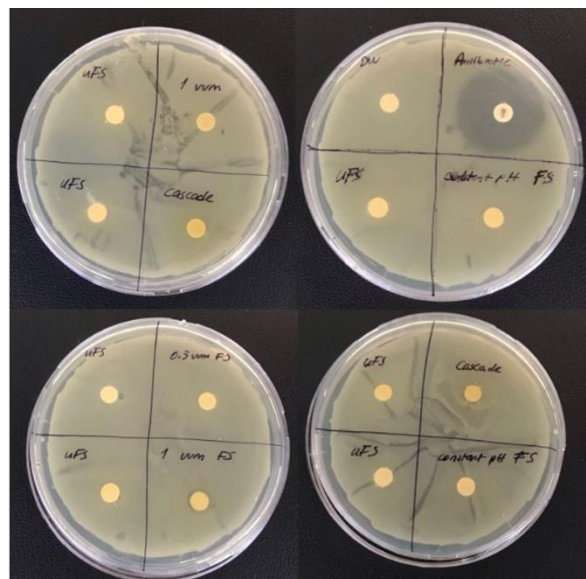


Figure 4.11: Investigation of antimicrobial activity of bioreactor products (150 mg/ml) and their unfermented counterparts (150 mg/ml) on *E. coli* by disc diffusion method. (uFS: unfermented *Spirulina*, DW: distilled water, antibiotic: standard chloramphenicol disc, FS: fermented *Spirulina*, 0.5 vvm FS: fermented *Spirulina* at 0.5 vvm, 1 vvm FS: fermented *Spirulina* at 1 vvm, cascade FS: fermented *Spirulina* at cascade mode, const. pH FS: fermented *Spirulina* at controlled pH production).

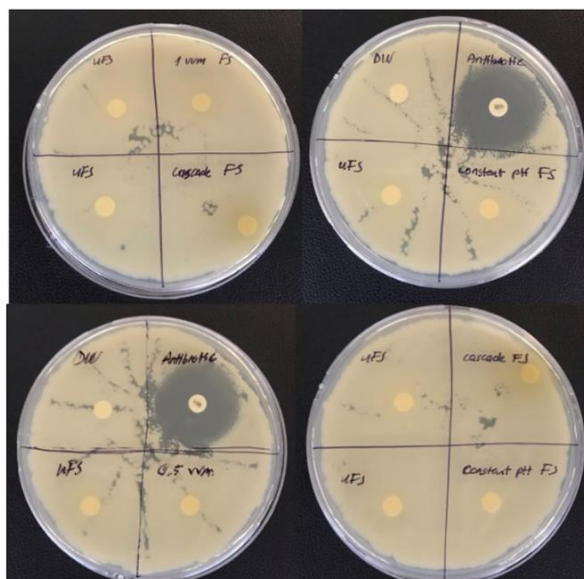


Figure 4.12: Investigation of antimicrobial activity of bioreactor products (150 mg/ml) and their unfermented counterparts (150 mg/ml) on *S. aureus* by disc diffusion method. (uFS: unfermented *Spirulina*, DW: distilled water, antibiotic: standard chloramphenicol disc, FS: fermented *Spirulina*, 0.5 vvm FS: fermented *Spirulina* at 0.5 vvm, 1 vvm FS: fermented *Spirulina* at 1 vvm, cascade FS: fermented *Spirulina* at cascade mode, const. pH FS: fermented *Spirulina* at controlled pH production).

4.5. Identification and Quantification of Volatile Organic Compounds (VOCs) of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions

The unique aroma and odor characteristics of microalgae can cause negative consumer perception towards microalgae-containing foods. The fermentation process may also provide the opportunity to improve the unique aroma and odor characteristics of microalgae. Volatile organic compounds (VOCs) can possess certain aroma notes such as phenyl ethyl alcohol having “floral/rose” and butanoic acid having “cheesy” aroma. In this part of the study, FS products obtained at different operating conditions as well as unFS were evaluated for their volatile organic compounds (VOCs) and seventy-three VOCs were detected in GC-MS and listed in Table 4.5. Acids, alcohols, esters, aldehydes, ketones, lactones, furan and pyrazines were the main VOCs group found in the products. The composition of these groups varied for different bioreactor products and for unFS. Surprisingly, acids were the dominating group of VOCs in all *Spirulina* products (Figure 4.13).

Hexanoic acid and acetic acid were the prominent acids found in the products on the contrary to Bao et al. (2018), Kurt et al. (2023), Sahin et al. (2022). Some acidic compounds were also found in these studies, but the types of acids and amounts varied. According to Bao et al. (2018) propanoic acid found in unFS and FS by *B. subtilis* at low relative content (0.80% and 1.57% respectively); succinic acid (0.03%), lactic acid (2.17%-3.06%) and palmitic acid (0.09%) were the other acid found in some FS products at low relative content. Butanoic acid, 2-methyl butanoic acid, 3-methyl butanoic acid, pentanoic acid, nonanoic acid and hexanoic acid were the acidic compounds found by Kurt et al. (2023) and Sahin et al. (2022). The amounts for these acidic compounds were low as well. For instance, unFS contained 3.2 µg/kg butanoic acid and this concentration decreased to 0.61 µg/kg for the FS product by *L. helveticus* B4526 [Kurt et al., 2023]. On the other hand, 3-methyl butanoic acid was not detected in unFS but its concentration increased to 3.04 µg/kg in the FS by *K. marxianus* and butanoic acid was 1.96 µg/kg and the amount was 6.65 µg/kg in the FS by *K. marxianus* [Sahin et al., 2022]. Acid content was substantially decreased in the cascade FS (Figure 4.13d) and alcohol (18%), furan and pyrazine (19%), lactone (3%) and ketone (4%) content were also higher compared to other FS products. The amount of tetramethylpyrazine (TMP) (cocoa, roasted nutty) was nearly doubled in the cascade FS compared to the unFS. TMP is associated with various functions such as antioxidant, anti-inflammatory and anti-apoptosis properties [Lin et al., 2022]. The high level of TMP may be one of the reasons for the higher antioxidant activity observed in the cascade FS. The principal component analysis (PCA) was conducted to see the relationship between aroma profile of the different FS and unFS products (Figure 4.14). The aroma profile of all FS products was different compared to unFS, while the controlled pH FS had the closest flavor profile to unFS among the other FS products. Cascade FS had the most distinctive aroma profile.

The capillary column used in this kind of analysis is important in the separation of the VOCs. DB-5MS was used in the studies performed by Kurt et al. (2023) and Sahin et al. (2022). However, in the present study HP-INNOWAX column was used. Thus, although the same methodology was used for the VOC analysis, the column difference may have caused some variation in the separation. For instance, in a study performed by Komthong et al. (2006) for evaluating the aroma and VOCs of Fuji

apple, differences were found between the VOCs detected by DB-Wax and DB-5MS columns. Butyl-, isopentyl- and hexyl-acetate were higher in the DB-WAX column, while isopentyl formate and 2-methyl-1-butanol were not detected by DB-WAX. For these two columns, the odor characteristics and relative concentrations of VOCs were also expressed by FD factors, and it was observed that the values of some components in DB-Wax were different from DB-5MS column; this was attributed to the difference in polarity between the columns. *K. marxianus* is known to produce various flavor compounds such as esters, carboxylic acid, ketone, and alcohol [Scharpf et al., 1986]. Among these, phenylethyl alcohol is a commercially important flavor component that can be found in various food groups (such as ice cream, candy and beverages) [Fonseca et al., 2008]. Sahin et al. (2022) showed that low content of this aroma in unFS (2.03 µg/kg) increased substantially to 354.72 µg/kg in the fermentation of *Spirulina* by *K. marxianus*. In the cascade FS amount of the phenyl ethyl alcohol was maximum compared to other FS products and unFS with a value of 107.63 µg/kg. Isoamyl alcohol was another compound that was found less in the FS products of bioreactors compared (2.94 µg/kg for 0.5 vvm FS and 0.38 µg/kg for 1 vvm FS) and to the FS by *K. marxianus* in Sahin et al. (2022)'s study (92.64 µg/kg). In a study by Kilmanoğlu et al. (2021) where the hydrolysis of tomato pulp was optimized and hydrolyzed tomato pulp was fermented with *K. marxianus* both at the flask level and in a 5 L bioreactor, it was suggested that the decrease in the amount of VOCs at the bioreactor level was due to the escape of VOCs with the exhaust gas, referred to as the "stripping effect". In addition, since the scale of the process was larger at the bioreactor level compared to the flask level and the larger volume of production and biomass accumulation can have an inhibitory effect on the culture and cause the desired VOCs not to be produced. In the literature, there are productions where an adsorption unit in the exhaust gas is used to prevent aroma escape in fermentations at the bioreactor level [Lukin et al., 2021], [H. Wang et al., 2011]. This suggests that aroma escape from the exhaust gas in bioreactors may be another parameter to consider for volatile compounds that were observed at both the flask level and the bioreactor level, but whose amount decreased in bioreactor production.

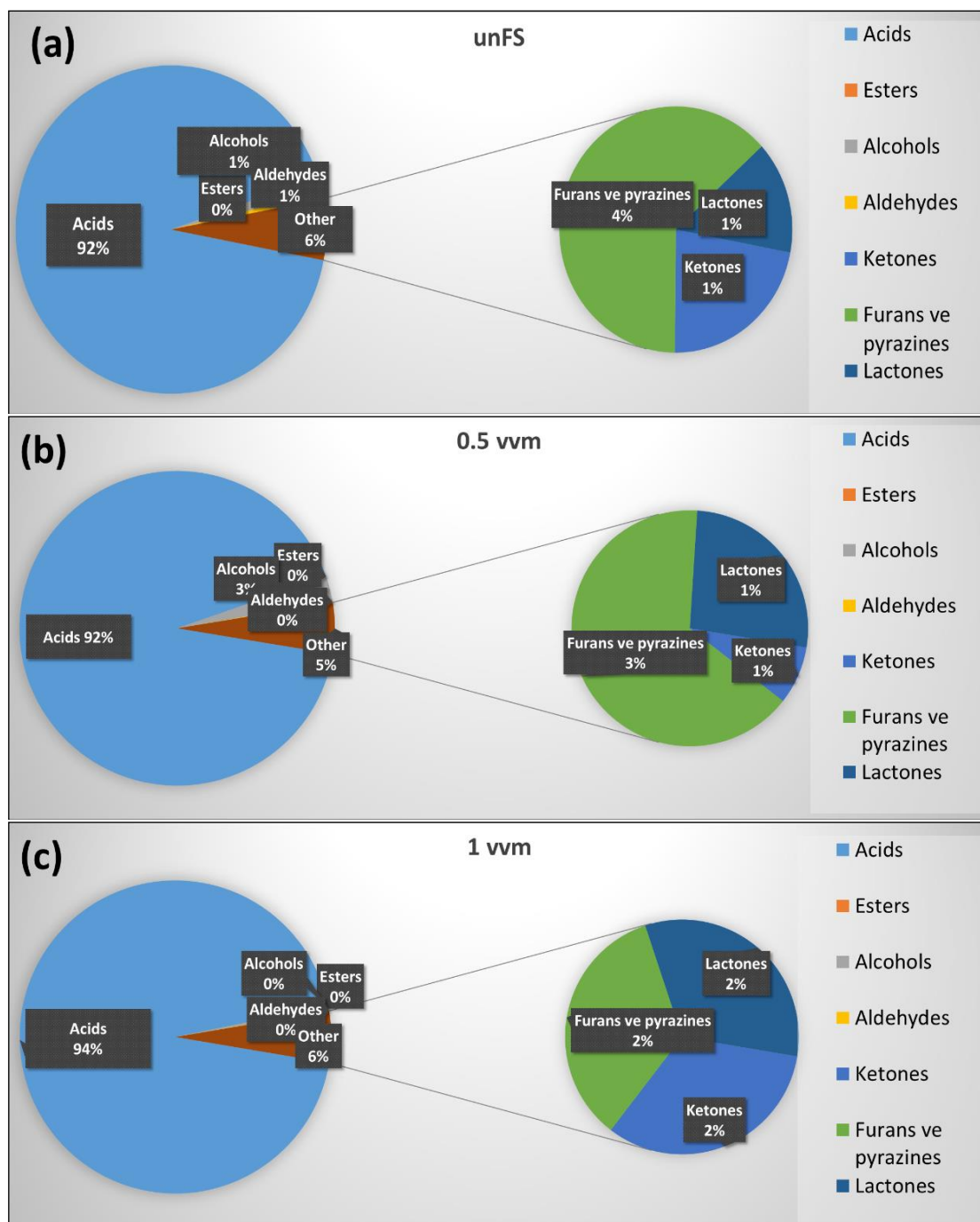
Table 4.5: Quantitative distribution of volatile organic compounds (VOCs) detected in cell-free supernatants of unfermented *Spirulina* and cell-free supernatants of fermented *Spirulina* products obtained at the end of 72 h fermentation of different parameters ("0.5 vvm" and "1 vvm" constant air, "cascade" mode and "controlled pH") at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus* expressed as µg/kg.

No	Volatile Organic Compounds (VOCs)	Volatile Organic Compounds (µg/kg) (Mean± SE)				
		unFS	0.5 vvm	1 vvm	Cascade	Controlled pH
1	2-methyl- butane	0.31±0.05	0.38±0.04	0.37±0.04	0.56±0.20	0.29±0.03
2	acetone	Nd.	Nd.	Nd.	10.25±2.90	Nd.
3	3-methyl-2-butanone	Nd.	Nd.	Nd.	2.08±0.77	Nd.
4	ethanol	Nd.	0.61±0.06	Nd.	Nd.	Nd.
5	2-pentanone	0.52±0.06	0.18±0.01	Nd.	Nd.	1.01±0.04
6	3-methyl-2-pentanone	Nd.	Nd.	0.26±0.02	3.88±1.22	Nd.
7	1,3,5-trimethylcyclohexane	0.19±0.02	0.18±0.02	0.21±0.05	0.39±0.13	0.14±0.01
8	toluene	0.28±0.03	0.27±0.05	Nd.	Nd.	Nd.
9	di methyl disulfide	0.41±0.07	Nd.	Nd.	Nd.	Nd.
10	hexanal	1.30±0.24	Nd.	Nd.	Nd.	Nd.
11	2-n-butylfuran	0.53±0.07	Nd.	Nd.	Nd.	Nd.
12	isoterpinolene	1.24±0.87	Nd.	Nd.	Nd.	Nd.
13	3-methylene-1,5,5,5-trimethylcyclohexene	1.73±0.7	Nd.	Nd.	Nd.	Nd.
14	octyl isobutyrate	Nd.	0.06±0.01	0.05±0.01	Nd.	0.07±0.01
15	3-pentene-2-one	0.77±0.01	Nd.	Nd.	Nd.	Nd.
16	2-heptanone	3.84±0.30	Nd.	Nd.	1.98±0.66	Nd.
17	isoamyl alcohol	0.73±0.05	2.94±0.23	0.38±0.02	Nd.	0.70±0.06
18	2-n-pentylfuran	3.66±0.23	Nd.	Nd.	Nd.	Nd.
19	6-methyl 2- heptanone	0.44±0.02	0.04±0.01	0.09±0.01	0.07±0.02	Nd.
20	3-octanone	Nd.	0.23±0.00	0.29±0.05	2.41±0.68	Nd.
21	1-pentanol	0.42±0.02	Nd.	Nd.	Nd.	0.28±0.01
22	hexyl acetate	0.76±0.03	Nd.	Nd.	Nd.	Nd.
23	methyl pyrazine	Nd.	Nd.	Nd.	0.46±0.13	Nd.
24	2-octanone	0.44±0.01	0.38±0.03	0.29±0.07	0.27±0.10	Nd.
25	isobutyl 2-methylvalerate	Nd.	Nd.	Nd.	0.38±0.06	0.39±0.01
26	2,2,6-trimethylcyclohexanone	2.99±0.46	Nd.	Nd.	0.30±0.08	Nd.
27	2-methyl-2-hepten-6-one	0.29±0.02	Nd.	Nd.		Nd.
28	2,3-octadione	0.37±0.03	Nd.	Nd.	0.16±0.05	Nd.
29	1-phenyl-2-pentanone	Nd.	0.23±0.04	0.22±0.06		0.26±0.02
30	2,5-dimethyl pyrazine	2.03±0.09	0.71±0.03	1.22±0.21	10.56±2.91	1.43±0.07
31	2-acetyl furan	Nd.	0.30±0.04	0.16±0.01	Nd.	Nd.
32	1-hexanol	3.86±0.52	0.58±0.07	0.13±0.05	0.62±0.21	1.61±0.14
33	2,3-dimethyl pyrazine	1.12±0.07	0.29±0.01	0.66±0.57	1.53±0.44	0.28±0.01
34	2-ethyl-5-methyl-pyrazine	0.43±0.03	Nd.	Nd.	Nd.	Nd.
35	5-methyl-3-heptanol	Nd.	Nd.	Nd.	Nd.	0.33±0.02
36	α-Isophorone	1.86±0.17	Nd.	Nd.	Nd.	Nd.
37	4-methoxy-3-ethyl phenol	1.39±0.21	Nd.	Nd.	Nd.	Nd.

Table 4.5 continued.

38	3,5-octadien-2-ol	0.29±0.01	Nd.	Nd.	Nd.	Nd.
39	linalool oxide	0.27±0.01	Nd.	Nd.	0.20±0.05	Nd.
40	1-octen-3-ol	2.71±0.11	0.46±0.01	0.23±0.01	0.50±0.14	Nd.
41	acetic acid	837.96±46.2	887.95±55.	881.00±210.	468.82±63.	560.69±229.
		5	17	83	53	47
42	2,3-dimethyl-5-ethyl-pyrazine	3.94±0.21	0.43±0.00	0.10±0.01	6.26±1.69	Nd.
43	2,6-dimethyl -4 -heptanol	Nd.	Nd.	Nd.	Nd.	1.06±0.11
44	furfural	1.48±0.031	Nd.	Nd.	Nd.	Nd.
45	tetramethyl pyrazine	69.59±6.07	33.30±1.35	14.41±2.49	126.83±31.	26.83±2.03
					62	
46	2,3,5-trimethyl-6-ethylpyrazine	Nd.	0.75±0.01	0.29±0.06	9.23±2.40	0.42±0.23
47	benzaldehyde	2.85±0.08	Nd.	0.12±0.04	9.74±3.35	0.43±0.01
48	2,5-furandicarboxyaldehyde	0.56±0.05	Nd.	Nd.	Nd.	Nd.
49	2,3, butanediol	Nd.	0.32±0.02	Nd.	Nd.	Nd.
50	5-ethyl-2-furan-carboxyaldehyde	0.23±0.02	Nd.	Nd.	Nd.	Nd.
51	anisole	0.68±0.01	Nd.	Nd.	Nd.	Nd.
52	2,6-dimethyl cyclohexanol	24.03±0.26	10.54±1.13	3.47±1.15	45.96±14.7	8.65±0.44
					9	
53	3-thujonone	1.77±0.04	0.38±0.01	0.20±0.05	Nd.	Nd.
54	β-cyclocitral	5.60±0.19	1.00±0.01	0.55±0.08	Nd.	Nd.
55	2-acetyl-5-methylfuran	1.16±0.01	0.34±0.01	0.27±0.06	1.75±0.51	
56	butyrolactone	4.97±0.32	0.28±0.00	0.19±0.04	0.81±0.22	0.23±0.02
57	2-acetylthiazole	0.51±0.01	Nd.	Nd.	Nd.	Nd.
58	2-furanmethanol	1.48±0.04	Nd.	Nd.	Nd.	Nd.
59	α-ionene	0.78±0.08	Nd.	Nd.	Nd.	Nd.
60	6-methyl-2-tridecanone	0.32±0.04	Nd.	Nd.	Nd.	Nd.
61	3,4-dimethyl benzaldehyde	0.83±0.01	0.10±0.00	Nd.	Nd.	Nd.
62	acetophenone	0.83±0.01	Nd.	3.35±1.07	Nd.	Nd.
63	p-acetyltoluene	1.18±0.04	Nd.	Nd.	0.99±0.30	Nd.
64	hexanoic acid	1159.92±88.	85.59±34.4	Nd.	Nd.	182.10±78.8
		15	0			2
65	phenylethyl alcohol	Nd.	11.98±0.45	Nd.	107.63±30.	37.51±2.76
					05	
66	β-ionone	13.72±0.42	2.96±0.35	11.57±2.54	13.02±4.05	2.59±0.09
67	2-acetylpyrrole	Nd.	0.26±0.01	1.41±0.33	0.83±0.24	Nd.
68	β-ionone epoxide	3.83±0.07	2.56±0.12	0.25±0.08	8.82±2.35	2.97±0.19
69	γ-nonolactone	0.33±0.07	0.20±0.08	1.85±0.52	0.82±0.23	0.56±0.04
70	γ-decalactone	Nd.	Nd.	Nd.	Nd.	1.51±0.08
71	α-cyclocitral	0.12±0.01	0.16±0.01	0.12±0.03	Nd.	Nd.
72	2,6-di-tert-butyl phenol	Nd.	0.10±0.01	0.12±0.04	0.16±0.04	Nd.
73	(2,6,6-Trimethyl-2-hydroxycyclohexylidene)acetic acid lactone	14.08±0.14	14.21±0.40	14.22±4.04	25.24±6.78	10.95±1.18

SE: standard error, Nd.: not detected.



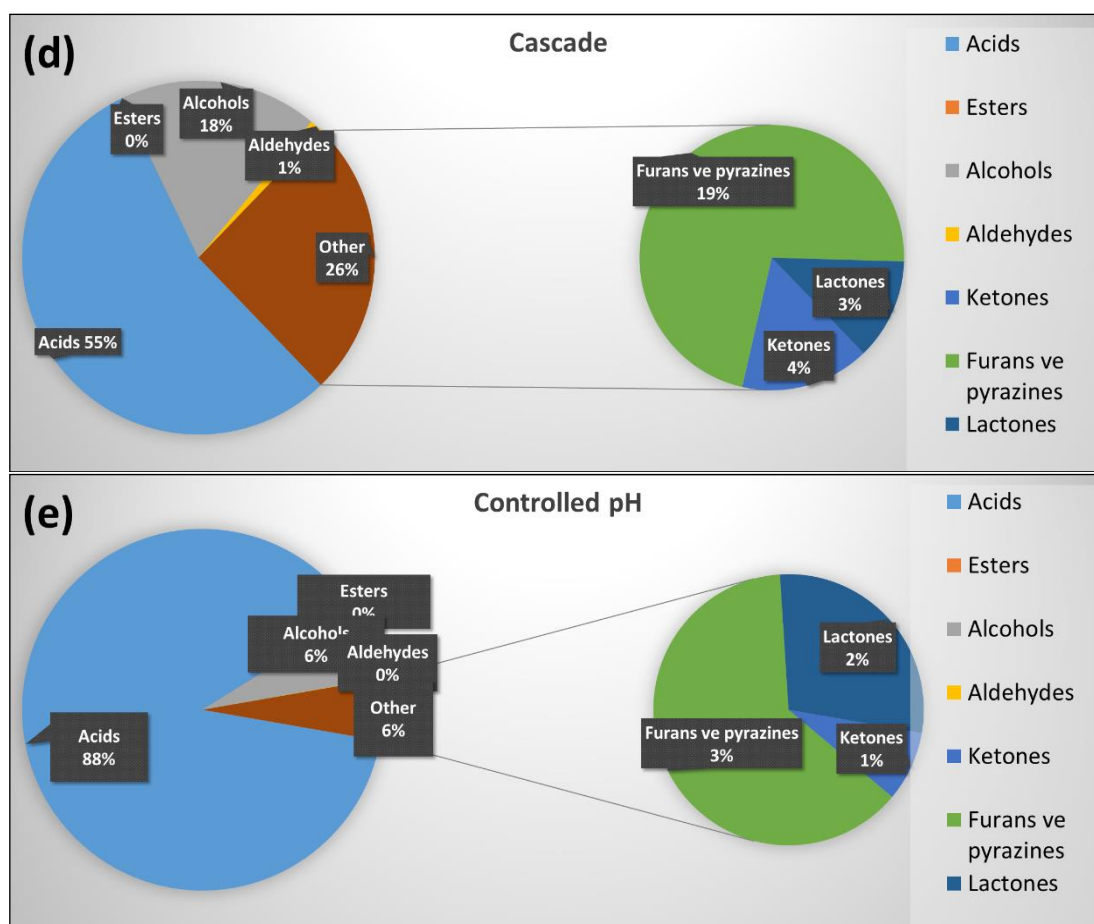


Figure 4.13: Distribution of volatile organic compound (VOC) content for (a) unfermented *Spirulina* (unFS) and fermented *Spirulina* (FS) obtained at the end of 72 hours of fermentation at different operating conditions (b) 0.5 vvm and (c) 1 vvm constant air, (d) cascade mode and (e) controlled pH at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus*.

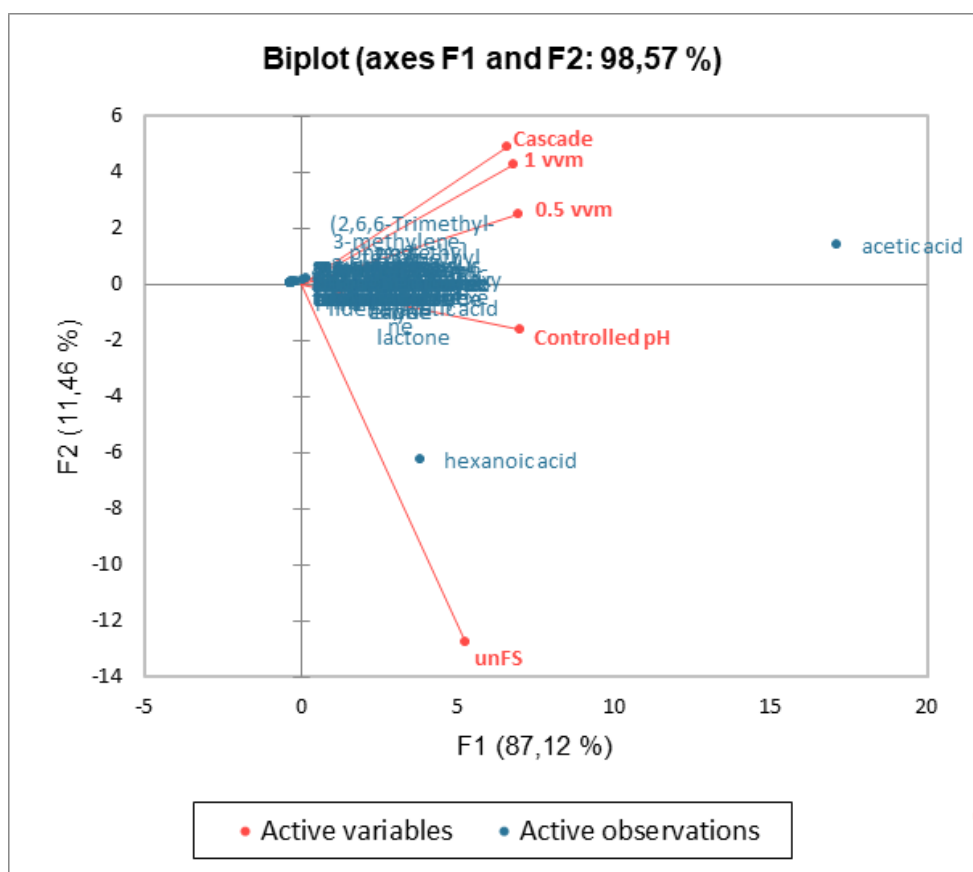


Figure 4.14: Principle Component Analysis (PCA) map for VOCs of unfermented *Spirulina* (unFS) and fermented *Spirulina* (FS) obtained at the end of 72 hours of fermentation at different operating conditions (“0.5 vvm” and “1 vvm” constant air, “cascade” mode and “controlled pH”) at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus*.

4.6. Determination of Sensory Profiles of Unfermented *Spirulina* and Fermented *Spirulina* Products Produced Under Different Bioreactor Conditions

In addition to bioactive properties, sensory and flavor attributes of a newly developed food product are quite important for consumer acceptance. To investigate the sensory attributes of the unfermented and fermented products, a descriptive sensory analysis was performed by panelists who first sniffed the products to evaluate the odor accumulated in the headspace of the jars, and then tasted the products. The products were evaluated for their basic taste and flavor attributes and depicted in Figure 4.15. The sensory analysis revealed that there were differences between different FS products in addition to their differences compared to unFS. “Seaweed” flavor (score 9.7) dominated in unFS product followed by “green/grassy”

(score 4.4) and “muddy/earthy” (score 4.4) flavors; “cooked” (score 3.6), “umami” taste (score 3.5), “astringency” (score 2.5) and “sweetness” (score 2.2) were found in some extent in terms of its basic taste. On the other hand, the intensity of “seaweed” and “muddy/earthy” flavors were significantly reduced in all bioreactor productions. In addition to that, each bioreactor condition provided unique flavor to the final product. The intensity of “spiciness” was high (3.2) in 1 vvm FS product whereas in controlled pH FS “fermented” (score 5.0), “blood/metallic” (score 6.3) and “salt” (score 3.4) flavors dominated. For the cascade FS products, “ammonia” (score 6.8) odor was detected, and also “wet towel” (score 4.6) and “bitter” (score 4.1) flavors were also the highest compared to the other products. Overall, sensory results showed that some of the flavors such as “rose” attribute of *K. marxianus* FS was not found at the bioreactor level and new flavors such as “cooked” and “blood/metallic” were observed [Sahin et al., 2022].

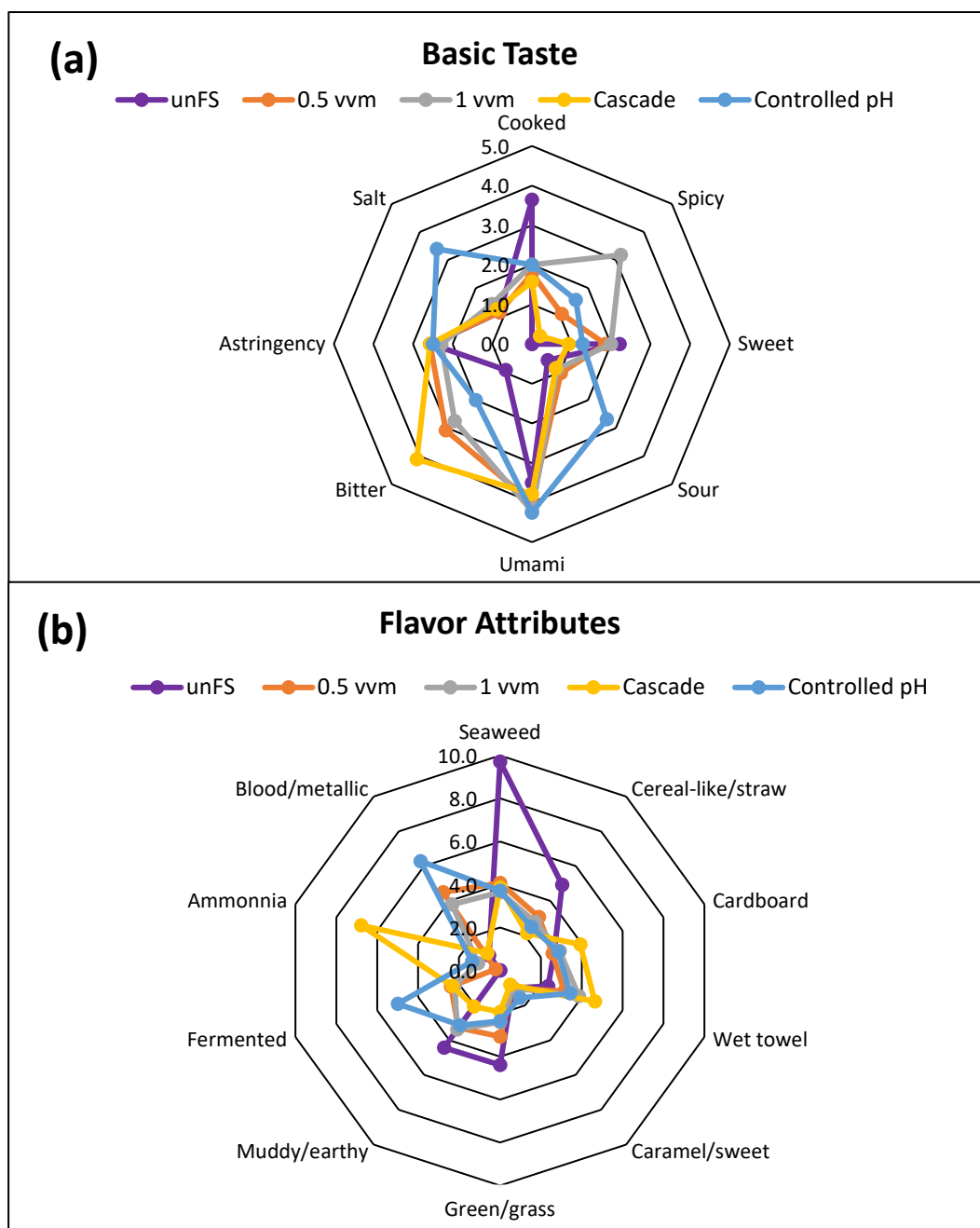


Figure 4.15: Descriptive sensory analysis of unfermented *Spirulina* (unFS) and fermented *Spirulina* products obtained at the end of 72 hours of fermentation with different parameters (“0.5 vvm” and “1 vvm” constant air, “cascade” mode and “controlled pH”) at 3-L bioreactor level with mixed culture of *L. helveticus* and *K. marxianus* in terms of their (a) basic taste, (b) flavor attributes. Attributes were scored on a 15-point Spectrum Density scale, where (0 indicates absence of the attribute and 15 indicates extremely high intensity of the attribute).

4.7. Determination of SDS-PAGE Protein Profile During Cascade Mode Fermentation

Considering the total soluble protein concentration, amino acid content, variety in the VOCs and bioactivities *Spirulina* fermented obtained through varying bioreactor conditions, the cascade mode was promising for further investigations. Previous SDS-PAGE profile (Figure 4.4) demonstrated the protein profile of the product obtained by cascade mode before and after the fermentation (72nd hour). Thus, in order to consider the changes in protein profile and thus to have an understanding of the metabolism of the microorganisms during the fermentation process, samples were taken at different time points during the fermentation. SDS-PAGE profile of the cascade mode production was shown in Figure 4.16. It clearly showed that starting from the 9th hour of fermentation, microorganisms hydrolyzed higher molecular weight proteins and formed a variety of smaller proteins, as indicated by the increase in bands between 25-75 kDa. Further hydrolysis was observed at the 48 and 72 h.

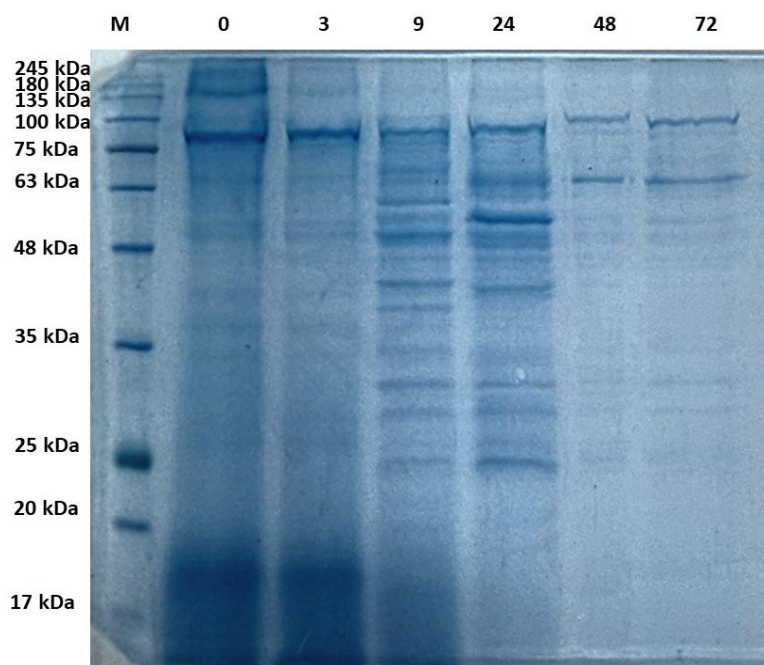


Figure 4.16: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles during 72 h fermentation of *Spirulina* with mixed culture of *L. helveticus* and *K. marxianus* at 3-L bioreactor level with cascade mode. M indicates the standard protein marker between 10.5 kDa-175 kDa. 0, 3, 9, 24, 48 and 72 show the fermentation hour.

4.8. Effect of *In Vitro* Gastrointestinal Digestion on Unfermented *Spirulina* and Cascade Fermented *Spirulina* Products

A newly developed food product may show good characteristics in terms of bioactive properties, but estimating its activity after digestion is another aspect that should be considered. In this case, the product produced under the most promising bioreactor conditions, cascade mode, was subjected to *in vitro* digestion by our collaborative research group led by Prof. Dr. Sibel Karakaya from Ege University Food Engineering Department and bioactive properties after gastric and gastrointestinal digestion were investigated.

4.8.1. Determination of Antioxidant Activity of unFS and Cascade FS Products After *In Vitro* Gastric and Gastrointestinal Digestion According to Total Phenolic Content, DPPH Radical Scavenging Activity and TEAC Analysis, and their ACE Inhibitory Activity

Changes in the total phenolic content and bioactivities (DPPH scavenging, TEAC, and ACE-I) after gastric and gastrointestinal digestion are shown in Table 4.6. Compared to the undigested unFS and cascade FS (Table 4.3 and 4.4) some changes were observed after *in vitro* digestion. The total phenolic content of unFS increased from 10.48 mg GAE/g (undigested) to 12.42 mg GAE/g after gastric digestion; however, some part of the phenolics were lost after gastrointestinal digestion as the total phenolic content decreased to 8.79 mg GAE/g. Such an increase after gastric digestion may be due to the bond hydrolysis of phenolic compounds, which facilitated the dissolution of phenolic compounds release of bounded phenolic compounds as a result of the low pH in gastric digestion; however, the alkaline pH in the intestinal digestion stage may have caused instability in phenolic compounds And degradation of phenolics [Dong et al., 2023]. On the other hand, TPC of the cascade FS, decreased from 49.02 mg GAE/g (undigested) to 27.71 mg GAE/g after gastric digestion. In contrast to the unFS, cascade FS was able to maintain its TPC after gastrointestinal digestion with a value of 26.82 mg GAE/g. In terms of DPPH scavenging activities, the EC₅₀ values increased, so the activity decreased for both unFS and cascade FS. However, this decrease was also higher for unFS. The EC₅₀ value of unFS increased from 6.97 mg to 11.01 mg after gastric digestion, and a

major increase was observed after gastrointestinal digestion, where the EC₅₀ value reached 28.98 mg. The EC₅₀ value also increased for cascade FS; in undigested cascade FS it was 4.55 mg and it reached 8.02 mg and 8.07 mg after gastric and gastrointestinal digestion, respectively. Thus, although the DPPH scavenging activity was lost to some extent, cascade FS was still able to maintain its remaining activity at the end of gastrointestinal digestion. The effect of *in vitro* digestion on the TEAC was different than what was observed in TPC and DPPH scavenging activity. TEAC values increased in both unFS and cascade FS in both gastric and gastrointestinal digestion compared to their undigested forms. The TEAC of unFS after gastric digestion was almost 4.5 times that of the undigested counterpart, and there was no significant difference in its TEAC after gastrointestinal digestion compared to the gastric digestion. Gastric digestion caused a 1.7-fold increase and gastrointestinal digestion caused a 2.3-fold increase in the TEAC value of cascade FS compared to its undigested form. Protein hydrolysis may have affected the protein-phenolic interactions and increased the free phenolic compounds as well as the formation of bioactive peptides with antioxidant activity [Ozdal et al., 2013], [Şanlıdere Aloglu and Öner, 2011]. Also, the transformation that the phenolic compounds underwent such as the conversion of glycoside forms to aglycones may have increased the antioxidant activity in terms of TEAC [L. Wang et al., 2018]. In conclusion, cascade FS also showed higher TEAC values after digestion and was also advantageous for this activity as well.

In a recent study investigating the effect of *in vitro* digestion and colonic fermentation on the total phenolic matter and antioxidant activity of 4 microalgae species (*Chlorella* sp., *Spirulina* sp., *Dunaliella* sp. and *Isochrysis* sp.), *Spirulina* sp. showed the highest total phenolic content (7.93 GAE/g) and DPPH scavenging activity (2.35 mg Trolox equivalent/g) after digestion. *Spirulina* sp. also showed the most increased short-chain fatty acids after 8 h of colonic fermentation, indicating that *Spirulina* sp. may be more beneficial to gut health [K. Li et al., 2022].

In a study investigating the effect of *in vitro* digestion of red and yellow pea hulls on phenolic content and antioxidant activity [Ma et al., 2021], a gradual decrease in both total phenolics and total flavonoids was observed in the model of digestion of red and yellow pea hulls after oral, gastric, small intestinal, and large intestinal digestion. While the greatest decrease occurred after oral and gastric

digestion, the fact that these amounts did not change much after small and large intestinal digestion was attributed to the fact that a small portion of the phenolic compounds remained in this part of the digestion after oral and gastric digestion. When the antioxidant activity of digestion was evaluated with ABTS radical scavenging activity, it was observed that the activity was similar to the change in total phenolic compounds and gradually decreased in digestion steps. On the other hand, it was observed that the antioxidant activity of yellow pea evaluated by DPPH radical scavenging was contradictory with the total phenolic content. This may be due to the interaction of phenolics with other dietary fibers, proteins and minerals released during digestion, as well as their molecular structure affected by digestion. Similar to that study, DPPH scavenging activity and TEAC were affected differently by digestion in this study, indicating that changes in the molecular structures of the phenolic compounds or the interactions may have positively affected the scavenging activity of ABTS, while may have hindered the scavenging activity of DPPH. In addition to the total amount of phenolics, phenolic composition may cause different results in different antioxidant activity methods. For example, in a study performed by Valero-Cases et al. (2017) investigating the effects of fermentation with different lactic acid bacteria (*B. bifidum*, *L. plantarum*, *B. longum* subsp. *infantis*, and *L. acidophilus*) and stimulated gastrointestinal digestion on pomegranate juices were investigated by different antioxidant methods. There was an increase in DPPH scavenging activity in all fermentations compared to unfermented pomegranate juice, while in the ABTS method, the activity did not change much with fermentation. They also found a catechin derived phenolic metabolite only in fermented samples. On the other hand, the antioxidant activity determined by DPPH and ABTS methods in fermented samples increased as a result of gastric digestion, while FRAP activity decreased. It was thought that gastric digestion conditions (low pH and pepsin activity) affected the release of bioactive compounds (such as epicatechin, catechin and α -punicalagin) that increased their bioaccessibility. According to the FRAP method, it was suggested that the decreased chelating activity as a result of digestion may be due to the conversion of catechin and epicatechin into a catechin-derived microbial metabolite. In a recent study, the structural stability and antioxidant activity of twenty-seven phenolic compounds (phenolic acids, flavonoids, and flavanones) were investigated during *in vitro* digestion [C. X. Li et al., 2023].

Performing the study on pure phenolics, which are independent of a food matrix can, on the one hand can eliminate the effects of the food matrix; on the other hand, it can help to understand the mechanisms of the changes during digestion, as it will directly show the effect of digestion on the compounds. In this study, it was found that the bioaccessibility of phenolics varies according to the structure of the phenolic substance and after oral digestion, most of the phenolic compounds were degraded and the antioxidant activity decreased. At the end of the intestinal digestion, phenolic compounds were largely degraded, and in general, the antioxidant activity of many compounds decreased after digestion. However, the antioxidant activity of most flavonoids increased after *in vitro* digestion. Overall, the effect of digestion is a complex phenomenon and more detailed work such as investigating the changes in the phenolic composition during different stages of *in vitro* digestion, may help to understand the changes in the bioactivity of FS products.

In the case of ACE-I activity, the changes were similar to those observed for DPPH inhibition activity. UnFS maintained its ACE-I activity after gastric digestion (66.32%) compared to the ACE-I activity of the undigested form (66.88%), however ACE-I activity decreased significantly after gastrointestinal digestion to an inhibition value of 38.06%. On the other hand, the ACE-I of cascade FS decreased from 86.53% to 56.22% after gastric digestion, and after gastrointestinal digestion, the ACE-I activity was maintained with a value of 55.25%. In a study of egg white hydrolysate (HEW) obtained with pepsin, ACE-I activity was evaluated after small intestinal digestion of the hydrolysate and the fraction of the hydrolysate less than 3 kDa (HEW<3 kDa) [Miguel et al., 2007]. When they examined ACE-I activity after digestion, they found a decrease in ACE-I activity due to a slight increase IC_{50} value of both HEW and HEW<3 kDa. They noted that despite the decrease, the levels detected were still strong indicators of ACE-I activity and attributed this decrease to the possibility that some ACE-I peptides were degraded during digestion. Similarly, the changes in ACE-I activity obtained by *in vitro* gastrointestinal digestion in this project support the possibility that peptides showing ACE-I activity in the fermented product are degraded during digestion. On the other hand, the preservation of activity in the fermented product in small intestinal digestion suggested that ACE-I peptides might have preserved in small intestinal digestion.

Table 4.6: Effect of the *in vitro* gastric and gastrointestinal digestion on the antioxidant activity of unFS and cascade FS in terms of total phenolic content, DPPH radical scavenging activity and Trolox equivalent antioxidant capacity (TEAC) and Angiotensin I Converting Enzyme Inhibitory (ACE-I) activity.

Sample	Total Phenolic Content (mg GAE/g)	EC ₅₀ (mg sample)	TEAC (mM Trolox/g sample)	ACE-I (%)
unFS gastric digestion	12.42±0 ^B	11.01±1.30 ^B	279.78±3.80 ^C	66.32±7.52 ^A
unFS gastrointestinal digestion	8.79±0.008 ^C	28.98±5.78 ^A	259.07±11.17 ^C	38.06±1.23 ^B
Cascade FS gastric digestion	27.71±0.18 ^A	8.02±0.28 ^B	547.87±18.71 ^B	56.22±4.81 ^{AB}
Cascade FS gastrointestinal digestion	26.82±0.56 ^A	8.07±0.34 ^B	722.21±21.46 ^A	55.25±0.93 ^{AB}

Mean± SD. GAE: gallic acid equivalent.

^{A-C} Means followed by different capital letters in each column indicate significant changes between different production conditions (p<0.05)

4.9. Fractionation of unFS and Cascade FS Products Using Ultrafiltration

As cascade FS seemed promising for bioactive properties to investigate whether fractionation can enhance these bioactive properties, cascade FS and unFS, were fractionated to different MW (<10 kDa, 5-10 kDa, <5 kDa) using ultrafiltration and evaluated for their bioactive properties.

4.9.1. Determination of Antioxidant Activity of Different MW Fractions (>10 kDa, 5-10 kDa and <5 kDa) of unFS and Cascade FS Products According to Their Total Phenolic Content, DPPH Radical Scavenging Activity and Trolox Equivalent Antioxidant Capacity (TEAC) and Angiotensin I converting Enzyme Inhibitory (ACE-I) Activity

Bioactive properties of the fractions of unFS and cascade FS are shown in Table 4.7. There was no significant difference in total phenolic content between different fractions of unFS. This observation was also seen for ACE-I activity in the unFS: as MW became smaller, the ACE-I activity tended to be lower. On the other hand, TPC increased with decreasing MW of the fractions for cascade FS. A similar

situation was observed for TEAC values, with the fraction <5 kDa had the highest TEAC value in the cascade FS product (793.20 mM Trolox/g sample). In contrast, the <5 kDa fraction of unFS had the lowest TEAC value (10.07 mM Trolox/g sample). In contrast to TPC and TEAC value, the lowest ACE-I activity belonged to the <5 kDa fraction of cascade FS and the 5-10 kDa and >10 kDa fractions had similar and higher ACE-I activity. For the DPPH scavenging activity, 5-10 kDa and <5 kDa fractions of unFS showed similar inhibitory activity as the >10 kDa and <5 kDa fractions of the cascade FS. The highest DPPH inhibitory activity belonged to the 5-10 kDa fraction of cascade FS with 2.75 mg, which was also better than the activity of undigested cascade FS (4.55 mg) and the activity after the *in vitro* digestion (8.07 mg) of cascade FS. He et al. (2013) investigated the effect of ultrafiltration fractions <1, 1-3, 3-5 and 5-10 kDa of enzymatic rapeseed protein hydrolysates (prepared by hydrolyzing rapeseed protein isolate with different proteases) on the antioxidant activities (DPPH radical scavenging activity, metal chelating activity, ferric reducing power, superoxide radical scavenging assay and inhibition of linoleic acid oxidation). Fractions <1 kDa showed higher DPPH scavenging activity for all the hydrolysates prepared with different protease treatments. This supported the fact that the smaller fraction (5-10 kDa) of the cascade FS had better DPPH activity than the unfractionated cascade FS. On the other hand, for Fe^{+2} chelating activity unfractionated rapeseed hydrolysate (RPH) and 5-10 kDa fractions exhibited good activities and the authors concluded that increased peptide chain length might have led to higher chelating activities due to synergistic effects of higher number of amino acids. Phongthai et al. (2018) investigated the effect of *in vitro* gastrointestinal digestion followed by fractionation on the antioxidant activities of rice bran protein concentrate (MW of <3 kDa, 3-5 kDa and 5-10 kDa). Their results showed that compared to rice bran protein concentrate, rice bran protein hydrolysate obtained by *in vitro* digestion showed higher antioxidant properties indicating *in vitro* digestion led to the formation of antioxidant peptides. While the antioxidant properties of the fractions varied for different antioxidant assays. The smaller fractions <3 kDa and 3-5 kDa showed higher ABTS radical scavenging activity even higher than the RBPH. Similarly, in the current study, TEAC values of cascade FS increased with fractionation to smaller MWs and the smallest fraction of

<5 kDa showed the highest TEAC value compared to both the unfractionated and digested cascade FS.

Regarding ACE-I activity, some studies have shown higher ACE-I activity associated with the lower MW fractions such as 1-3 and <1 kDa [Campos et al., 2010], [Herrera Chalé et al., 2014]. However, in the current study the <5 kDa fraction of both cascade FS and unFS showed the lowest ACE-I activity. This could be due to the synergistic effect of smaller peptides with the bigger peptides or amino acid composition of the peptides.

Table 4.7: Antioxidant activity of unFS and cascade FS fractions (>10 kDa, 5-10 kDa and <5 kDa) in terms of total phenolic content, DPPH radical scavenging activity and Trolox equivalent antioxidant capacity (TEAC) and Angiotensin I Converting Enzyme Inhibitory (ACE-I) activity.

Sample	Total Phenolic Content (mg GAE/g sample)	EC ₅₀ (mg)	TEAC (mM Trolox/g sample)	ACE-I (%)
unFS >10 kDa	11.44±0.12 ^D	13.49±0.74 ^A	189.73±8.00 ^D	83.37±0.012 ^{AB}
unFS 5-10 kDa	12.98±0.22 ^D	8.85±0.11 ^B	204.30±15.76 ^D	69.76±0.61 ^{BC}
unFS <5 kDa	10.07±0.14 ^D	7.89±0.011 ^B	100.02±6.23 ^E	55.29±5.73 ^{CD}
Cascade >10 kDa	27.48±1.47 ^C	7.87±0.019 ^B	276.81±2.44 ^C	84.52±1.51 ^{AB}
Cascade 5-10 kDa	37.88±2.63 ^B	2.75±0.0008 ^C	571.67±16.10 ^B	86.41±0.48 ^A
Cascade <5 kDa	59.85±0.21 ^A	7.85±0.11 ^B	793.20±27.38 ^A	52.42±4.05 ^D

Mean± SD. GAE: gallic acid equivalent.

^{A-E} Means followed by different capital letters indicate significant changes between different production conditions (p<0.05)

5. CONCLUSION

Spirulina platensis has a long history of consumption due to its nutritious characteristics and are among alternative protein sources. Therefore, it is a great sustainable source for the development of innovative ingredients and food products. In this thesis, the fermentation of *Spirulina* by mixed culture of *L. helveticus* B-4526 and *K. marxianus* Y-329 was optimized at the bioreactor level by applying various operating parameters such as aeration rate, adjustment of dissolved oxygen level and pH control. And the effect of fermentation was investigated from different perspectives: microbial growth, total soluble protein content, degree of hydrolysis and protein profile, amino acid composition, volatile organic compounds, antioxidant properties and effect of in vitro digestion.

Microbial growth at different operating conditions showed that the bioreactor conditions supported the growth *L. helveticus* B-4526 and *K. marxianus* Y-329. The number of viable *L. helveticus* cell counts started with 6.5-6.8 log CFU/mL and exceeded 9 log CFU/mL as a result of 24 h fermentations in 4 different batch bioreactor operation conditions, and the highest number of viable cells was reached with 9.6 log CFU/mL in a bioreactor performed with “controlled pH” condition and the viable cell number of *L. helveticus* was above 9 log CFU/mL at the end of the fermentation for all operating conditions. On the other hand, there were differences in the initial counts of *K. marxianus* at different operating conditions in which “cascade” production started with less viable cell numbers of 5.1 log CFU/mL whereas at the end of 24h the highest increase with a 3.1 log CFU/mL was observed in the “cascade” operating condition.

Total soluble protein concentration was higher in “cascade” and “1 vvm” FS which was also supported by high degree of hydrolysis and SDS-PAGE profile. Total amino acid and free amino acid content was higher in all productions compared to unFS. The essential amino acid content also increased in all fermentations, with the cascade having the highest content of essential amino acids. Arginine, tyrosine, valine, methionine and phenylalanine were abundant in the cascade FS. “1 vvm” FS was also rich in essential amino acids but in terms of relative essential amino acid content (essential amino acids/total amino acids) “controlled pH” was also

promising. Thus, fermentation of *Spirulina* increased the quality of *Spirulina* for the diet.

In terms of bioactive properties (DPPH radical scavenging and TEAC) and total phenolic content, fermentation generally had a positive effect by increasing the bioactivities. However, fermentation did not affect the DPPH radical scavenging activity, while cascade FS significantly increased the total phenolic content, TEAC and ACE-I activity. FS of “controlled pH” production was also promising for ACE-I activity. FS of “1 vvm” was also rich in TPC and had high TEAC. On the other hand, antimicrobial activity was not observed in any of the FS products, while unFS had a weak static effect against *E. coli*. Thus, it can be said that the bioactive properties of *Spirulina*, except the DPPH scavenging activity, were improved in all the productions, while cascade FS showed the best performance.

Seventy-three VOCs were detected in GC-MS. Acids, alcohols, esters, aldehydes, ketones, lactones, furans, and pyrazines were the major groups of VOCs found in the products. Acids were the dominant group of VOCs in all *Spirulina* products, while the acid content is greatly reduced in cascade FS and cascade FS had the most diversified VOCs as the content of the alcohols, furan, pyrazines, lactones and ketones increased significantly compared to other FS products as well as unFS product. PCA analysis showed that aroma profile of all FS products was different compared to unFS, while the controlled pH FS had the closest flavor profile to unFS among the other FS products. Cascade FS had the most distinctive aroma profile.

Fermentation of *Spirulina* significantly reduced the intensity of “seaweed” and “muddy/earthy” flavors present in unFS in all bioreactor productions and each bioreactor condition provided unique flavor to the final product. The intensity of “spiciness” was high (3.2) in 1 vvm FS product whereas in controlled pH FS “fermented” (score 5.0), “blood/metallic” (score 6.3) and “salt” (score 3.4) flavors dominated. For the cascade FS products, “ammonia” (score 6.8) odor was detected, and also “wet towel” (score 4.6) and “bitter” (score 4.1) flavors were also the highest compared to the other products.

Since the fate of bioactive compounds in the digestion is crucial to show their biological effect, the bioactivity of the unFS product and the cascade FS product, the most promising FS product in terms of bioactivity, were evaluated after *in vitro* gastric and gastrointestinal digestion. In general, *in vitro* digestion studies showed

that FS had maintained its activity at the end of the gastrointestinal digestion compared to the unFS.

Fractions (>10 kDa, 5-10 kDa and <5 kDa) of cascade FS had different TPC and antioxidant activities. While the 5-10 kDa showed the highest DPPH radical scavenging activity and good ACE-I activity, the smallest fraction, <5 kDa, had the highest TPC content and TEAC and the lowest ACE-I activity.

Overall, this study showed the different aspects of the development of *Spirulina*-based fermented products and the cascade FS was the most optimum production mode to enhance the bioactive properties. To have a detailed understanding related to the changes as a result of fermentation and during the *in vitro* digestion, the composition of phenolics can be evaluated by an HPLC method in the future. Also, studies related to the shelf-life of the FS such as viable cell numbers of *L. helveticus* and *K. marxianus* during shelf-life experiments and the optimum form of the FS that reaches to the consumer or the FS in the new food formulations can be investigated.

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BIOGRAPHY

Cansu Yay completed her bachelor's degree at Marmara University, Department of Bioengineering as the highest ranked student. During her undergraduate studies, she completed an Erasmus+ internship at the Universidade de Aveiro, Portugal. Currently, she is a master student and also a research assistant at Gebze Technical University, Institute of Biotechnology. During her master's program, she was a guest researcher at the Technical University of Denmark for 3 months working on the antioxidant and emulsifying properties of potato protein hydrolysates.

APPENDICES

Appendix A: Standard calibration curve to determine total soluble protein concentration according to Lowry et al. (1951) with bovine serum albumin (BSA) as standard.

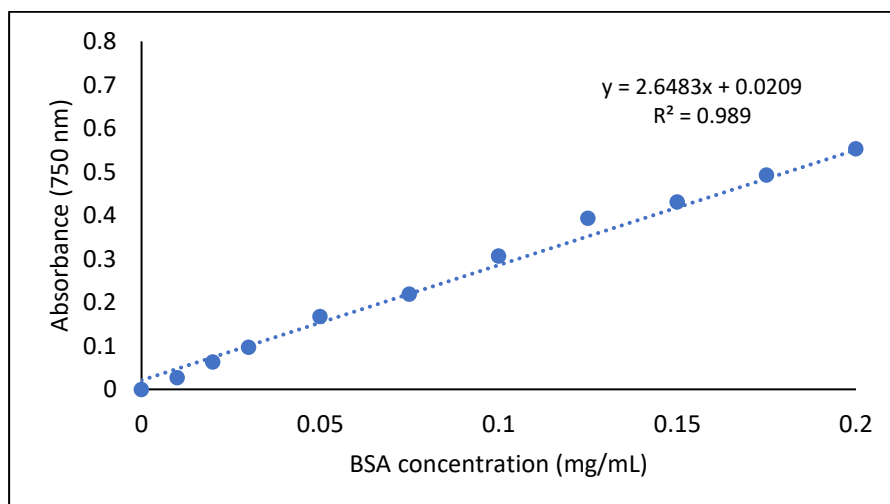


Figure A1.1: BSA (0-0.2 mg/mL) standard calibration curve to determine total soluble protein concentration by Lowry method. Epoch2 well-plate reader was used for this assay.

Appendix B: Standard calibration curve used to determine degree of hydrolysis by cadmium-ninhydrin method.

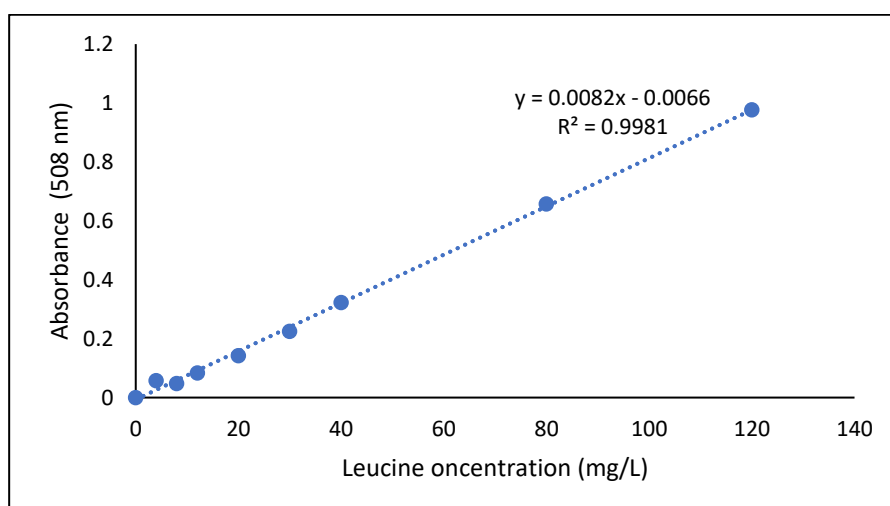


Figure B1.1: Leucine (0-120 mg/L) standard calibration curve to determine the degree of hydrolysis by cadmium-ninhydrin method. Epoch2 well-plate reader was used for this assay.

Appendix C: Standard calibration curve used to measure total phenolic content using gallic acid as standard.

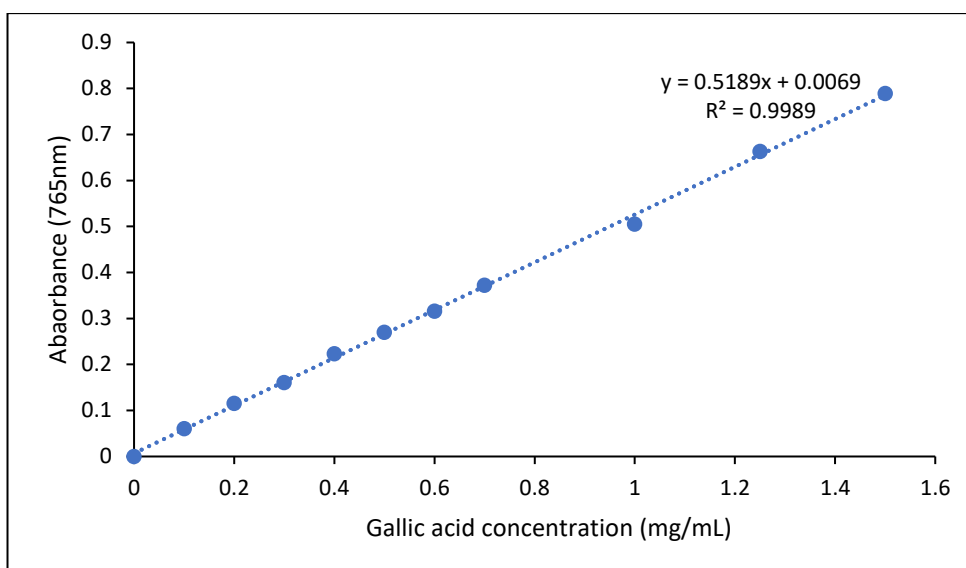


Figure C1.1: Gallic acid (0-1.5 mg/mL) standard calibration curve to determine total phenolic content for the measurements performed on Epoch2 well-plate reader.

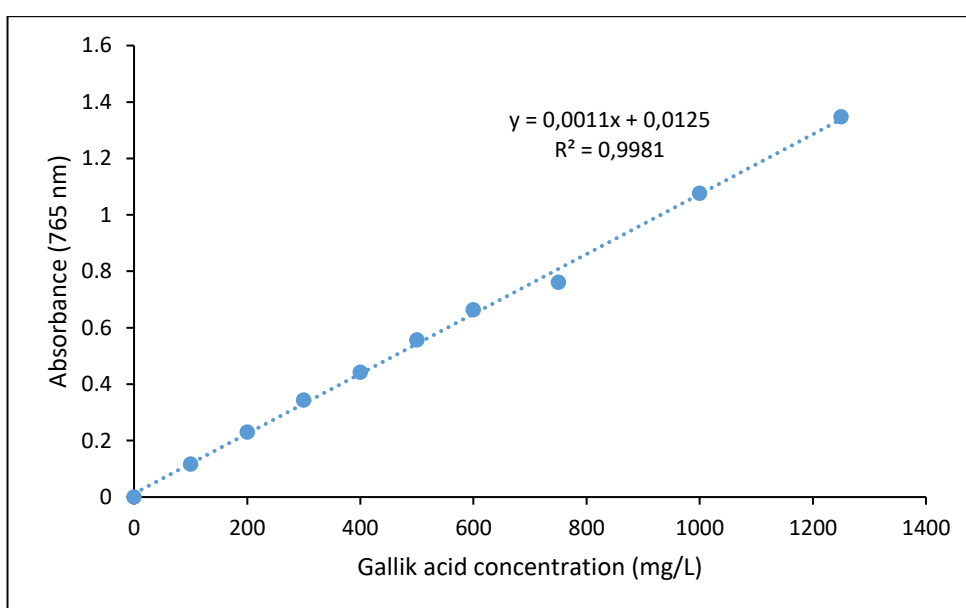


Figure C1.2: Gallic acid (0-1250 mg/L) standard calibration curve to determine total phenolic content for the measurements performed on Shimadzu UV-1280.

Appendix D: Graphs used in linear regression to calculate EC₅₀ values for DPPH radical scavenging activity.

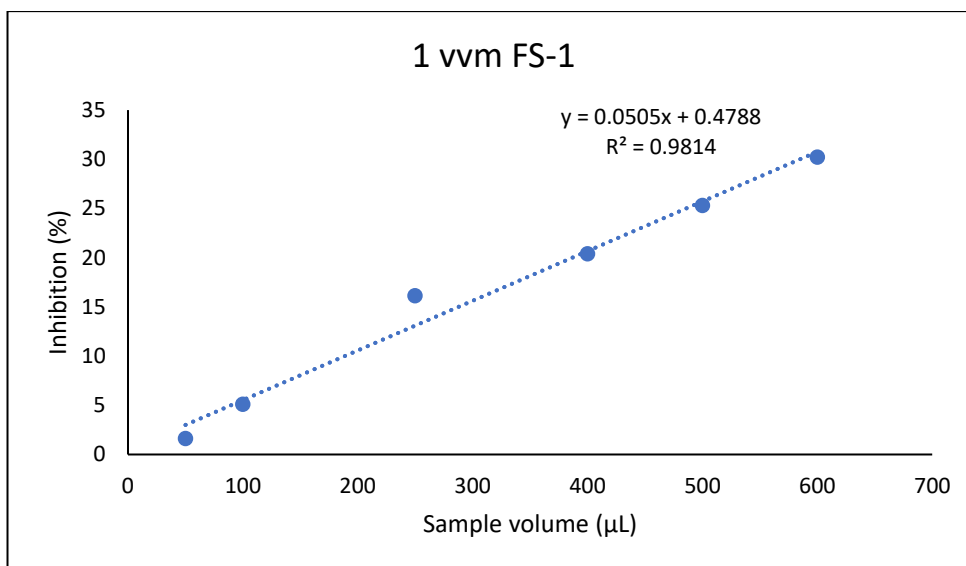


Figure D1.1: Inhibition of DPPH radical versus sample volume of the 1 vvm FS product to calculate EC₅₀ value.

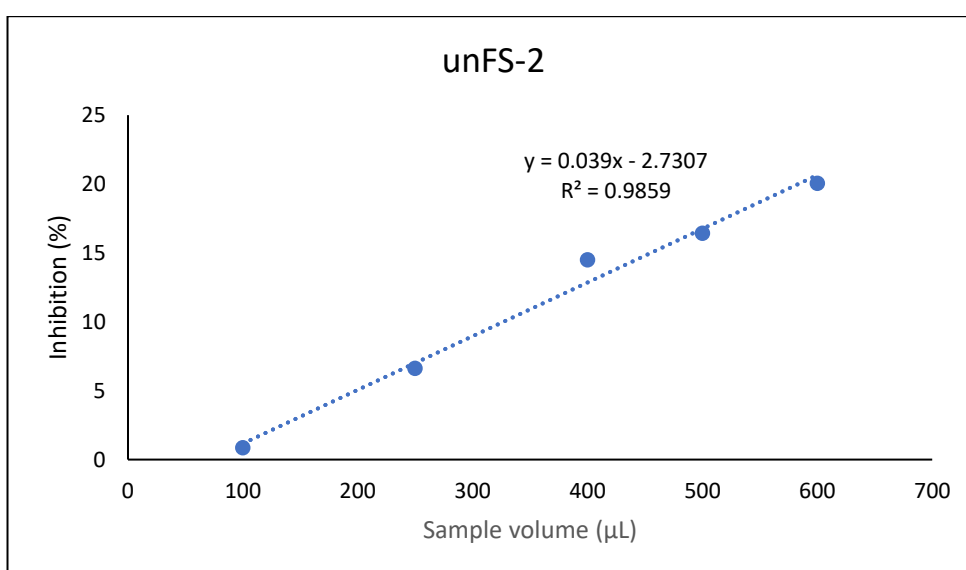


Figure D1.2: Inhibition of DPPH radical versus sample volume of the unFS product to calculate EC₅₀ value.

Appendix E: Trolox standard calibration curve to determine the TEAC of the products.

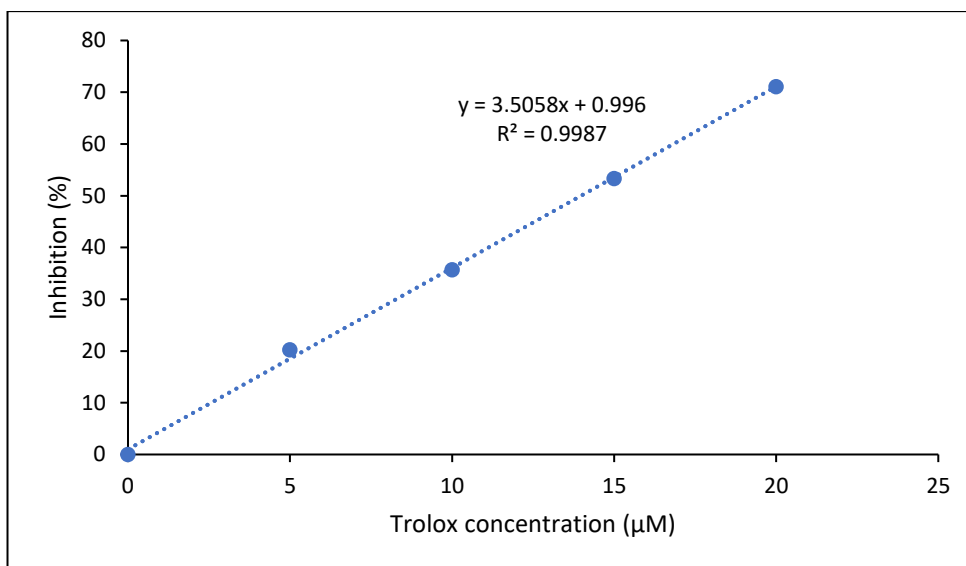


Figure E1.1: Trolox standard calibration curve prepared to measure the TEAC value of products. The measurements performed on Shimadzu UV-1280.

Appendix F: Example graphs showing the ABTS inhibition activity versus the sample volume that was used to calculate the TEAC value.

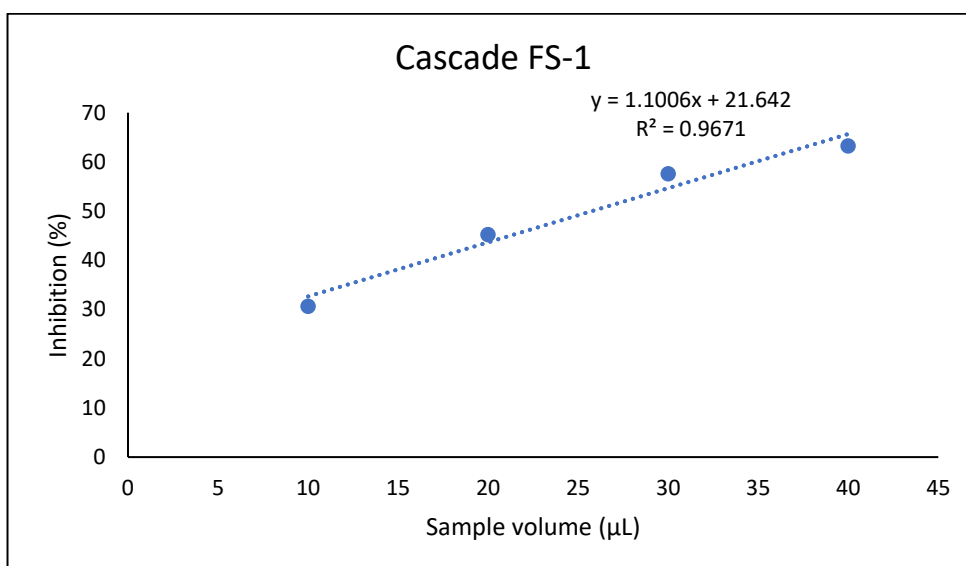


Figure F1.1: ABTS inhibition activity versus the sample volume of cascade FS. The TEAC value is calculated by dividing the slope of the line to the slope of the Trolox standard curve.

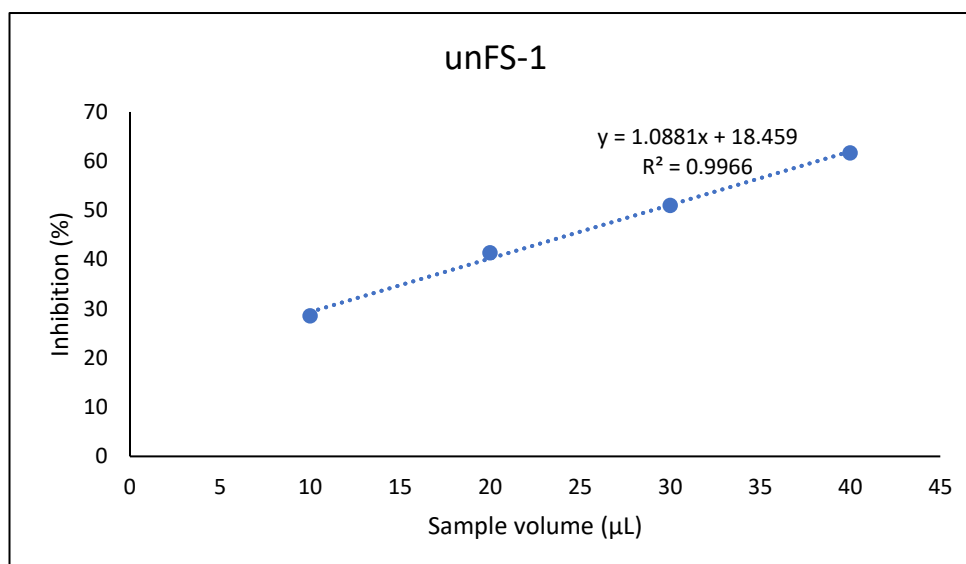


Figure F1.2: ABTS inhibition activity versus the sample volume of unFS. The TEAC value is calculated by dividing the slope of the line to the slope of the Trolox standard curve.

Appendix G: Total soluble protein concentration with the statistical analysis (ANOVA, Tukey's HSD $p \leq 0.05$).

Table G1.1: Total soluble protein concentration determined by Lowry et al. (1951) with statistical analysis.

Bioreactor	Time (h)			
	0	24	48	72
0.5 vvm	4.68±0.07 ^{AB,ab}	4.12±0.47 ^{B,a}	5.04±0.02 ^{AB,b}	5.75±0.20 ^{A,c}
1 vvm	4.93±0.30 ^{B,ab}	4.51±0.18 ^{B,a}	5.66±0.41 ^{B,b}	8.65±0.47 ^{A,ab}
Cascade	3.66±0.12 ^{C,b}	5.79±0.41 ^{B,a}	9.48±0.23 ^{A,a}	9.10±0.47 ^{A,a}
Controlled pH	5.55±0.42 ^{A,a}	5.97±0.23 ^{A,a}	5.97±0.06 ^{A,b}	6.23±0.51 ^{A,bc}

Means ±SD. ^{A-C} Means in the same production condition followed by different capital letters indicate significant changes during fermentation. ^{a-c} Means in the same fermentation time followed by different lowercase letters indicate significant changes between different production conditions ($p < 0.05$).

Appendix H: Publications related to thesis.

Yay, C., Yağız, N., Kurt, H., Şahin, B., Güneşer, O., Karagül Yüceer, Y., İşleten Hoşoğlu, M. (2023). Fermented microalgae: changes in bioactive properties and sensory characteristics. Algae Nexus Conference, İstanbul, 9-10 February 2023. (Oral presentation).

Yay, C., Yağız, N., İşleten Hoşoğlu, M. (2021). The Impact of Fermentation on Antioxidant and Antimicrobial Activities of *Spirulina platensis*. 13th International Conference on Protein Stabilization (ProtStab), Online, 7-9 October 2021. (Oral presentation).