

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

**USE OF ARTIFICIAL INTELLIGENCE-BASED APPROACHES FOR
DEVELOPING MICROALGAE-BASED NUTRIENT MEDIA AND MODELLING
THE GROWTH OF *B.SUBTILIS* IN THESE ALTERNATIVE MEDIA**

ŞEVVAL ÇİMYAPAN

**A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY**

ADVISOR: ASSOC. PROF. DR. MÜGE İŞLETEN HOŞOĞLU

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**MİKROALG BAZLI BESİN ORTAMLARI GELİŞTİRMEK VE *B.SUBTİLİS*'İN BU
ALTERNATİF ORTAMLARDA BÜYÜMESİNİ MODELLEMELİK İÇİN YAPAY
ZEKA TEMELLİ YAKLAŞIMLARIN KULLANILMASI**

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ABSTRACT

This study was carried out for optimization of the parameters for extraction of proteins from different microalgal biomass (*Spirulina platensis*, *Auxenochlorella protothecoides* and defatted *Schizochytrium limacinum* biomass) by using Ultrasound Homogenization (USH) method with the aid of Artificial Intelligence tools.

Then, the growth of *Bacillus subtilis* (B-3387) in such protein and other nutrients rich algae-based three different media were optimized by using both Response Surface Methodology (RSM) and Artificial-Intelligence approaches. The resulting fermented products (FPs) were evaluated in terms of their microbiological and chemical properties. Accordingly, the Random Forest (RF) AI Algorithm gave the best prediction result in the USH extraction optimization ($R^2=0.9921$).

The highest viable cell count of $9.05 \log \text{CFU.mL}^{-1}$ was obtained in *S.limacinum* biomass based (SBB) for 72 h fermentation. According to the SDS-PAGE profile, more protein bands were observed in SBB fermented product compared to unfermented counterparts. Antioxidant values of all FMs were investigated. Accordingly, SBB fermented products had the highest DPPH ($\text{EC}_{50}=171.39 \mu\text{L}$), TEAC (75.42 mM.mL^{-1}) and TPC (Total Phenolic Content Analysis) (0.71 mg.mL^{-1}) results. Besides, SBB had also the best results according to its ACE inhibitor activity potential (77.74%). Moreover, the total soluble protein contents of all FPs decreased at the end of fermentations. It was interpreted that *B. subtilis* could be able to utilize them for its growth and its metabolic activities.

Gaussian processes (GP) gave the best prediction result ($R^2=0.843$) among the Artificial Intelligence based prediction models based on the results obtained according to both pre-fermentation and post-fermentation contents of all microalgae based media.

In this work, different microalgae biomass based fermentation processes were developed. And also, it was revealed that the use of artificial intelligence algorithms offered promising approaches for understanding non-linear and complex data, model prediction of models in experiments and the acceleration of laboratory work.

Keywords: Microalgae-based media, Artificial intelligence, Fermentation, Physico-chemical analysis, Response surface methodology (RSM)

ÖZET

Bu çalışmada, farklı mikroalgal biyokütle tabanlı fermente ürünlerinin üretilmesi amacıyla, hem fermentasyon ortamı hazırlığında hem de *Bacillus subtilis* (B-3387) ile gerçekleştirilen fermentasyonlarda mikrobiyal gelişimlerin Yapay Zeka yaklaşımları ile optimizasyonu hedeflenmiştir. Bu amaçla ilk olarak, farklı mikroalgal biyokütlelerden (*Spirulina platensis*, *Auxenochlorella protothecoides* ve yağı alınmış *Schizochytrium limacinum*) Ultrason homojenizasyonu (USH) yöntemi kullanılarak protein ekstraksiyonu optimizasyonlarına odaklanılmıştır. Ardından, *B.subtilis*'in (B-3387) başta çözünür proteinler bakımından zengin üç farklı mikroalg tabanlı besiyerinde gelişimi, hem yanıt yüzey yöntemi hem de Yapay Zeka yaklaşımları kullanılarak optimize edilmiştir. Elde edilen ürünler mikrobiyolojik ve kimyasal özellikleri bakımından incelenmiştir. Buna göre, Rastgele Orman (RF) Yapay Zeka Algoritması USH ekstraksiyon optimizasyonunda en iyi tahmin sonuçlarını vermiştir ($R^2=0,9921$). En yüksek *B. subtilis* canlı hücre sayısına $9,05 \log \text{CFU.mL}^{-1}$ *S. limacinum* bazlı fermente ürünlerde (ScBF) 72 saatlik fermentasyon sonunda ulaşılmıştır. SDS-PAGE profiline göre, ScBF ürünlerde, fermente edilmemiş (ScB_unF) benzerlerine kıyasla daha fazla protein bandı olduğu saptanmıştır. Bununla birlikte, ScBF ürünlerin antioksidan aktivite açısından DPPH ($\text{EC}_{50}=171.39 \mu\text{L}$), TEAC (75.42 mM.mL^{-1}) ve TPC (Toplam Fenolik İçerik Analizi) (0.71 mg.mL^{-1}) en yüksek potansiyele sahip olduğu ortaya konmuştur. Benzer olarak, ScBF, ACE inhibitör aktivite (%77.74) özelliği ile de ön plana çıkmaktadır. Ayrıca, tüm ürünlerin fermentasyonlar sonucunda toplam çözünür protein konsantrasyonlarının azaldığı tespit edilmiştir. Fermentasyon sonrasında hem fermente edilmemiş hem de fermente ürünlerin protein konsantrasyonlarından oluşturulan Yapay Zeka tabanlı tahmin modelleri arasında en iyi tahmin sonucunu Gauss süreçleri (GP) vermiştir ($R^2=0,843$). Bu çalışma ile, farklı mikroalgal biyokütle tabanlı fermente ürünlerin geliştirilme potansiyeli ortaya konmuş olup, aynı zamanda yapay zeka algoritmalarının kullanımının doğrusal olmayan ve karmaşık verilerin anlaşılması, deneylerde model tahmini ve laboratuvar çalışmalarının hızlandırılması için umut verici sonuçları olduğu ortaya konmuştur.

Anahtar Kelimeler: Mikroalg, Fermentasyon, Yapay Zeka, Yanıt Yüzey Yöntemi, Kimyasal Analizler

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	: Celsius
%	: Percentage
h	: Hour
β	: Beta
γ	: Gamma
g	: Gram
w	: Weight
v	: Volume
mL	: Mililiter
M	: Molar
mg	: Miligram
μg	: Microgram
μl	: Microliter
L	: Liter
μm	: Micrometer
p	: P value
ACE	: Angiotensin-I converting enzyme
Appx.	: Appendix
ANN	: Artificial neural networks
ABTS	: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
Cd	: Cadmium
cfu	: Colony forming unit
CO ₂	: Carbon dioxide
R ²	: Coefficient determination
DPPH	: 2,2-diphenyl-1-picrylhydrazyl
DHA	: Docosahexaenoic acid
DT	: Desicion Tree
Eq	: Equation
GC–MS	: Gas chromatography-mass spectrometry
GRAS	: Generally regarded as safe
GAM	: Generalised Additive Model Regression
GP	: Gaussian processes
GA	: Genetic algorithm
FM	: Fermented Microalgae
HPLC	: High-performance liquid chromatography
HA	: Hippuric acid
kDa	: Kilodaltons
log	: Logarithm
min	: Minutes
MM	: Molecular Mass
MSLE	: Mean Squared Logarithmic Error
nonFM	: NonFermented Microalgae
PUFAs	: Polyunsaturated fatty acids
PBS	: Phosphate buffer saline
RSM	: Response Surface Methodology
RF	: Random Forest
RMSE	: Root mean square error

spp.	: Subspecies
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
USH	: Ultrasound homogenization
TPC	: Total phenolic contents
TEAC	: Trolox Equivalent Antioxidant Capacity
TGY	: Tryptone Glucose Yeast Extract Medium
SVM	: Support vector machine
WHO	: World Health Organization



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1. INTRODUCTION

Nowadays, it is recognised that developing healthy and alternative nutritional habits would be much better for both public health and the planet. While foods are modified to prevent the excessive consumption of animal-based products in developed countries, consumption of cholesterol-free, low-fat foods and adoption of healthy nutritional approaches rich in prebiotic, bioactive components is encouraged in developing countries. In this context, obtaining protein, one of the most important nutritional needs has been carried out on obtaining from alternative sources. Among these protein sources, microalgae are of interest as an alternative protein source due to their balanced amino acid ratio. However, extraction of proteins from algal biomass depend on various factors like type of microalgae, methods used for cell disruption and extraction, time (**Abomohra et al., 2016; Bleakley & Hayes, 2017**). There are several cell disruption techniques such as mechanical, chemical, enzymatic and some emerging Technologies (**Alam et al., 2020; Halim et al., 2010**). To preserve the functionality of algal metabolites during cell disruption is one of the important factors. And also, a feasible and an energy-efficient cell disruption method should be applied to ensure both low operating cost, and high product recovery. Ultrasound homogenisation (USH), the simplest, most efficient and scalable alternative, is one of the most preferred methods used for extraction purposes (**Günerken et al., 2015**). However, in order to promote the lowest energy consumption and avoid protein denaturation due to the shear force and time applied, the USH process should be optimised for different microalgae species and metabolite extractions. In the last few years, machine learning techniques have grown in popularity thanks to increasing computational capacity and the large volumes of data that can be produced and managed (**Jordan & Mitchell, 2015**).

In this way, predicting the best extraction conditions for USH process with machine learning could be considered to provide more efficient results for future studies and to deal with such operational problems. To solve these types of complex issues, machine learning algorithms, could be successfully applied to predict the determination of best conditions (**Jordan & Mitchell, 2015**). For example, in one study, the extraction and recovery of phenolic compounds from photosynthetic organisms was modelled using artificial neural networks (ANN), a machine learning algorithm, to predict ideal values

and standards for the process **(Dahmoune et al., 2015)** . Despite the consumption of algal proteins in humans is currently underdeveloped, their high protein content, favourable essential amino acid profile and being sustainable and natural alternative make them a promising source of protein for future expansion **(Bleakley & Hayes, 2017)** .The most important feature that limit the use of microalgal metabolites as food or feed additives is their distinct aroma, flavor, and color properties **(Isleten Hosoglu, 2018; Lafarga, 2019; Milovanović et al., 2015; Yamamoto et al., 2014)** .Fermentation can be an alternative way in creating unique flavor characteristics to raw materials. Fermentation may also leads to increasing functionality of raw material and formation of innovative ingredients. Recently, there is an interest to use different micro/macroalgae species in different fermentation processes **(Bao et al., 2018a; de Marco Castro et al., 2019a)**. The development of microalgae based fermentation processes is a new and challenging phenomenon. Studies has shown that the type of microorganism chosen and fermentation conditions, such as the concentration of algal biomass, fermentation time, and temperature, can significantly impact the bioactive properties as well as the taste and flavor of the end product. Thanks to these studies, it provides a perspective on the industrial use of bioactive compounds and post-fermentation products **(Bao et al., 2018a; de Marco Castro et al., 2019a)**.

1.1. Aim and Contribution of Thesis

This study, firstly, investigated the protein extraction efficiency from various microalgal biomass, including *Spirulina platensis*, *Auxenochlorella protothecoides*, and defatted *Schizochytrium limacum* using the Ultrasound Homogenization (USH) method. Response Surface Methodology (RSM) was used to reveal the optimum extraction conditions depending on biomass concentration (g/L) and time (h). After establishing the optimum extraction conditions by using USH method, Viscozyme enzyme was also applied to improve the protein extraction efficiencies from three different algal biomass. Finally, these three algal extracts were used as fermentation media for growing *B. subtilis*. A data set was created with the results obtained from the experiments performed depending on the parameters from the experimental design created with RSM. Prediction models were created to evaluate the joint performance of biomasses with the data sets obtained from protein extractions with also Artificial

Intelligence tools. Random Forest (RF), Support Vector Machine (SVM) and Generalised Additive Model Regression (GAM) models were used. Unlike other methods, the Artificial Intelligence (AI)-algorithm used also allowed us to make a prediction approach for the protein content to be obtained as a result of a common extraction process of three different microalgae biomasses.

Bacillus subtilis (B-3387), *Spirulina platensis*, *Auxenochlorella protothecoides* and defatted *Schizochytrium limacum* biomasses were used to develop functional products. Some of the chemical and microbial properties of the fermented products were investigated. As a result, the advantages of the fermentation process related to the products formed were revealed. In addition, protein content was determined by taking samples from microalgal media at certain periods during the fermentation process. As a result of the determinations, a dataset was created for three algal biomass media depending on the presence of inoculation and time parameters. Random Forest (RF), Decision Tree (DT), Generalised Additive Model Regression (GAM), Support Vector Machine (SVM) and Gaussian processes (GP) artificial intelligence tools were used to create prediction models to evaluate the joint performance of the biomasses on the observed changes in protein concentration in the fermentation process.

To the best of our knowledge, there are no studies on optimisation of protein extraction of *Spirulina platensis*, *Auxenochlorella protothecoides* and defatted *Schizochytrium limacum* biomasses by USH and artificial intelligence based prediction modelling. In addition, there are no studies on the growth of *B.subtilis* species in different microalgae biomasses, their evaluation in terms of bioactivity, and artificial intelligence based on protein concentration in fermentation processes.

With this study, both physicochemical and microbial analyses of microalgae, which is an alternative product source, were carried out and it was aimed to provide benefits such as time, cost and labour force by shedding light for future studies by using artificial intelligence based prediction modelling.

1.2. Microalgal Proteins: The Healthy and Sustainable Protein Source of the Future

The increase in the global population and the decrease in food resources have led to an increase in research on sustainable and alternative healthy food sources. When it comes to new alternative food sources, single cell proteins such as microalgae, fungi, yeasts and bacteria can be counted **(Becker, 2007a)**. In this context, microalgal proteins stand out among alternative protein sources. As an alternative and sustainable food sources, microalgae constitute an important alternative with their high protein content, aminoacid profile and easy production processes **(Bleakley & Hayes, 2017)**. As a result of studies on healthy protein sources, microalgae are rich in amino acids and also provide an important potential against this global problem with their nutritional properties **(Samarasinghe et al., 2012)**. Microalgae are photosynthetic organisms. Microalgae include members of prokaryotic, eukaryotic or cyanobacteria. They contain high amounts of nutrients such as protein, vitamins, minerals and essential fatty acids **(Alam et al., 2020)**. They are also valuable for health as they contain bioactive compounds such as carotenoids and chlorophylls. Due to their rich nutrient content and health benefits, the World Health Organisation (WHO) recommends the consumption of microalgae by humans. Microalgae are considered to be of significant value because they naturally contain various active substances, pigments, proteins, polysaccharides, fatty acids and many metabolites **(Barkia et al., 2019; Tang et al., 2020)**. Thanks to these metabolic products they produce, they are involved in many sectors such as food, feed, health and cosmetics.

Table1.1: Protein content (% dry weight basis), class, and kingdom of different microalgae

Microalgae	Protein Content (%)	Class	Domain	Reference
<i>Anabaena cylindrica</i>	43-56	Cyanophyceae	Procaryota	(Becker, 2007b)
<i>Aphanizomenon flos-aquae</i>	62	Cyanophyceae	Procaryota	(Becker, 2007b)
<i>Chlorella vulgaris</i>	51-58 53.3	Trebouxiophyceae	Eukaryota	(Becker, 2007b) (Tibbetts et al., 2015)
<i>Dunaliella salina</i>	57	Chlorophyceae	Eukaryota	(Tibbetts et al., 2015)
<i>Schizochytrium limacinum</i>	50-68	Labyrinthulomycetes	Eukaryota	(García-Ortega et al., 2016)
<i>Spirulina platensis</i>	60-71	Cyanophyceae	Procaryota	(Tibbetts et al., 2015)

Total protein content in microalgae biomass varies according to microalgae species. This total protein content varies between 30% and 70% of dry weight (García-Ortega et al., 2016). For example, the protein content of *Spirulina platensis*, which is used in many industrial areas, is 24% higher than chicken, 24% higher than fish, 22% higher than beef and 37% higher than soya flour (Moorhead K. & Capelli B., 2011. *Spirulina Nature's...* - Google Akademik, n.d.) Due to their high protein content, microalgae species such as *Spirulina platensis*, *Auxenochlorella protothecoides*, *Chlamydomonas reinhardtii*, etc. are used for industrial ways.

1.2.1. *Spirulina platensis* as an Alternative Microalge for Protein Demand

Spirulina (Arthrospira) platensis is one of the blue-green algae known today as superfoods. The reason why it is called superfood is its high nutritional value and health benefits. *S.platensis* is a free-floating filamentous cyanobacterium. **Figure 1.** shows the microscopic image of *S.platensis* microalgae (**Seyidoglu et al., 2017**). It is a microalgae species known in the past in human nutrition. It is known to be used as food since 1521 recorded in history (**Q. Hu, 2007**). The biomass obtained from microalgae cultivation on an industrial scale has a high protein content (60-70%). Every 100g of *S.platensis*, which has high protein content, contains essential amino acids such as 3.209g isoleucine, 4.947g leucine, 5.793g aspartic acid, 8.386g glutamic acid (**C. Y. Chen et al., 2011**). *S. platensis* is also an important source of phycobiliproteins, including c-phycocyanin, a natural blue pigment. It also contains allophycocyanin and phycocyanin, a class of phycobilins, which are blue in colour and together with chlorophyll-a give algae their blue-green colour (**Seyidoglu et al., 2017**). The fact that it contains phycocyanin reveals its anti-inflammatory, anticancer and antioxidant properties (**Su et al., 2014**).



Figure 1.1: (A) Microscopic image of *S. platensis* and (B) Scanning electron micrograph of *S. platensis*. (Seyidoglu et al., 2017)

With its high values, *S. platensis* offers an alternative at many points in terms of its use in the field of health, as a protein supplement or as a food component. Various applications are carried out on *S. platensis* to ensure its use in these areas. Mechanical extraction and chemical applications are used for the extraction of proteins in its content. Methods that provide mechanical disintegration, such as the use of homogenizers, enable the emergence of bioactive compounds and proteins thanks to the effective disintegration of the cell wall (Samarasinghe et al., 2012). In addition, various methods such as solvent extraction are used (Bleakley & Hayes, 2017). As a result of the studies, the protein content of *S. platensis* biomass can be increased, and

with its high values, it offers alternatives at many points in terms of its use in the field of health, its use as a protein supplement or its evaluation as a food component.

1.2.2. *Auxenochlorella protothecoides* as an Alternative Microalga for Protein Demand

A. protothecoides belongs to the class of green algae (Chlorophyta). It is also known as *A. protothecoides protothecoides*. **Figure 2** shows the microscopic image of microalgae *A. protothecoides* (**Broady, 1984**).

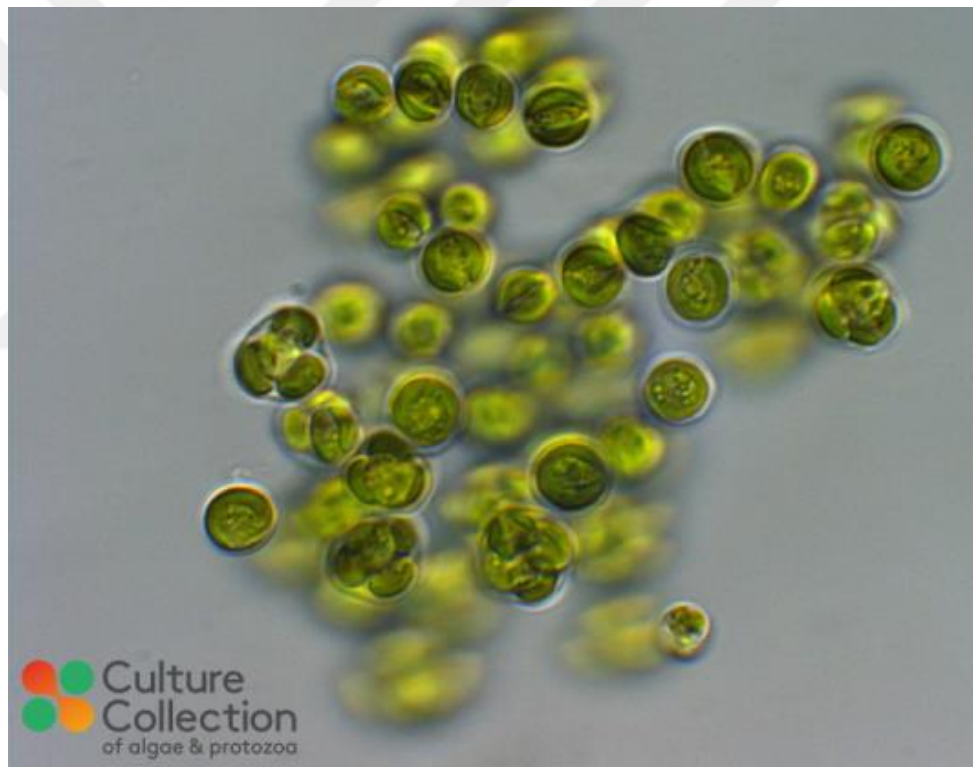


Figure 1.2: Microscopic image of *A. protothecoides* (Broady, 1984)

It can produce energy photosynthetically. It utilises sunlight to produce organic compounds by using carbon dioxide and water. The most important organic compounds produced by *A. protothecoides* are lipids. It also has a high potential for lipid accumulation. For this reason, it is an industrially important microalgae species. The oil accumulated in the majority of microalgae consists mainly of triglycerides with a fatty acid profile rich in C16 and C18 (**Y. H. Chen et al., 2012**). *A. protothecoides* is

very rich in C20 arachidate fatty acids (**Monlau et al., 2021**). For use as a biodiesel feedstock, *A.protothecoides* has therefore proved to be the most promising microalgae species (**Monlau et al., 2021**). This characteristic is considered a great advantage nowadays, when the demand for energy sources such as biodiesel and biofuel production is increasing. In addition, the proteins present in *Auxenochlorella protothecoides* show approaches to the development of functional food products. For example, protein production of similar species such as *C. vulgaris* and *C. sorokiniana* has been optimised (**Lai et al., 2019**). It was concluded that the microalgae proteins obtained consisted of 40% essential amino acids. As a result, the usability of the obtained product as animal feed was mentioned and it was revealed that it has the potential to be a protein-rich raw material (**Lai et al., 2019**). The study also evaluated the antibacterial and prebiotic activities of *A.protothecoides* proteins and provided promising results. In addition, the safety of consumption of *A.protothecoides* protein has been proven and the health effects of its consumption have been shown to reduce high blood pressure and cholesterol levels (**Chia et al., 2019**). Alkaline ultrasound assisted single solvent extraction method was applied for extraction to reveal the protein content in *A.protothecoides* (**Hildebrand et al., 2020**). The amount of protein content obtained was high. For this reason, the evaluation of *A.protothecoides* in terms of nutritional value, examination of its bioactive properties and scalability are of great importance.

1.2.3. *Schizochytrium limacinum* as an Alternatif Microalge for Protein Demand

Schizochytrium limacinum is a marine thraustochytrid, a species of heterotrophic microorganism belonging to the class Labyrinthulomycetes. It has a rich content of polyunsaturated fatty acids (PUFA). It has the capacity to produce docosahexaenoic acid (DHA), an important omega-3 fatty acid, at high capacity (**Song et al., 2007**). Its high lipid content has applications in biodiesel and biofuels. The use of *S.limacinum* in the food industry is becoming widespread. In addition to its use as a food supplement, a study on enrichment of consumer eggs with n-3 PUFAs by adding it to the feed of chickens in animal feed has shown that the content is enriched (**Kralik et**

al., 2020). The use of *Schizochytrium* in chicken feed has been shown to be nutritionally beneficial to consumers. In addition, *S.limacinum* contains rich compounds such as enzymes, pigments, carbohydrates. The proteins in its content exhibit sustainable and promising approaches to alternative food and biocompound production. Since it has been observed that extracts obtained from microalgae contain peptides with high bioactivities (**X. Hu et al., 2019a**). These peptides are considered as food components that exhibit activities such as antihypertensive, antioxidant and beneficial to human health (**Moaveni et al., 2022a**).



Figure 1.3: Microscopic view of *S. limacinum* (**Abad & Turon, 2015**)

It is also possible to use *S.limacinum* residue by-product obtained from oil extraction (**X. Hu et al., 2019b**). The content of *Schizochytrium limacinum* contributes to its potential applications in the production of functional foods and nutraceuticals. The *Schizochytrium* residue contains more than 40 proteins (**X. Hu et al., 2019b**). In this field, *Schizochytrium* has a low economic value as it plays the role of a residue. However, the presence of valuable bioactive components such as phycoerythrin, phycocyanin and allophycocyanin in its content has revealed that it may exhibit antioxidant and anticancer properties (**X. Hu et al., 2019b**).

1.3. Extraction Methods used to Increase the Yield from Microalgae

Microalgae have an important place in bioprocessing applications. The extraction of bioactive components such as proteins, carbohydrates, lipids, vitamins from microalgae and the utilisation of these components in areas such as biofuel production and food industry are among the common topics of study today. There are some important considerations in determining extraction methods. Firstly, the techniques used differ depending on the characteristics of the cell wall of the microalgae species. Extraction conditions may differ in microalgae species with high polysaccharide levels such as *Algaenan*, *Chlorella* spp. and microalgae species with less rigid cell walls such as *Arthorspira* spp. or in the use of a common method, optimisation should be achieved with extra parameters such as time, intensity, temperature, pH (**Günerken et al., 2015**). Mechanical and chemical extraction methods are available. Mechanical and physical methods can promote extraction by many means, such as high-speed or high-pressure homogenisation or microwave irradiation, ultrasonication, pulsed electric fields, autoclaving, freeze/thaw cycles (**Günerken et al., 2015**). For example, ultrasound homogenisation methods play an important role in increasing protein yield from microalgae and offer several advantages in the extraction process. One of the most important advantages is the efficient disruption of cell walls and the optimal release of intracellular proteins. This mechanical disruption at high speeds ensures complete homogenisation by breaking down cellular barriers and facilitating access to valuable protein components (**Corrêa et al., 2020**). Non-mechanical methods include chemical methods that may use acids, detergents or enzymatic processes. Enzymatic extraction differs from other methods in that it uses less energy and is selective-targeted. For example, vizcozyme, thanks to its enzyme complex, increases starch productivity and facilitates the extraction of bioactive compounds, making it a viable extraction method in bioprocesses (**R. Zhang et al., 2018**). Together with the examples given, the importance of extraction methods is emphasised by the fact that microalgae are often used for the extraction and efficient recovery of their biochemical components by disruption or disruption of the cellular membrane.

1.4. Importance of Fermentation for such Functional Ingredients Development Issues

Fermentation is an old method used from past to present. Fermentation is divided into two in terms of ATP production. It occurs aerobically in the presence of oxygen and anaerobically in its absence by the breakdown of organic compounds by microorganisms. Fermentation is shown as an important technique in the development of functional algae-based products. Studies show the comprehensive effect of algae-based fermentation in many fields such as health, food and fuel production. During fermentation, microorganisms are utilised to obtain content-rich products. *Bacillus sp.* can be given as an example of the microorganism species used (Gu et al., 2018). *Bacillus* species are characterised by their ability to secrete protein (Aguilar et al., 2007). In this way, in the industrial production of various products, their easy growth conditions, their robustness against endogenous metabolisms have made them the preferred microorganism especially in industrial protein production (Zweers et al., 2008). In addition, *B.subtilis* is one of the microorganisms defined as a GRAS strain by the US Food and Drug Administration (Gu et al., 2018). In this way, *B.subtilis* has an important role in the development of algae-based commercial products. There are studies showing that the use of microorganisms during the fermentation of algae is beneficial, such as the conversion of organic compounds such as lipids and proteins in the algae content into bioactive compounds with high antioxidant capacity, anticancer and anti-inflammatory potential (Samarakoon & Jeon, 2012). For example, the potential of fermentation in the production of new antioxidants from enzymatic hydrolysates from *S.limacinum*, a microalgae species with widespread industrial use, has been supported by a study (Moaveni et al., 2022a). In the study, the potential of *S. limacinum* as an alternative protein source with its high protein content and essential amino acid diversity was emphasised. At the same time, the importance of regulatory approval is emphasised and the full utilisation of microalgal biomass is emphasised (Moaveni et al., 2022a).

1.5. Artificial Intelligence in Bioprocess Technology

The importance and application areas of the bio-based economy are increasing day by day. The trend towards industrial bioprocesses to produce biofuels, materials and health products is also increasing (**Mol et al., 2021**). Bioprocesses are processes in which living cells or living cell compounds are used to produce value-added products. We can say that bioprocesses are affected by many factors such as the viability of the microorganism used, its metabolic properties and external environmental conditions. These factors have a significant impact on the scaling up of processes. The development and optimisation of processes is important for process efficiency and product quality. Therefore, optimisation studies should be carried out to obtain better performances from bioprocesses (**G. Wang et al., n.d.**). In this context, AI modelling addresses an important point in optimisation and control processes. Machine learning (ML) is one of the AI disciplines. Machine learning is a learning process based on available data. ML, which creates the concept map in the process with the available data, increases its performance with the increase in data (**Helleckes et al., 2023**). Thanks to this approach, it is possible to model the non-linear relationships of the variables in the process and to use the models created for process optimisation. For example, an artificial neural network (ANN) was applied to model and optimise the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by fermentation. Genetic algorithm (GA), artificial neural network (ANN) and response surface methodology (RSM) simulation were compared and the predictions of ANN were better than those of RSM and in good agreement with experimental findings (**Zafar et al., 2012**). ANNs have also been utilised to improve the efficiency of fermentation processes. ANN modelling was used for ale beer production. The data obtained from the operation parameters were taught to AI. As a result of this modelling, the production of beer that meets the quality specifications with a correlation coefficient higher than 0.85 has been successfully achieved (**Takahashi et al., 2019**). The advantages and economic benefits of hybrid machine learning in the optimisation of production processes have been mentioned in different studies (**Saini et al., 2021**). If it is necessary to mention the benefits emphasised in the studies; AI enables bioprocesses to be optimised accurately by creating a data-driven model. In addition, the joint evaluation of different data groups is an important benefit. It has the ability

to create models and predictions with a small number of data. In this way, it has the capacity to create models even in data sets containing missing values (**Cheng et al., 2023**). However, since the studies carried out in bioprocesses are dealt with very specific issues, it is difficult to use and apply ready-made data sets by creating common data sets. By detailing the appropriate data sets in the studies, more opportunities can be provided for studies in this field in the future.



2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Microorganisms and Materials

Dried form of *Spirulina platensis* biomass was obtained from a local manufacturer Algae Ltd. (Canakkale, Türkiye). *Auxenochlorella protothecoides* and de-oiled *Schizochytrium limacinum* biomass was obtained from the company Marinbio (Aydın, Türkiye). *B.subtilis* (B-3387), GRAS microorganism used for fermented microalgal products, were obtained from NRRL Culture Collection, USDA Agricultural Research Service (IL, USA).

2.1.2. Chemicals

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 ($\text{C}_5\text{-C}_{30}$) (Aldrich), Amino acid mix (Sigma-Aldrich), Methanol (Sigma-Aldrich), 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, (Acs Reagent, Anhydrous, Acros), Ethanol (Isolab), Acetic Acid (Glacial, 100 %, Isolab), Sodium Chloride (Isolab), Yeast Extract (Biolife), 39 Peptone Bacteriological (Biolife), Glucose (Dextrose), Bacteriological (Biolife), Lactose (Biolife), Sucrose (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).

2.1.3. Laboratory Devices

All laboratory equipment used was as shown in **Table 2.1**.

Table 2.1: The list of laboratory devices used in this study

LABORATORY DEVICES	BRANDS
Micropipettes	Rainin Pipet-Lite XLS
Vortex stirrer	N-Biotek
Autoclave	Nuve NC 90M
Magnetic stirrer	Witeg MSH-20D
pH meter	Hanna
Biological Safety Cabinet (Class 2)	SafeFast Classic
Water-bath	Biorad
SDS electrophoresis	Biorad
Ultrasonic homogenizer	Bandelin HD 2070
Shaking incubator	N-Biotek
Spectrophotometer	Shimadzu UV-1280
Centrifuges	Nuve, NF 1200R; Sigma 1-16K
Precision balance	Wisestir
Microscope	XSZ-107BN of Biological Microscope
Ice-maker	Labcold Flake
HPLC System	Agilent Technologies INC., Folsom, CA.(Infinity 1260 Autosampler, 1260 Multiple Wavelength UV detector)

2.1.4. Software for Analysis

Two-way ANOVA was performed using GraphPad Prism version 9.0 for MacOS.

2.2. Methods

2.2.1. Extractions Performes with Three Different Microalgal Biomass

As for cell disruption and increasing the efficiency of soluble protein extractions from three different microalgae biomass *A. protothecoides*, *S.limacinum* and *S.platensis*, a probe type ultrasound homogenizer (Bandelin HD 2070) was used. Extractions were carried out with USH technique at 9*10% cycle and 45-55 % power at the respective times. RSM with a Box-Behnken approach was used for the optimisation of biomass concentration (g/L) and extraction time (min), which were extraction parameters that may affect the total soluble protein concentration of each extract obtained from three different microalgae biomasses. Considering three different microalgal biomass, a total of 13 runs (**Table 3.1**, **Table 3.2** and **Table 3.3**) were set using Design Expert® software (version 13.0.0, Stat-Ease Inc., Minneapolis, MN) for each. Biomass used for the experimental series were dissolved in deionized water based on the concentrations determined in the experimental design. Supernatants were obtained by centrifugation at 10,000 rpm for 40 minutes and kept in freezer until used for related experiments. Supernatants were analyzed in terms of total soluble protein content using the Lowry method (**Lowry et al., 1951**). (AppendixA)

2.2.2. Data Analysis

2.2.2.1. Response Surface Methodology for Investigating Optimum Extraction Conditions

RSM experimental designs were used to optimise dry weight concentration and time-dependent conditions using Box-Behnken.

RSM was used to investigate interaction of two extraction parameters (Time and Concentration). For each microalgae biomass species, 13 sets of experiments were set up using Design Expert® software (version 13.0.0, Stat-Ease Inc., Minneapolis, MN). Extraction parameters were normalised as coded parameters. Response Surface 2FI (2nd-Order Full Factorial) Model were used in Response Surface Methodology (RSM)

analysis. Analysis of variance (ANOVA) was used to analyse model accuracy, regression analysis and statistical significance. Three-dimensional (3D) contour plots were created and the interaction between the parameters was expressed by utilizing the responses.

The experimental design can be expressed by the general equation of the two-factor interaction (2FI) model as shown in Equation 2.1 to develop a model to be used for each biomass to analyse the interaction of parameters (Ahmad et al., 2013)

$$\gamma = b_0 + \sum(b_i X_i) + \sum(b_{ij} X_i X_j) + e_i \quad (2.1)$$

In the equation, γ is the response to be estimated; n is the number of factors; X_i and X_j are coded variables; b_0 is the constant coefficient; b_i and b_{ij} are the first-order and interaction coefficients, respectively; i and j are the index numbers for the factors; and e_i is the residual error.

For all three biomasses, models were constructed based on the equation expressed.

2.2.2.2. Artificial Intelligence Based Prediction Model Development for Extraction of Protein from Different Microalgal Biomass

In order to characterise the soluble protein contents obtained by USH extraction from *S.platensis*, *A.protothecoides* and *S.limacinum* , 39 data points were collected. Data were categorised according to microalgae species. Various machine learning based regression methods (SVM, RF, GAM, etc.) were compared and evaluated according to the best prediction accuracy. During this evaluation; root mean square error (RMSE), normalised-root mean square error (N-RMSE) coefficient of determination (R^2), adjusted- R^2 and Mean Squared Logarithmic Error (MSLE) were considered. All these procedures were performed in Matlab 9.10.0.1710957 (R2021a) software (MathWorks Inc., Natick, MA, USA).

The prepared data set was firstly utilised using Random Forest, a machine learning based regression method. RF is a regression method in which many decision trees are formed together (Elmaz et al., 2020). In the bagging method, the training data are separated by bootstrap sampling method. While training each decision tree, bootstrap

sampling was utilised. With bootstrap sampling, new data sets are created by selecting data from the data set and taking random samples. Afterwards, these data sets were brought together and decision trees were formed. The decision trees enabled us to predict the target responses by using the response expressions in the data set. Random Forest was created by combining each decision tree. Validation tests were taken into account to evaluate the performance of the model. The model graph was created.

$$f_{rf}^{-K}(x) = \frac{1}{K} \sum_{k=1}^K \{T(x)\}^k \quad (2.2)$$

In the equation 2.2, the forecasts are averaged from the results of the decision trees and represent the RFR Model. Where K is the number of trees, and $\{T(X)\}^k$ is the output of the k^{th} tree.

The data were evaluated with a different regression method, Support Vector Machine (SVM). SVM is a popular machine learning algorithm. SVM, which is also used in regression problems, solves learning problems with fewer samples (**Suykens et al., n.d.**) The SVM model thickens the optimisation process by creating linear equations (Suykens & Vandewalle, 1999). Since it is a model that benefits from the use of kernel functions, it minimises the error. Since it performs better on smaller datasets, it performed well on our dataset of 39 data. The features were first mapped to a high dimensional space (F) with a kernel function and linearly related to the outputs, thus creating a Support Vector Machine model for regression on our dataset. (**Dashti et al., 2019**). The SVM Equation utilised is given in 2.3

$$f(x) = \sum_{i=1}^n a_i K(x_i, x_j) + b \quad (2.3)$$

where, $K(x_i, x_j)$ is the kernel function, b is the bias term and (i = 1, 2, 3 ..., n) are constant parameters.

Generalized Additive Model Regression (GAMR) is widely used to model non-linear relationships. It contains combined, flexible functions called splines, which combine linear and additive models (**Y. Zhang et al., 2021**) The sum of these splines is the GAM. As in RF, bagging and boosting methods are utilised. Due to this reason, the

working performance is high. As mentioned with the data in this study, a shape function consisting of splines is used. This makes the model easy to interpret.

Evaluation methods are used in machine learning approaches. One of them is the k-fold cross validation method and it is used in our study to evaluate the approaches we have obtained. The data set is divided into k equal parts (folds) and each fold is used as a test data set in turn, while the model is trained using the remaining k-1 folds. This process is repeated k times and for each iteration, the performance measures of the model (e.g. accuracy rate or mean squared error) are calculated. Finally, the overall performance of the model is obtained by averaging the results of k repetitions (**Elmaz et al., 2020**) In this study, the data set was divided into 10 equal parts and the replications were calculated accordingly. 10-fold cross validation is a cross validation method in which the data set is divided into 10 equal parts and repeated 10 times. In this case, the data set was divided into 10 folds and each fold was used as a test data set in turn, while the model was trained using the remaining 9 folds. This process was repeated 10 times and the performance of the model was measured for each repetition. The results were averaged to obtain the overall performance of the model.

2.2.3. Enzymatic Treatment

After optimization of USH conditions for each microalgal biomass, enzymatic treatment was also performed using Viscozyme® L cellulolytic enzyme mixture (Sigma-Aldrich) (1.2 %; v/w) at pH 4.0, 50 °C and 2 h as described by (**Deenu et al., 2013**). Then, the solutions were kept in a 75 °C water bath for 15 min to deactivate the enzyme and samples were analyzed for total soluble protein content using the Lowry method (**Lowry et al., 1951**).

2.2.4. Preparation Inoculum of *B.subtilis* and Fermentations with Three Different Microalgae Media

Following extractions performed with USH at optimized conditions and then enzymatic application, three different culture media to be used in fermentations with *B.subtilis* were obtained from those three microalgae biomass. The pH value of three

different microalgae-based culture media was fixed at 7.00 ± 0.2 , before autoclaving. Then, all media were autoclaved at 121°C for 15 min and made ready for fermentations. The portion of sterilized unfermented microalgae (nonFM) media for each microalgae biomass was used as a control group.

B. subtilis culture was activated at 28°C in a rotary shaker and agitated at 130 rpm. Two consecutive transfers were performed in Tryptone Glucose Yeast Extract Medium (TGY) (Tryptone 5 g.g.L^{-1} ; Yeast Extract 5 g.L^{-1} ; Glucose 1 g.L^{-1} ; K_2HPO_4 1 g.L^{-1}) which was a suitable medium to be used for obtaining the sufficient inoculum amount for fermentation experiments. Before inoculation, aliquots from the culture were centrifuged at $6000\times g$ for 15 min, then resuspended in sterile physiological solution ($0.9\% \text{ w/v}^{-1} \text{ NaCl}$). After the *B.subtilis* was activated by two consecutive transfers, McFarland densitometer was used to adjust the initial cell concentrations. *Bacillus* strain 1 MacFarland, i.e. approximately $3.0 \times 10^8 \text{ log CFU.mL}^{-1}$ initial inoculum was obtained. Microalgae media were prepared in 100 mL erlenmayer flasks with a working volume of 30 mL in 3 replicates and bacterial inoculum was performed under aseptic conditions. Fermentation were carried out at 28°C on a rotary shaker and agitated 130 rpm.

2.2.5. Monitoring Microbial Growth of *B.subtilis*

Microbial fermentations were monitored by withdrawing samples at 0, 4, 8, 12, 24, 48, 72 and 96 h of fermentations using pour plate method with Tryptone Glucose Yeast Extract (TGY) agar, followed by incubation at 28°C for 24–48 h. Changes in viable cell counts were determined as log CFU mL^{-1} for each fermented microalgae products. After completion of all fermentation studies with three different microalgae media, 72 h was determined as an appropriate fermentation time for all products. For validation purpose and revealing the differences regarding their chemical properties, the same fermentation conditions were repeated with three different microalgae media and the samples collected at 0 and 72 h for the analysis mentioned below.

2.2.6. Chemical Analysis

Prior to chemical analysis, in order to remove other residues (e.g. bacterial biomass and microalgae lysates) from the culture medium, nonFM and FM products were subjected to centrifugation at $6000 \times g$ for 40 min at 4°C. The supernatants were accumulated, and stored for chemical analysis at -20°C.

2.2.6.1. Determination of Total Soluble Protein Content of Nonfermented (nonFM) and Fermented Microalgae (FM) Products

The soluble protein contents of nonFM and FM products were determined by Lowry protein assay. Samples from control groups and 4, 8, 12, 24, 48, 72 and 96 h for FM products were collected. Standard solutions of bovine serum albumin (BSA, Sigma-Aldrich) at concentrations ranging from 0 to 0.25 (mg. mL⁻¹) were used to prepare the standard calibration curve for protein measurements (Appendix A). Optical density measurements were performed at a wavelength of 750 nm.

2.2.6.2. Determination of Total Phenolic Content (TPC) of Products

Total phenolic contents (TPC) of samples belong to nonFM (control-T0) and FM (T72, 72 h fermentations) products were determined using Folin-Ciocalteu assay (Singleton et al., 1999).

The method was based on the principle that the phenolic compounds in the samples are reduced by Folin-Ciocalteu reagent in alkaline medium and the blue color resulting from the reaction is measured using a spectrophotometer.

A 100 µL of sample was mixed with 900 µL of deionized water, 4 mL of saturated sodium carbonate (75g of sodium carbonate was prepared in a 100 mL flask by first dissolving in a little deionized water and then adding to 100 mL) and 5 mL of 0.2 N Folin-Ciocalteu reagent. The samples were vortexed. Incubated for 2 h in the dark at room temperature. Absorbance values were measured at 765 nm using a spectrophotometer (Model Shimatzu). Total phenolic contents were expressed as

milligrams of gallic acid equivalent (mg GAE.L⁻¹). The calibration curve prepared using gallic acid used to determine the total phenolic content can be found in Appendix B.

2.2.6.3. Determination of Antioxidant Activity of Products by TEAC (Trolox Equivalent Antioxidant Capacity) Method

The method was based on the determination of antioxidant activity as a result of the reduction of the radical by adding our antioxidant-containing samples to the radical solution produced by the oxidation of ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (Re et al., 1999). 7 mM ABTS solution containing 2.45 mM potassium sulfate is prepared by adding 0.0384 g ABTS to 2 mL 12.25 mM potassium persulfate in a 10 mL balloon joje and filling the remaining volume with deionized water. The prepared radical is made ready for use by keeping it at room temperature and in a dark environment for 12-16 h. The radical solution prepared in this way remains stable for 2-3 days.

PBS (Phosphate buffer saline; 0.1 M, pH 7.4) is prepared by mixing 19 mL of 0.2 M monobasic sodium phosphate solution and 81 mL of 0.2 M dibasic sodium phosphate solution and adding to 1L. This solution is used for the dilution of the radical solution and for the construction of the trolox calibration graph. The calibration curve prepared using trolox used for TEAC measurement can be found in Appendix C.

The absorbance at 734 nm was adjusted to 0.7 ± 0.2 by dilution of ABTS radical with PBS. A 20X dilution was made using PBS to maintain the desired measurement range on the samples. Absorbance values were measured at 734 nm using a spectrophotometer (Model Shimatzu) at every 6 minutes for each sample at 4 different concentrations of 10 μ L, 20 μ L, 30 μ L and 40 μ L with the prepared ABTS radical diluted with PBS so that the total volumes were completed to 1000 μ L.

TEAC values were calculated by dividing the slope of the percent inhibition curve of the samples by the slope of the standard curve of trolox. This process was repeated three times for three different biomasses, one for each nonFM samples and one for each FM samples, and the inhibition values were calculated.

2.2.6.4. Determination of Antioxidant Activity of products by DPPH (2,2-diphenyl-1-picrylhydrazyl) Method

The antioxidant activity was determined by following (Benvenuti et al., 2004) relying on the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical's scavenging activity. The results of the analysis indicate the EC₅₀ (Efficient Concentration) value, that is, the concentration of the antioxidant substance that inhibits 50% of the DPPH radical in the environment. Briefly, 0.03943g of DPPH was weighed and dissolved in a small amount of methanol and then added to the total volume in a 100 mL flask. It is stored wrapped in aluminum foil at +4°C. For each sample, 5 test tubes were used and 600 µL of DPPH radical was added to each. Different volumes (250-300-350-400-450 µL) were taken from the samples and added to the tubes containing the radical. Methanol solution was added to bring the total volume of the tubes to 6 mL. The tubes were vortexed and incubated at room temperature and in the dark for 15 minutes. For Blank, 5.4 mL of methanol was added in 600 µL of DPPH solution and incubated with the other samples. At the end of the incubation period, absorbance values were read at a wavelength of 517 nm in a spectrophotometer.

$$\%Inhibition = \frac{[A_{DPPH} - A_{EKSTRACT}]}{A_{DPPH}} * 100 \quad (2.4)$$

Percent inhibition values corresponding to each sample volume were calculated using the equation 2.4. A is mean of absorbance form spectrophotometer values. The inhibition values determined according to the equation were transferred to a graph against the sample volumes and linear regression analysis was applied to obtain the curve for the sample and the equation completing this curve. EC₅₀ value was calculated for each sample.

2.2.6.5. Imaging of protein fragmentation by SDS-Page Method

SDS-Page Analysis of nonFM and FM products was carried out on 4% stake and 12% running polyacrylamide gels using a Mini Protean BioRad branded electrophoresis system. Lowry assay was performed to set equal protein concentration prior to loading. Samples equalized at 89 µL were first heated at 95 °C for 5 minutes for a total of 30

uL and then loaded into the wells on the gel. After running, the gel was stained with Commasie Blue. 11.0-265 kDa molecular marker (GangNam-Stain) was used to determine the extent of protein diversity in each microalgae fermentation.

2.2.6.6. Determination of Free Amino Acids for nonfermented Microalgae (nonFM) and Fermented Microalgae (FM) Products

Free amino acid profiles of all FM and nonFM products were determined by liquid phase chromatography. Chromatographic separation of amino acids was performed by using ZORBAX Eclipse-AAA column (4.6×150 mm, 3.5μ PN 963400–902) and the Agilent 1260 Infinity HPLC System (Agilent Technologies Inc., Folsom, CA, USA). The method described by (Sahin et al., 2022) and (Kurt et al., 2023) was used to identify the free amino acids contents of all products. The amino acids were quantified by performing the external standard method. Quantities of amino acids were represented in μM units (Kurt et al., 2023).

2.2.6.7. Determination of Angiotensin I Converting Enzyme (ACE) Inhibitory Activity of Products

The ACE-I activity of the products of (Martínez-Alvarez et al., 2016) was based on the determination of the amount of hippuric acid (HA) released from N-Hippuryl-Histidyl-Leucine (HHL) as a result of ACE activity using HPLC and in the presence or absence of inhibitor in the samples. The solution was prepared at a concentration of 25 milliUnit mL⁻¹ and the substrate (HHL) was diluted in 0.1 M borate buffer pH 8.3 containing 0.3 M NaCl (5 mM HHL solution). Then, 40 μL of sample was mixed with 200 μL of HHL solution and 40 μL of ACE solution in a glass test tube and the test tube was incubated at 37 °C for 60 min. The reaction was stopped by adding 100 μL of 1 M HCl. Hippuric Acid (HA) released from HHL was determined by HPLC on a Gemini C18 column (Phenomenex, 110Ao, 150x 46 mm, particle size of 5 μm 5μm, Phenomenex Co., Torrance, CA, USA). The mobile phases used were solvent A (water

containing 0.1% (v/v) TFA) and solvent B (acetonitrile containing 0.1% (v/v) TFA). Elution conditions and flow rates were: 0-5 min, solvent B 20% and 5-15 min, solvent B 20% to 60%; solvent B 60% was held for 15-19 min and then returned to the initial elution conditions (solvent B 20%) for 19-21 min. The flow rate was 0.8 mL min⁻¹ and the column temperature was set to 25°C. Elution peaks of Hippuric Acid (HA) and HHL were visualized at 228 nm (**Kurt et al., 2023; Sahin et al., 2022**).

ACE-I was calculated as follows:

$$ACE - 1 (\%) = \left[1 - \left(\frac{A_{INHIBITOR}}{A_{CONTROL}} \right) \right] * 100 \quad (2.5)$$

$A_{inhibitor}$: peak area of HA in the sample; $A_{control}$: peak area of HA in the unextracted control sample.

The percentage inhibition values obtained based on the equation were calculated.

2.2.7. Artificial Intelligence Based Prediction Model Development for Post-Fermentation and nonFM Soluble Protein Concentrations

A fermentation process of 96 h was carried out for the extraction of growth curves for all three biomasses. During this process, 200 µL samples were taken at 0-4-8-12-24-48-72 and 96 h. Soluble protein amounts were measured. Total 24 data from each biomass and 72 data in total were collected from the fermentation processes carried out in 3 replicates. For validation, the fermentation process of each biomass was repeated for 72 h. During the validation, 6 data were collected from each biomass in three replicates at 0 and 72 h, totalling 18 data. The same procedures were repeated for control (nonFM) biomasses at both 96 hours and 72 h of fermentation. The collected data were averaged in their repetitions within themselves. The data obtained were categorised as Duration and Inoculation Present / Absent. In this way, an Excel file consisting of 60 data was created for new evaluations.

As evaluated in the results obtained from the FM and nonFM performance of biomasses, models were constructed with various machine learning based regression methods and evaluated according to the prediction accuracy. During this evaluation;

root mean square error (RMSE), normalised-root mean square error (N-RMSE) coefficient of determination (R^2), adjusted- R^2 and Mean Squared Logarithmic Error (MSLE) were considered. All these procedures were performed in Matlab 9.10.0.1710957 (R2021a) software (MathWorks Inc., Natick, MA, USA).

The machine learning based regression methods used for evaluation at this stage were Random Forest (RF), Desicion Tree (DT), Generalised Additive Model Regression (GAM), Support Vector Machine (SVM) and Gaussian processes (GP). Unlike extraction modelling, models were created with Desicion Tree (DT) and Gaussian processes (GP) at this stage. Decision Tree (DT) is a widely used machine learning method. It is based on minimising the prediction error by dividing the data set into small data sets. The basic directed decision tree consists of a root node, internal nodes and terminal nodes (Zhao & Zhang, n.d.). Since DTR is not a parametric model, it implies that the output model can be informed about the data. The use of decision trees in classification and prediction is progressed as the creation of a decision tree model based on training data, the evaluation of this model with test data using appropriate test criteria, and the use of the relevant model to predict future values (Quinlan, 1986).

$$Gain(C, A) = S(C) - \sum_{v \in A} \frac{|C_v|}{|C|} S(C_v) \quad (2.6)$$

The DT Equation utilised is given in equation 2.6 where C is the cluster set, A is attribute, and S(C) is the entropy of the set C.

Gaussian Process Regression (GPR) is a commonly used statistical regression method. GPR is based on Gaussian processes. Gaussian processes are flexible mathematical tools. They adapt to large data sets, generating an output value probability for each input point (Makridakis et al., 2018).

$$f(x) \sim GP(m(x), k(x, x')) \quad (2.7)$$

A Gaussian process is completely specified by its mean function, $m(x)$, and covariance function, $k(x, x')$, of a real process $f(x)$, expressed as in equation 2.7 (Shuzan et al., 2021). If the data is properly scaled, the $m(x)$ may be taken as zero (Birgen et al., 2021).

3. RESULTS AND DISCUSSION

3.1. Characterization of nonFM and FM Products According to Their Total Protein Content with Two Different Optimization Approaches.

In recent years, optimisation of the extraction of proteins from different raw materials has gained importance to meet the growing demand for sustainable protein. In this context, Response Surface Methodology (RSM) has been widely used approach (Q. Yang et al., 2019).

For example, in a study performed by *Hadiyanto et al.* (Hadiyanto & Adetya, 2018) on protein recovery from *Arthrospira platensis* could be a good example demonstrating the efficiency of RSM in this field. However, there are not many studies in this field using Artificial Intelligence optimisation approaches for such purposes. The use of AI techniques, through regression methods such as Random Forest (RF), Support Vector Machine (SVM) and Generalised Additive Models (GAM), allows these optimised processes to be further improved. In this context, the experimental design using RSM has been carried out experimentally in a real environment. . In this context, the evaluation of the results obtained from RSM approach for extraction of protein from three different microalgae biomass by using USH method in a real environment by both RSM and AI methods can be considered as an important progress in this field. The results of this study provide critical information for further optimisation of protein extraction processes from different microalgae biomass.

3.1.1. Response Surface Methodology Analysis to Define Optimum Process Conditions

In the current study, microalgae biomass (*A. protothecoides*, *S. limacinum* and *S. platensis*) concentration and extraction time were considered as variables to optimize soluble protein production using the response surface method (RSM). Total soluble

protein concentration for different experimental conditions based on the experimental design was estimated and results of each have also been included in **Table 3.1**.

Table 3.1: Experimental range and levels of the independent variables (Biomass concentration (g/L), extraction time (min)) for *S.platensis* according to Box–Behnken experimental design in actual value and observed response (soluble protein content, g/L).

Run	Variables				Response
	Biomass concentration (g/L)		Extraction Time (min)		Total Soluble Protein Concentration (g/L)
1	30,0	0	12.30	0	1,210
2	30,0	0	12.30	0	0,923
3	1,72	-1	12.30	0	0,101
4	30,0	0	12.30	0	0,976
5	30,0	0	12.30	0	0,944
6	30,0	0	12.30	0	0,955
7	58,28	+1	12.30	0	1,773
8	50,0	+1	20.00	+1	2,050
9	30,0	0	23.11	+1	1,422
10	50,0	+1	5.00	-1	1,493
11	10,0	-1	20.00	+1	0,700
12	30,0	0	2.29	-1	0,838
13	10,0	-1	2.00	-1	0,374

After the extraction process of the sets formed in the experimental design, the soluble protein amount responses were collected by applying Lowry. The responses were regression analysed to determine the interactions of the factors. This analysis provided ANOVA (Analysis of Variance) statistical outputs. In this way, the optimum points of the three biomasses in the experimental design were determined.

The effects of quadratic terms, interactions between factors and non-linearities were evaluated with the Response Surface 2FI Model for *S.platensis* biomass. This analysis was significant with a model p value <0.0001 in the ANOVA table results. The model

was able to accurately predict the model responses for all variables, i.e. extraction efficiency with $R^2=0.95$.

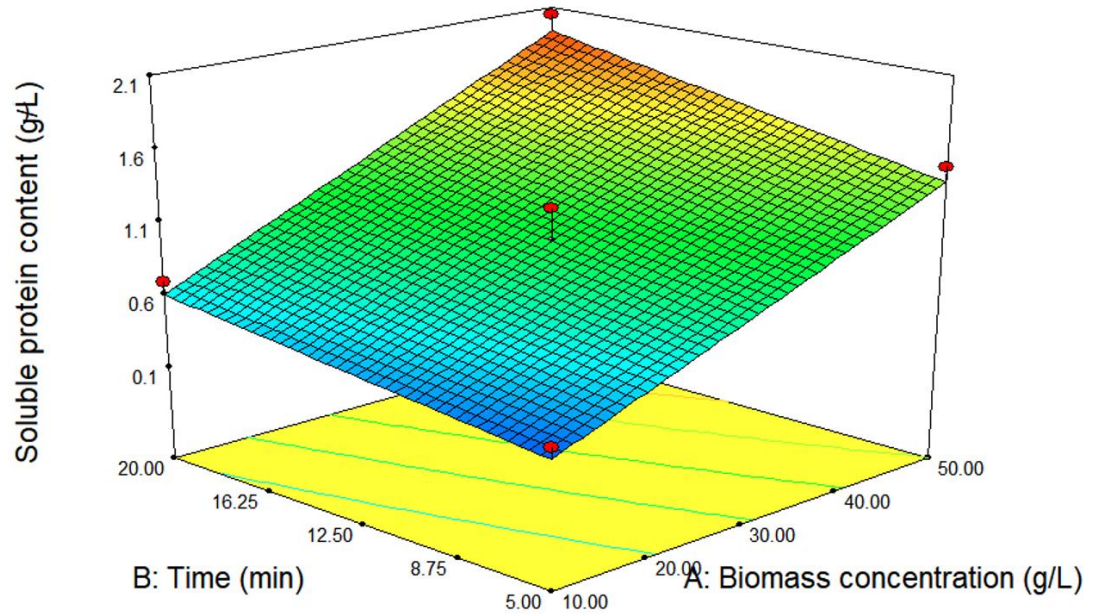


Figure 3.1: 3D response surface plot and contour line of Box–Behnken Design showing the mutual effect of biomass concentration (g/L) and extraction time (min.) on soluble protein content of *S. platensis* based media

When the factors used in this study are considered, only the effect of biomass concentration was significant (p value < 0.0001).

The relationship of the response variable with the coded parameters is expressed in Equation 3.1 for *S. platensis* biomass.

$$\text{Soluble Protein Content}_{\text{spirulina}} = -0.06063 + 0.025407 * \text{Dry cell weight concentration} + 0.017240 * \text{Time} + 3.83333\text{E-}004 * \text{Dry cell weight concentration} * \text{Time} \quad (3.1)$$

A model of the responses from the extraction of *S. platensis* biomass reveals experimentally optimised results. The coefficients in this equation show the effect of the independent variables (biomass concentration and time) on soluble protein content. The coefficient of dry cell weight concentration (0.025407) expresses the positive effect of unit increase of dry cell weight concentration on the amount of soluble protein. This means that as the dry cell weight concentration increases, the amount of

soluble protein also increases. When the coefficients are compared, the dry cell weight shows the greatest effect in the model.

Table 3.2: Experimental range and levels of the independent variables (Biomass concentration ((g/L), extraction time (min)) for *A.protothecoides* biomass and Box–Behnken design in actual value and observed response (soluble protein content, g/L).

Run	Variables				Response
	Biomass concentration (g/L)		Extraction Time (min)		Total Soluble Protein Concentration (g/L)
1	30,0	0	27.50	0	0,613
2	30,0	0	27.50	0	0,666
3	1,72	-1	27.50	0	0,0001
4	30,0	0	10.22	-1	0,647
5	50,0	+1	40.00	+1	1,008
6	30,0	0	27.50	0	0,663
7	30,0	0	27.50	0	0,723
8	58,28	+1	27.50	0	1,116
9	50,0	+1	15.00	-1	0,978
10	30,0	0	48.18	+1	0,640
11	10,0	-1	40.00	+1	0,185
12	10,0	-1	15.00	-1	0,168
13	30,0	0	27.50	0	0,564

The experimental design for the optimisation of protein extraction from *A.protothecoides* biomass was significant with a p value <0.0001. The model was able to accurately predict the model responses for all variables such as extraction efficiency with $R^2=0.97$.

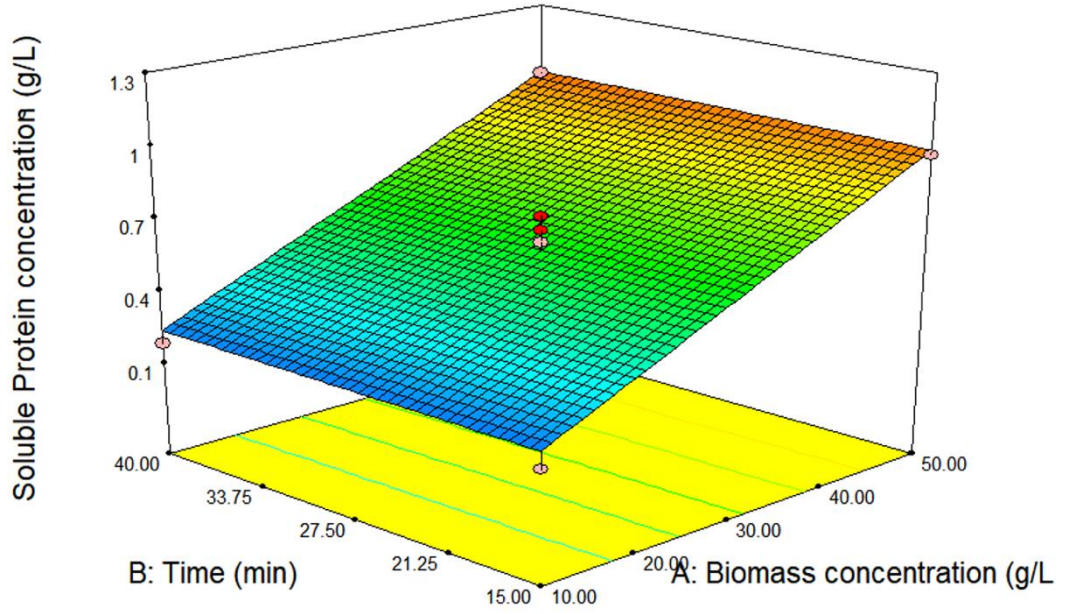


Figure 3.2: 3D response surface plot and contour line of Box–Behnken Design showing the mutual effect of biomass concentration (g/L) and extraction time (min.) on soluble protein content of *A. protothecoides* based media

The relationship of the response variable with the coded parameters was expressed in the equation for *A. protothecoides* biomass coded parameters is expressed in equation 3.2 for *A. protothecoides* biomass.

$$\text{Soluble Protein Amount}_{\text{chlorella}} = 0.62 + 0.38 * \text{Dry cell weight concentration} + 6.250E-003 * \text{Time} + 2.500E-003 * \text{Dry cell weight concentration} * \text{Time} \quad (3.2)$$

A model of the responses from the extraction of *A. protothecoides* biomass reveals experimentally optimised results. We can say that the coefficient of dry cell weight, which has a positive and high coefficient, has a higher efficiency in the model. When we make a comparison from a different direction, in the (R. Zhang et al., 2018) study, high amount of protein extraction was tried to be obtained from *Chlorella pyrenoidosa* species using high pressure homogenisation and other techniques. In this *Chlorella pyrenoidosa* study, 72.4% protein extraction was obtained under optimal conditions, indicating that homogenisation is an effective cell disruption method.

Table 3.3: Experimental range and levels of the independent variables (Biomass concentration ((g/L), extraction time (min)) for *S.limacinum* biomass and Box–Behnken design in actual value and observed response (soluble protein content, g/L).

Run	Variables				Response
	Biomass concentration (g/L)		Extraction Time (min)		Total Soluble Protein Concentration (g/L)
1	30,0	0	27.50	0	1,099
2	30,0	0	27.50	0	1,114
3	1,72	-1	27.50	0	0,033
4	30,0	0	10.22	-1	1,008
5	50,0	+1	40.00	+1	1,610
6	30,0	0	27.50	0	1,133
7	30,0	0	27.50	0	1,119
8	58,28	+1	27.50	0	1,752
9	50,0	+1	15.00	-1	0,523
10	30,0	0	48.18	+1	1,246
11	10,0	-1	40.00	+1	0,485
12	10,0	-1	15.00	-1	0,447
13	30,0	0	27.50	0	0,108

The experimental design for the optimisation of protein extraction from *S.limacinum* biomass ; the model generated by the Response Surface 2FI Model based on the responses among the factors was significant with a p value <0.0001. The R^2 value was determined as 0.96 in this model. This high R^2 value indicated that the model fit the data well and explained a large proportion of the factors used to explain the amount of soluble protein. That is, Dry cell weight concentration, Time and the interaction term showed a high success in explaining the amount of soluble protein.

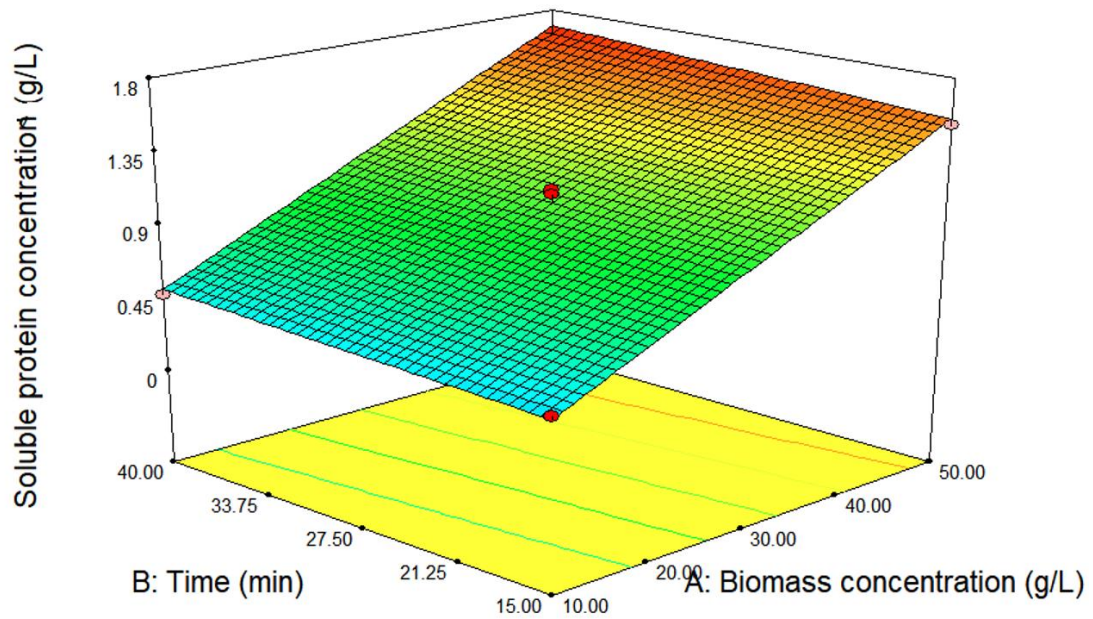


Figure 3.3: 3D response surface plot and contour line of Box–Behnken Design showing the mutual effect of biomass concentration (g/L) and extraction time (min.) on soluble protein content of *S.limacium* based media

Equation 3.3 obtained with the Response Surface 2FI Model was used to predict the amount of soluble protein and determine the optimum value.

$$\text{Soluble Protein Content}_{\text{schizochytrium}} = 1.05 + 0.58 * \text{Dry cell weight concentration} + 0.059 * \text{Time} + 0.012 * \text{Dry cell weight concentration} * \text{Time} \quad (3.3)$$

The coefficients in the equation represent the effects of the factors. In this case, since the Dry cell weight concentration factor had a positive coefficient (+0.58), increasing the biomass concentration increases the amount of soluble protein. Likewise, since the Time factor also had a positive coefficient (0.059), increasing the time also increases the amount of soluble protein. The interaction term (0.012) of Dry cell weight concentration and Time factors was also included in the equation. However, when the interaction of the parameters on the model was compared by considering the difference between the coefficients, the p value of dry cell weight concentration was <0.0001 and significant. Time is not as significant as Dry cell weight concentration on the model (p=0.1440). There is an optimisation study on the antioxidant activity of *Schizochytrium* feed protein hydrolysate using RSM (Q. Yang et al., 2019) This study investigated the

effects of independent variables such as different protease dose, hydrolysis time and pH and determined the optimum conditions. This study demonstrates the use of RSM in the optimisation of lipid and protein extraction from *S.limacinum* biomass. However, different parameters were evaluated.

In this way, the optimum points of the three biomasses in the experimental design were determined. Optimum extraction conditions for 3 different microalgal biomasses were revealed using USH extraction technique.

Table 3.4: Optimum conditions (biomass concentration (g/L), extraction time (min)) for protein extractions from *S.platensis*, *A.protothecoides* and *S.limacinum* biomasses by using ultrasound homogenizer according to observed responses (soluble protein content, g/L).

Microalgal Biomass	Variables		Response
	Biomass concentration (g/L)	Extraction Time (min)	Total Soluble Protein Concentration (g/L)
<i>S.platensis</i>	50.0	20.0	2,005
<i>A.protothecoides</i>	50.0	40.0	1,053
<i>S.limacinum</i>	50.0	40.0	1,939

Similar studies (Ores et al., 2016) had shown that the protein concentrations obtained in USH assisted extraction of biomasses such as *S.platensis* was high. The protein amounts obtained due to higher strength support the efficiency in experimental processes. The use of RSM was played a critical role in determining the interactions and importance of factors affecting protein solubility and recovery. The analysis equations can serve as an efficient guide during the optimisation of process conditions and in this way determine the optimal process conditions for maximum protein recovery. Consequently, RSM represents a modelling tool for optimising process parameters for protein extraction from *S.platensis* biomass and can be used as a tool to determine the optimal combination of specific process variables. Unlike other methods, the AI-algorithm used also allowed us to make a prediction approach for the protein content to be obtained as a result of a common extraction process of three different microalgae biomasses. In this context, the RSM method was recognised as an important optimisation tool in industrial biotechnology applications.

3.1.2. Artificial Intelligence Based Prediction Model Development

Soluble Protein content data obtained by USH extraction of *S.platensis*, *A.protothecoides* and *S.limacinum* were collected. A data set of 39 data was created.

Table 3.5: Performance evaluation of the proposed machine learning algorithms for interaction effect between Extraction Time and Biomass Concentration on protein content of the biomass extracts at constant USH.

Models	R^2	a- R^2	RMSE	N-RMSE	MSLE
RF	0,9921	0,9914	0,0429	0,0529	0,0001
SVM	0,9718	0,9694	0,0811	0,6377	0,0004
GAM	0,9282	0,9221	0,1294	1,7225	0,0013

The data set was evaluated with RF, SVM and GAM machine learning based regression methods. As a result of this evaluation, models were created. Root mean square error (RMSE), normalized-root mean square error (N-RMSE) coefficient of determination (R^2), adjusted- R^2 and Mean Squared Logarithmic Error (MSLE) were considered. The best prediction models were obtained by comparing statistical regression methods.

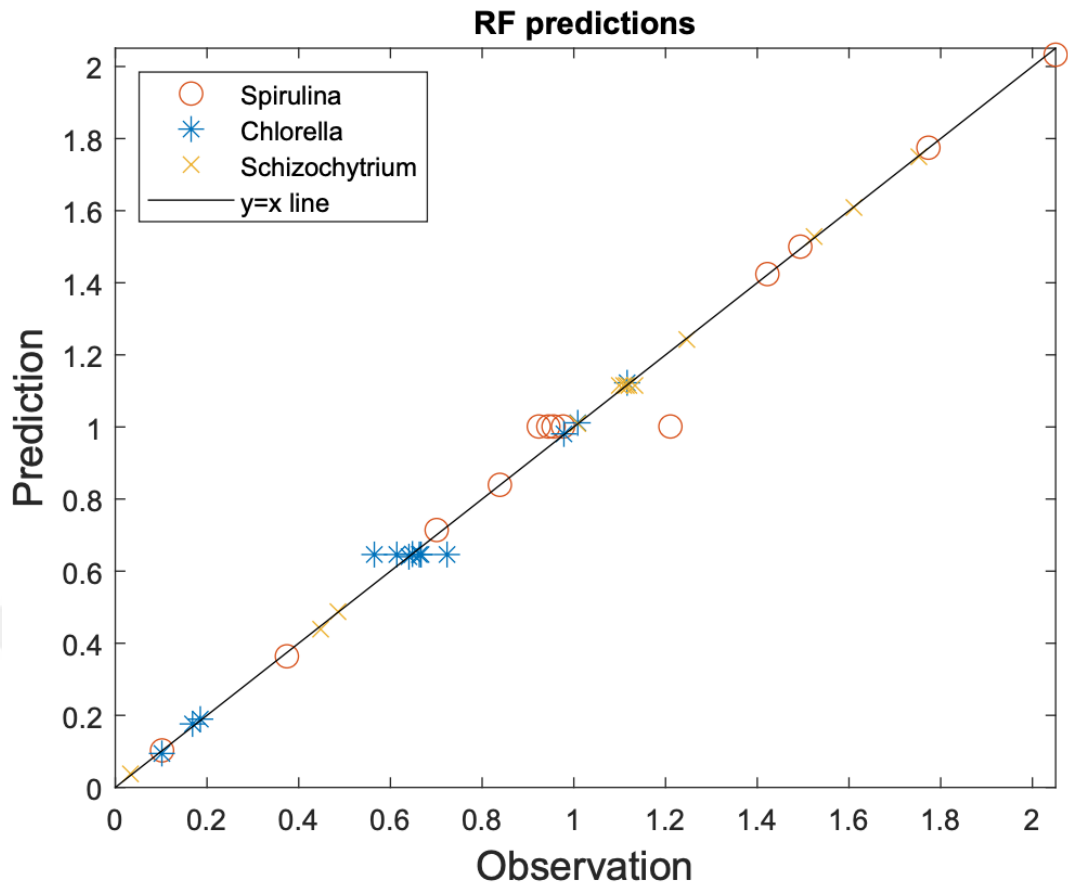


Figure 3.4: Random Forest Model. AI-Based Protein Content Prediction Analysis with in common with *S.platensis*, *A. protothecoides* and *S.limacium* biomasses .The interaction effect between Extraction Time and Biomass Concentration on protein content of the biomass extracts at constant USH.

The model obtained with RF gave R^2 0.99 and adjusted- R^2 0.99 values. These expressions show that the model predicts the observed data quite well. In addition, RMSE (Root Mean Square Error) is 0.04 and Normalised-RMSE value is 0.05. This indicates that the prediction errors of the model are low. The MSLE (Mean Squared Logarithmic Error) value of 0.0001 indicates that the model performs well on a logarithmic scale. We observe that the points expressed for all three biomasses are located very close to the y=x axis. This means that the model predictions are very close to the real data.

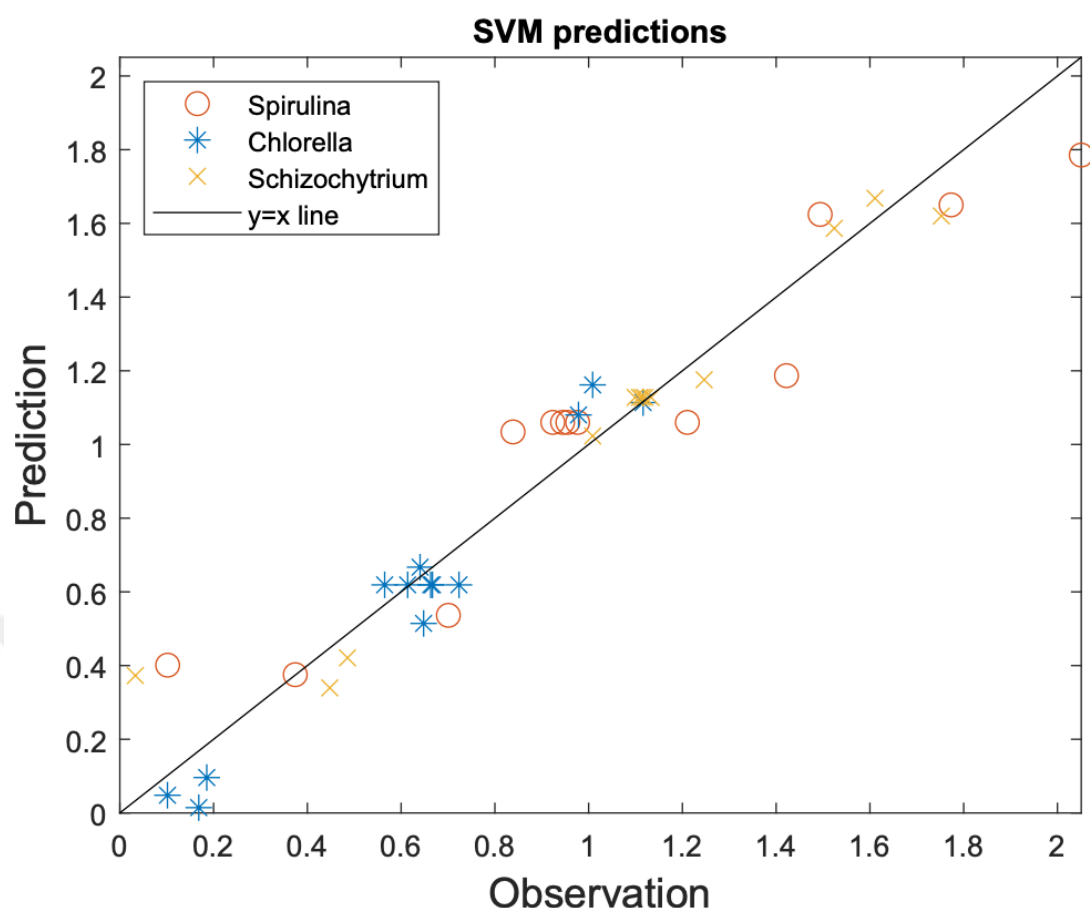


Figure 3.5: Support Vector Machine Model. AI-Based Protein Content Prediction Analysis with in common with *S.platensis*, *A. protothecoides* and *S.limacium* biomasses. The interaction effect between Extraction Time and Biomass Concentration on protein content of the biomass extracts at constant USH.

The high R^2 0.97 value and adjusted- R^2 0.96 value of the model built with Support Vector Machine (SVM) indicate that the model provides the prediction data quite well. The RMSE value of 0.08 and N-RMSE value of 0.06 indicate that the model has low mean square errors between observations and predictions. The MSLE value is low with 0.004. It can show a good performance in logarithmic scale. When the $y=x$ axis in the graph is analysed, we observe that some predictions deviate from the actual values. We can say that the model does not capture every data point, but it performs well in the general trend. In addition, it is inferior in performance compared to the RF model. However, we observe that SVM is a powerful tool for modelling complex relationships from complex data sets. In this way, it can be stated that it is a model that can be used in protein extraction optimisation depending on the parameters.

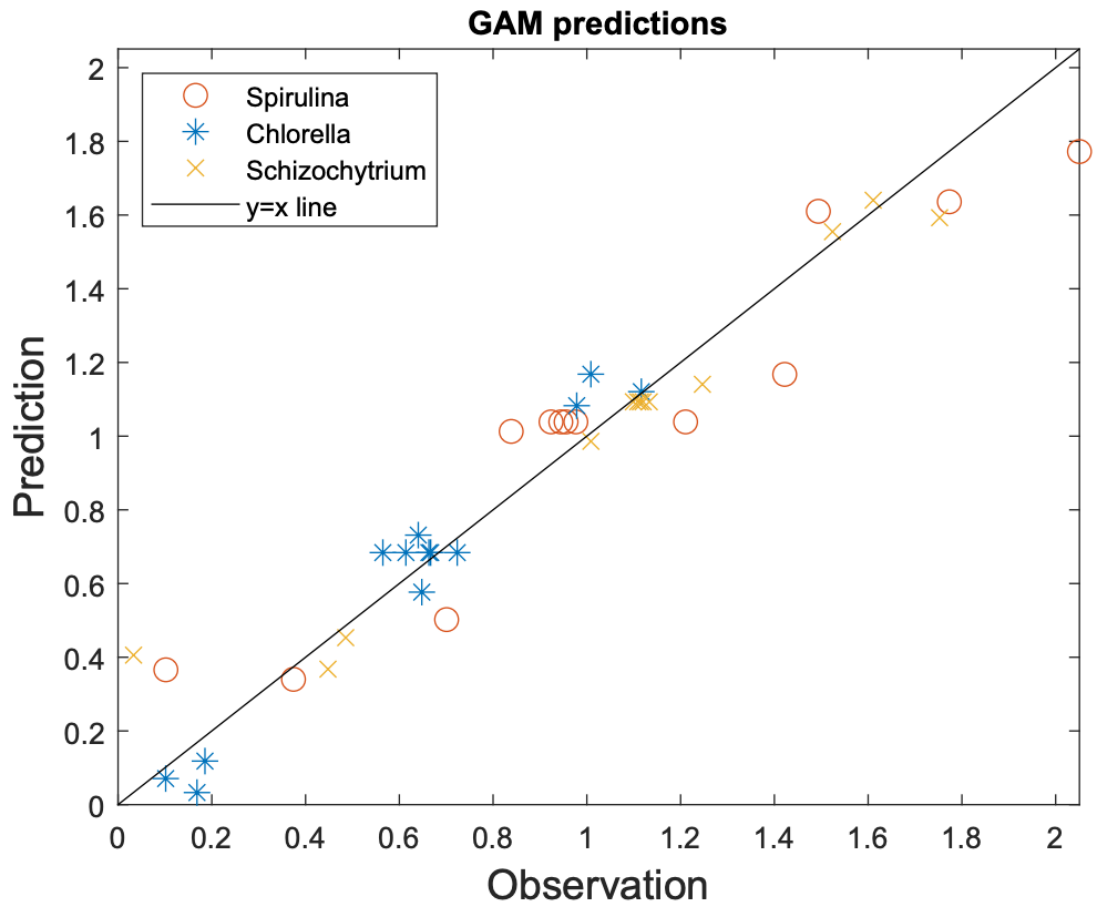


Figure 3.6: Generalized Additive Model. AI-Based Protein Content Prediction Analysis with in common with *S.platensis*, *A. protothecoides* and *S.limacium* biomasses. The interaction effect between Extraction Time and Biomass Concentration on protein content of the biomass extracts at constant USH.

In the model built using GAM, we observe the AI predictions on the graph. The R^2 and $a-R^2$ values of the model are 0.92 and 0.92, respectively. It shows that the values are quite high. The RMSE value of 0.12 and the N-RMSE value of 0.72 indicate that the mean square errors between the actual experimental data and the predictions are acceptable but not as reliable as RF and SVM. The MSLE value is 0.0013, we can say that the model provides an average performance on a logarithmic scale. When we examine the model graph, we observe that there are deviations from the actual values. We can say that this model captures the general trend, but the predictions may be less accurate at specific points.

Table 3.6: Evaluation of performance predictions of machine learning algorithms and Response Surface Methodology predictions for the interaction effect between optimum Extraction Time and Biomass Concentration on the protein content of biomass extracts at constant USH.

Models	<i>S.platensis</i>	<i>A.protothecoides</i>	<i>S.limacium</i>
RSM	1,93	1,007	1,699
RF	2,032	1,011	1,608
SVM	1,758	1,043	1,619
GAM	1,785	1,161	1,668

RESULT	<i>S.platensis</i>	<i>A.protothecoides</i>	<i>S.limacium</i>
Experimental	2,005	1,053	1,939

According to the values given, the predictions generated in all three biomasses were evaluated. According to our values, on average, we obtain the highest success in the Random Forest Model. RSM is a method generally used for process optimisation and design. RSM is widely used to model the interactions of parameters in bioprocesses (**Baranitharan et al., 2019**). AI algorithms are more advantageous in modelling more complex relationships and making higher predictions than RSM. In RSM, a single model is created for each biomass, while in AI models, a common model is created for three biomasses. This gives us a common assessment for more than one biomass at the same time. AI models have the ability to learn non-linear relationships in data sets. This is evident from the high correlation coefficient (R^2) and low root mean square error (RMSE) compared to RSM (**Baranitharan et al., 2019**). Depending on the specific process requirements, amount of data and experimental design, the selected method may differ.

3.2. Enzymatic Treatment

Viscozyme enzyme was applied to *S.platensis*, *A.protothecoides* and *S.limacium* biomasses after determining the optimized conditions. After enzyme treatment, total soluble protein increases were calculated using the Lowry method, and the increase

was observed as 19.29% in *S.platensis* (2,39 g.L⁻¹) , 161.94% in *A.protothecoides* (2,41 g.L⁻¹) and 63.57% in *S.limacinum* (3,17 g.L⁻¹). As a result of enzyme treatment, the best result was obtained from *A.protothecoides* , which has the lowest soluble protein concentration. When the effective behaviour on *A.protothecoides* was examined, (Mahdy et al., 2014), the interaction of *C.vulgaris* with viscozyme was observed with increasing of protein content. Viscozyme is a carbohydrylic enzyme complex. The use of this enzyme increases carbohydrate and protein solubility. This improves anaerobic degradation of biomass. This shows that the cell wall of *C.vulgaris* is degraded by viscozyme and thus proteins can be extracted more easily. This study supports the successful result obtained from viscozyme treatment with *A.protothecoides* . As a result of the effective performance of enzyme, successful results were obtained in protein extraction in all three biomass.

3.3. Fermentation

3.3.1. Growth of *B.subtilis* in *S.platensis*, *A.protothecoides* and *S.limacinum* Media

S.platensis, *A.protothecoides* and *S.limacinum* biomasses and *B.subtilis* B3387 fermentation were investigated. Improvement of protein hydrolysis were observed. Live cell growth was analysed with plate counting. It was carried out without any carbon supplementation. USH and enzymatically treated biomasses were sterilised by autoclaving and inoculated with 1 MacFarland, i.e. approximately 5.0 log CFU.mL⁻¹ *B.subtilis* per 30 mL of biomass volume in each bottle and incubated at 28 °C, 130 rpm. Autoclaved but not inoculated biomass samples (control) were also included in the experimental set to control the risk of contamination. During the fermentation process in each biomass, samples were taken at designated times and analysed for the determination of advanced physicochemical properties.

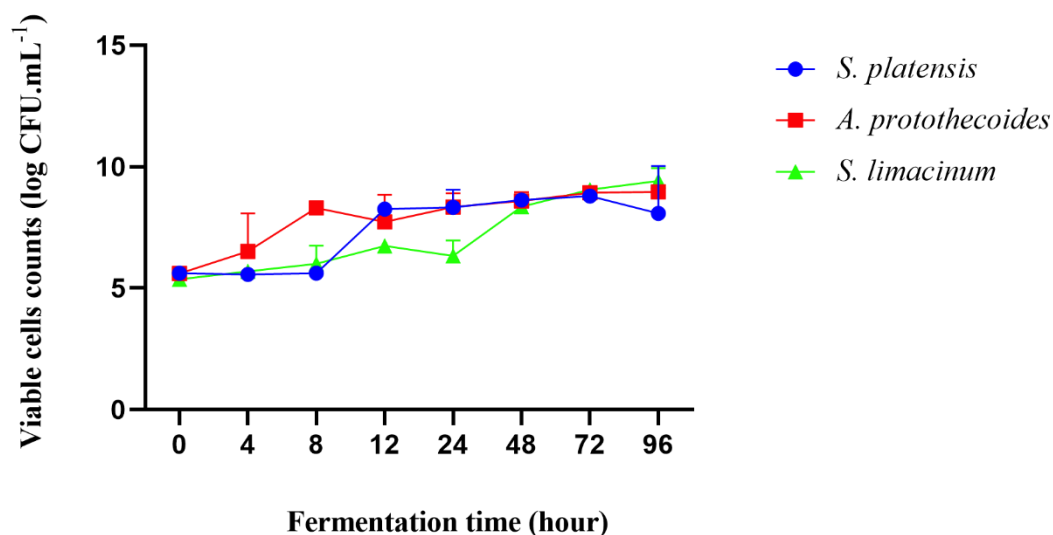


Figure 3.7: Live cell counts due to fermentation of *B.subtilis* strain on *S.platensis*, *A. protothecoides* and *S.limacinum* mediums (5 %, w/v) for 96 hours.

The interaction between biomass difference and fermentation time was significant ($p=0.0003$). Organic components in *S.platensis* biomass can serve as both carbon and nitrogen sources for *B. subtilis* (Kurt et al., 2023; Sridevi, 2018). In the fermentation process, *B. subtilis* can metabolise these components to produce energy and increase its growth rate. In *S.platensis* biomass, cell growth was significantly observed at 12, 24, 48, 72 and 96 hours depending on the initial time ($p<0.0005$), but there was no significant increase in growth after 12 h, approximately the same values were observed. *B.subtilis*, growth was observed in *S.platensis* biomass significantly after 12 h. Organic components in *S.platensis* biomass supported the growth of *B.subtilis*. This situation is supported in similar studies as (Bao et al., 2018b). In *A.protothecoides* and *B.subtilis* fermentation, the interaction of hours 0 and 4 with the following hours was significant ($p<0.0001$). Although, no significant interaction was observed in cell viability at hour 8 and onwards. Cell viability continued and growth was observed. When *B.subtilis* growth was observed in *A.protothecoides* medium, its growth was supported by metabolising the organic components in the medium. *B.subtilis* produces cellulase and haemicellulase enzymes that help to break down the *A.protothecoides* cell wall (Gu et al., 2018). In this way, the cell wall of *A.protothecoides* is broken down. With the breakdown of the cell wall, the nutrients of *A.protothecoides* become more easily accessible. In this way, an environment that supports the growth of

B.subtilis is created. There are not many studies on *B.subtilis* and *Chlorella* fermentation.

As a result of *B.subtilis* and *S.limacinum* fermentation, cell growth was significantly observed at 48, 72 and 96 hours compared to the initial time ($p < 0.0001$). cell growth was significantly observed at 48, 72 and 96 hours compared to the initial time ($p < 0.0001$). *S.limacinum* is a microalgae species that has been significantly used in the literature for the production of docosahexaenoic acid (DHA). However, oil was taken from the *S.limacinum* biomass used. Although its content is not known exactly, preliminary protein determinations showed that it contains protein close to *S.platensis* (**Figure 3.7.**). *B.subtilis* is a gram positive bacterium. It has features such as fast growth and low-cost carbon utilisation (**Gu et al., 2018**). It is also resistant to environmental stress conditions (**Q. Li et al., 2018**). It is advantageous for producing biochemicals on biomass substrates. It has high robustness thanks to its endogenous metabolic pathways and heterologous pathways compatibility (**Gu et al., 2018**). According to its properties, *B.subtilis* is frequently used in industrial bioprocesses. It is also adapted to the environment as a result of fermentation with *S.limacinum*. In this way, it showed 8.35 CFU.mL^{-1} growth at 48th hour in the growth curve. At 48 hours, $8.63 \log \text{ CFU.mL}^{-1}$ growth was observed in *S.platensis* biomass and 8.58 CFU.mL^{-1} growth was observed in *A.protothecoides*. Similar performances were noted for all microalgae biomasses. It is a bacterial species used quite frequently on an industrial scale. It is used in fermentation processes to produce enzymes, antimicrobial compounds, biosurfactants, etc. (**Nakano et al., 1997**).

3.3.2. Growth validation of *B.subtilis* in *S.platensis*, *A.protothecoides* and *S.limacinum* Media

After the fermentation process, it was decided to use 72 hours for validation due to reaching the consistency point and the production was repeated. Microbial growth was followed by pH monitoring, analytical methods and artificial intelligence based prediction based on total soluble protein concentrations.

3.3.3. The Change in pH of During Fermentation with *B.subtilis* in *S.platensis*, *A.protothecoides* and *S.limacinum* Media

The pH values were evaluated at the beginning and at the end of the fermentation process since low volumes were used. The average pH of the prepared biomass media was set as 7.02 ± 0.03 for *S.platensis*, 6.98 ± 0.03 for *A.protothecoides* and 7.02 ± 0.03 for *S.limacinum* in the experimental setup performed in 3 replicate flasks at the beginning of the fermentation. Figure 3.8 shows the pH measurements of the samples taken at the beginning and end of the fermentation.

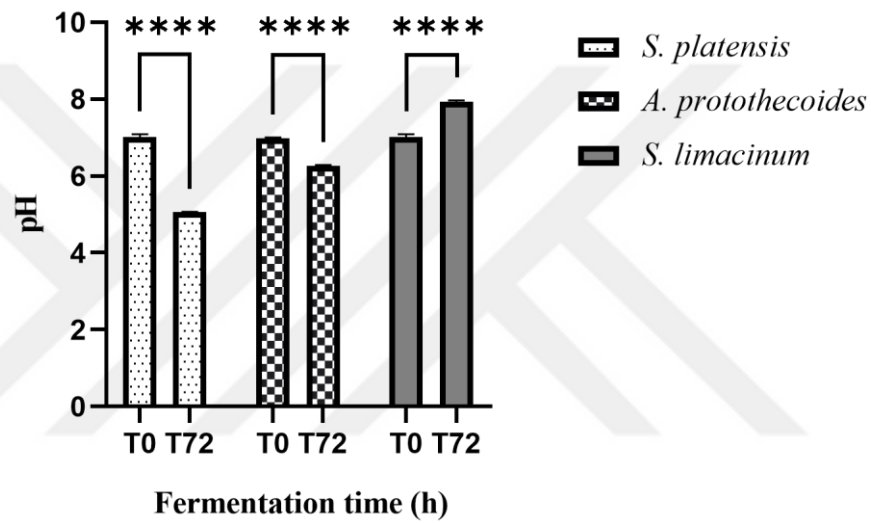


Figure 3.8: The pH values of *B.subtilis* strain on *S.platensis*, *A. protothecoides* and *S.limacinum* mediums (5 %, w.v-1) for 72 hours fermentation. (The values are presented as mean $\pm$ SD. **** indicates the significance level at $p < 0.0001$ compare with other varieties.)

2-way ANOVA analysis variance showed that the interaction between biomass and media and fermentation time was significant ($p < 0.0001$). At the end of fermentation, *S.platensis* biomass showed an average decrease of 1.95 units in pH value at 72 hours (pH= 5.06). When the result of the fermentation process in *A.protothecoides* medium (pH= 6.27) was evaluated, an average decrease of 0.71 units was observed. When evaluated for *S.limacinum* , unlike the other biomass media, an upward trend was observed. In *S.limacinum* , an average increase of 0.91 units was observed in the 72nd hour behavior (pH= 7.93) and therefore, we can say that *B.subtilis* species varies depending on the biomass medium used in the fermentation process. This variability

can be explained with some examples. Generally *B.subtilis* tends to increase the pH level during fermentation. For example **Sarkar & Tamang**, study , during the fermentation of soya beans with *B.subtilis*, it has been observed that the pH level increases up to 8.5. This was attributed to the alkalinity of the enzymes produced by *B.subtilis* during fermentation. In another study which **Wang & Shih, 1999** , *B.subtilis* used for keratinase production increased the pH from 7.0 to 8.5. However, there is a difference in the behaviour of *B.subtilis*. In this case (**Kurt et al., 2023**), a behaviour of *B.subtilis* in *S.platensis* medium was observed as a decrease in pH from 6.18 to 5.13. This supports the fermentation of *S.platensis* and *B.subtilis* in our study. We can associate this situation with the properties of the metabolites produced by *B.subtilis* depending on the biomass used.

3.3.4. Analytical Methods Performed During Fermentation Studies with *B.subtilis* in *S.platensis*, *A.protothecoides* and *S.limacinum* Media

Since, different FM products were produced by fermentations with *B.subtilis* and three different FM products by 72 h of fermentation were considered as final products in the current study. The insoluble substances (each bacterial biomass together with microalgae lysates) in the fermentation medium were separated by centrifugation (10000 rpm, 60 minutes). Then, the following analysis was carried out on each sample supernatant was analyzed in triplicate.

3.3.4.1. Determination of Total Phenolic Content (TPC)

Total phenolic compound (TPC) content was determined according to the (**Ainsworth & Gillespie, 2007**) analysis method. At the beginning of fermentation, the TPC values of the samples reserved for the control were *S.platensis* (0.31 mg/mL), *A.protothecoides* (0.33 mg/mL) and *S.limacinum* (0.47 mg/mL).

After 72 h of fermentation with *B.subtilis*, **Figure 3.9** shows that the total phenolic content in the fermented products generally increased in the fermentation of all three

biomasses. As a result of the fermentation, only *S.limacium* end product (0.71 mg/mL) showed a statistically significant ($p=0.0003$) difference with the control. *S.platensis* (0.33 mg/mL) and *A.protothecoides* (0.39 mg/mL) end-products showed an increase but no significant difference.

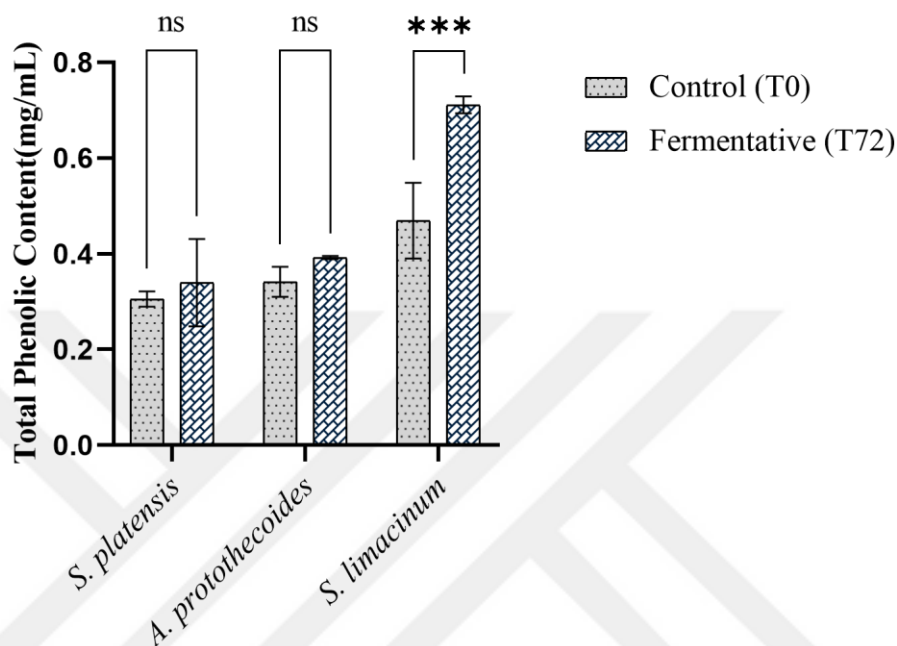


Figure 3.9: Total Phenolic Content (TPC) of fermented microalgae (*S.platensis*, *A. protothecoides* and *S.limacium* mediums (5 %, w.v⁻¹)) products due to fermentation *B.subtilis* and nonfermented counterparts (control) for 72 h. (The values are presented as mean $\pm$ SD. *** indicates the significance level at $p = 0.0003$ compare with other varieties.

The differences for *S.platensis* ($p>0.999$) and *A.protothecoides* ($p=0.7597$) were not statistically significant. TPC analysis provides us with information about the antioxidant activity of the end products. At this point, we can say that the antioxidant activity of *S.limacium* end product has a better activity compared to the end products of other biomasses both in the control sample and in the fermentative sample. Studies for *S.platensis* fermentation show that the fermentation process is curative in terms of total phenolic content. For example, (de Marco Castro et al., 2019b) observed that fermentation with *L.plantarum* increased the total phenolic content of *S.platensis* by 112% at 36 hours of fermentation, but the total antioxidant capacity decreased after 36 hours. This was explained as phenolic content was affected by heat and may cause

decomposition. In our study, a similar situation may have been encountered because the sample was taken at the 72nd hour. Since the sample was not taken at the intermediate hour, we can explain the situation in this way. There are not many studies on the positive or negative effect of fermentation on the total phenolic activity of *A.protothecoides*. However, there are studies on fermentation of herbal products (Kapasob et al., 2018; Somdee et al., 2023). These studies show that total phenolic activity increases after fermentation. In our study, no significant increase in total phenolic activity was observed. This may be associated with the biochemical properties of the biomass used. From a different point of view, as in the case of *S.platensis*, no increase in phenolic content may not have been observed due to the effect of heat depending on the time of sampling. There are no examples in the literature on the effect of fermentation of *S.limacinum* on total phenolic activity. However, in our study, a high increase in total phenolic activity was observed (0.71 mg/mL). In this case, studies of similar microalgal biomasses and fermentation studies with *B.subtilis* fermentation have been examined, but sufficient information could not be reached. The effect of fermentation on the total phenolic activity of biomass varies in the studies. In the publications, it was observed that the activity of β -glucosidase and proteases increased as a result of the fermentation of *B.subtilis* with soybean, resulting in the release of bioactive compounds such as phenols (Hyeon et al., 2020). In addition, in another study with soya beans, the positive effect of conversion to beneficial polyphenols showed the ability to increase the phenolic content of fermented soya beans (Ali et al., 2017). In another study using ginseng and black rice as substrates, it was observed that the phenolic activity of fermented products increased as a result of fermentation with *B.subtilis* (Hwang et al., 2019; Yoon et al., 2015). These studies have shown the potential of *B.subtilis* to favourably affect phenolic content and antioxidant activity in various substrates.

3.3.4.2. Determination of Antioxidant Activity by TEAC (Trolox Equivalent Antioxidant Capacity)

TEAC (Trolox Equivalent Antioxidant Capacity) analysis was determined according to the (Van Den Berg et al., 1999) analysis method. In this analysis, the calibration graph was previously prepared using the antioxidant compound called Trolox. The

antioxidant capacity of the control and fermentative products was measured and the results were expressed in Trolox equivalent.

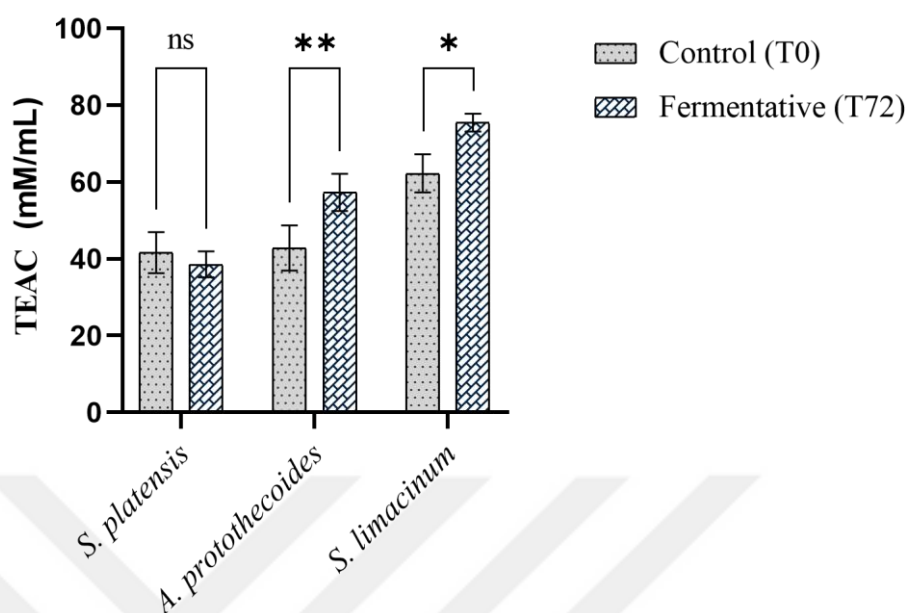


Figure 3.10: TEAC of fermented microalgae (*S.platensis*, *A. protothecoides* and *S.limacinum* mediums (5 %, w.v⁻¹)) products due to fermentation *B.subtilis* and nonfermented counterparts (control) for 72 h. (The values are presented as mean $\pm$ SD. * indicates the significance level at $p = 0.0130$, ** indicates the significance level at $p = 0.0070$ and ns indicates the not significance level at $p > 0.9999$ compare with other varieties according to Bonferroni's multiple comparison test.

After 72 hours of fermentation with *B.subtilis*, **Figure 3.10** shows the values of antioxidant capacity in the fermented products for the controls and fermentative products. As a result of the fermentation of *A.protothecoides* (57,28 μ M/mL) and *S.limacinum* (75,42 μ M/mL) biomasses, we can say that the activity of antioxidant compounds in the final products increased compared to the controls. At the same time, the Bonferroni's multiple comparisons test analysis for *A.protothecoides* ($p=0,0070$) and *S.limacinum* ($p=0,0130$) biomass showed a statistically significant difference in the increase in antioxidant capacity compared to the controls. *Chlorella* has a natural antioxidant potential, as it contains high-value active compounds such as α -tocopherol, β -carotene and ascorbic acid, which are effective against free radicals (El-Sheikh et al., 2018) In addition, its antioxidant potential is positively affected by the fact that it

contains high levels of substances that contribute to health benefits such as carotenoids (Serra et al., 2021). In a study investigating the antioxidant activities of 12 different microalgae species isolated from soil, *Chlorella vulgaris* water extract showed antioxidant levels ranging from $31.06 \pm 4.00 \mu\text{mol Trolox g}^{-1}$. It was considered as a high antioxidant level compared to other microalgae (Hajimahmoodi et al., 2010). When the TEAC activity of *A. protothecoides* after fermentation is evaluated, there are no specific studies directly addressing its effect. However, we know that fermentation increases the bioavailability of nutrients and bioactive compounds in microalgal biomasses. In our study, a significant positive effect of fermentation on TEAC activity of *A. protothecoides* was observed. However, this may give different results depending on the use of different strains or the fermentation method applied. The antioxidant activity of *Schizochytrium* can be attributed to the presence of compounds such as carotenoids, vitamin E, phenolics, astaxanthin pigments (X. Hu et al., 2019a; J. Li et al., 2019a; Lv et al., 2015). The presence of these compounds contributes to the antioxidant capacity of *S. limacinum*. It has also been mentioned that the lipid composition of *Schizochytrium* sp. contributes to its antioxidant activity (Ding et al., 2022; J. Li et al., 2019b; Ma et al., 2023). However, the defatting of the biomass used does not allow us to evaluate it in this respect. A significant increase in the antioxidant content of *S. limacinum* was observed after fermentation. However, there are no direct studies in this field. When the effect of fermentation on antioxidant compounds was examined on microalgae in general, it was emphasised that potential changes in TEAC values occurred (Coulombier et al., 2021).

The result observed in the fermentation of *S. platensis* ($38.57 \mu\text{M/mL}$) was not statistically significant ($p > 0.9999$) and a decrease was observed unlike other biomasses fermentation. The antioxidant capacity of *S. platensis* has been previously investigated in literature. The rich compound contents of *S. platensis* such as beta-carotene, tocopherol and phycocyanin support the presence of antioxidant activity (Samani et al., 2017). Fermentation significantly increases the bioactive properties of *S. platensis*. Lactic acid fermentation affects the TEAC value of *S. platensis* and antioxidant activity is affected as a result of fermentation (Niccolai et al., 2019). Beta-carotene, vitamin A and tocopherol contents, which are known for their health benefits, increased. These antioxidants show an important approach in terms of the benefit of *S. platensis* food products (Palanisamy et al., 2019). In our study, no significant

increase in TEAC activity was observed in *S.platensis*. However, an increase was observed. This may have been observed in this way due to the fermentation time or the type of bacteria used. As TPC analysis and TEAC analysis are complementary methods and we can obtain more comprehensive information about the amount and activity of antioxidant compounds in the final products, we can say that *S.limacinum* showed the best performance for both tests.

3.3.4.3. Determination of Antioxidant Activity by DPPH (2, 2-difenil-1-pikrilhidrazil)

DPPH (2,2-diphenyl-1-picrylhydrazyl) method is a method by which we determine the amount of antioxidants by spectrophotometric measurement of the formation of purple color by inactivating the DPPH radical of antioxidant samples.

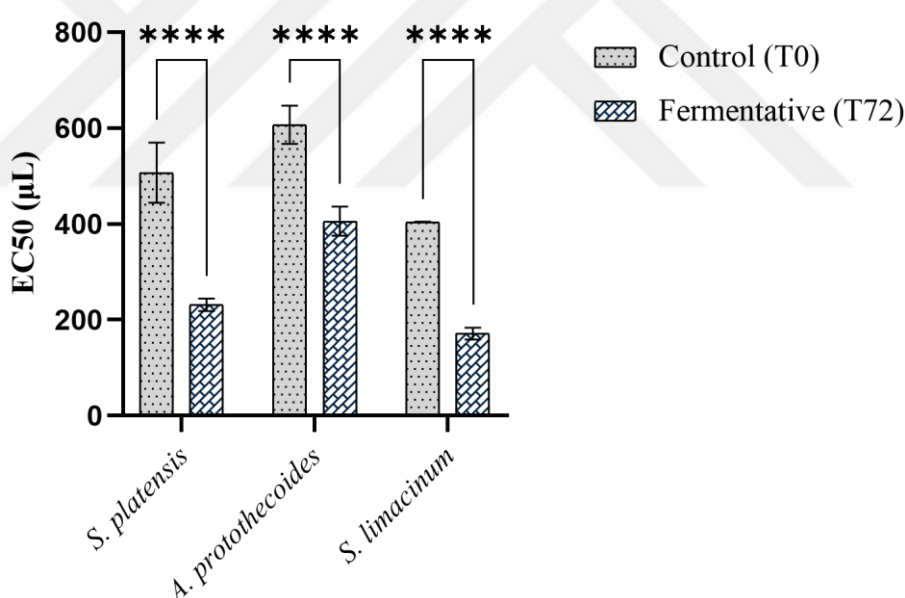


Figure 3.11: Determination of Antioxidant Activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) of fermented microalgae (*S.platensis*, *A. protothecoides* and *S.limacinum* mediums (5 %, w.v⁻¹)) products due to fermentation *B.subtilis* and nonfermented counterparts (control) for 72 h. (The values are presented as mean $\pm$ SD. **** indicates the significance level at $p < 0.0001$, compare with other varieties according to Bonferroni's multiple comparison test.

The results obtained by DPPH analysis with the EC50 value. The EC50 value gave us the half maximum activity concentration, which is the expressed antioxidant capacity. The EC50 value is the minimum concentration of the sample that should inactivate DPPH radicals at half the rate (Alexandrakis et al., 2014). A high EC50 value indicates a low antioxidant capacity, while a low EC50 value indicates a high antioxidant capacity.

The results of antioxidant determination by DPPH analysis of the end products of the three biomasses obtained as a result of fermentation with *B.subtilis* are evaluated in **Figure 3.11** Bonferroni's multiple comparisons test - 2-way ANOVA analysis of the EC50 values of *S.platensis* (507,01 μ L), *A.protothecoides* (607,03 μ L) and *S.limacinum* (548,197 μ L) biomasses compared to the initial controls and the final products obtained after 72 hours of fermentation, we can say that the antioxidant level increased. Considering that the lower EC50 value in DPPH analysis indicates higher antioxidant capacity, we can say that the increase in the antioxidant levels of the products has a statistically significant ($p < 0.0001$) difference. Bioactive substances such as chlorophyll, phycocyanin and carotenoids are known to contribute to the antioxidant properties of *S.platensis* (Karimzadeh et al., 2023; Papalia et al., 2019). In the study of *S.platensis* fermentation with lactic acid bacteria, the highest capacity was observed in DPPH analysis after 24 hours of fermentation (de Marco Castro et al., 2019c). In longer fermentations, DPPH radical scavenging capacity showed a tendency to decrease, probably due to the degradation of heat-sensitive antioxidant compounds. These results indicate that fermentation increases the antioxidant capacity of *S.platensis* (de Marco Castro et al., 2019c). However, unlike TEAC and TPC analyses, an increase in antioxidant activity was observed in this study. DPPH, TEAC and TPC are usually used together to comprehensively measure antioxidant capacity. In *S.platensis* biomass, a higher difference was observed between control and FM than in the other analyses. However, an increase after fermentation was observed in all analyses, although in fact significant/nonsignificant results were obtained. When the DPPH activity of *A.protothecoides* after fermentation is evaluated, there is no specific study directly addressing its effect. In our study, a significant positive effect of fermentation on the DPPH activity of *A.protothecoides* was observed. In the study in which the extraction efficiency of *C.vulgaris* was investigated in terms of antioxidant level by DPPH analysis, it was determined that the major phenolic compounds were

gallic acid, caffeic acid, p-coumaric acid and ferulic acid (Wan Mahmood et al., 2019). These phenolic compounds emphasised the importance of *Chlorella vulgaris* biomass in terms of antioxidant activity (Wan Mahmood et al., 2019). There are not many studies on how the antioxidant capacity of *Schizochytrium sp.* is directly affected by the fermentation process. However, it is known that essential compounds such as DHA, squalene and carotenoids in *Schizochytrium sp.* contribute to the antioxidant level (K. Zhang et al., 2017). In this way, fermentation has been shown to have a positive effect on antioxidant levels. The antioxidant activity provided by *Schizochytrium* is supported by the presence of amino acid components such as Methionine, Histidine, Tryptophan, Cysteine, Phenylalanine and Tyrosine (Moaveni et al., 2022b). In this study, *S. limacinum* protein hydrolysates were found to have high antioxidant activity. It was emphasised that the high DPPH radical scavenging activity of hydrolysates is important for the production of probiotic peptides and the development of cellular antioxidant defence systems. When we compare the final values with the EC50 values of *S.platensis* (273.42 μ L), *A.protothecoides* (405.94 μ L) and *S.limacinum* (171.39 μ L), as in the other antioxidant assays, the best effect of the fermentation process was observed on the final product composed of *S.limacinum* biomass. When we want to compare with TEAC and TPC analysis, different results were obtained due to the use of different radicals in each method and measuring different antioxidant capacities as a different point of DPPH analysis.

3.3.4.4. Imaging of Protein Fragmentation by SDS-PAGE of Fermented *S.platensis*, *A.protothecoides* and *S.limacinum* Products

After 72 hours of fermentation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) analysis was performed to observe the breakdown of large proteins into proteins with smaller molecular weights by hydrolysis during the fermentation process for separation and characterization of proteins. SDS-PAGE profiles of Coomassie Brilliant Blue stained protein fractions obtained from 72 h

fermentation of *S.platensis*, *A.protothecoides* and *S.limacinum* media are shown in **Figure 3.12**, **Figure 3.13** and **Figure 3.14** separately using protein markers in the range of 245-17 kDa. SDS-PAGE images of the final products provided information on the expression levels of proteins, post-translational modifications and changes in protein composition during the fermentation process.

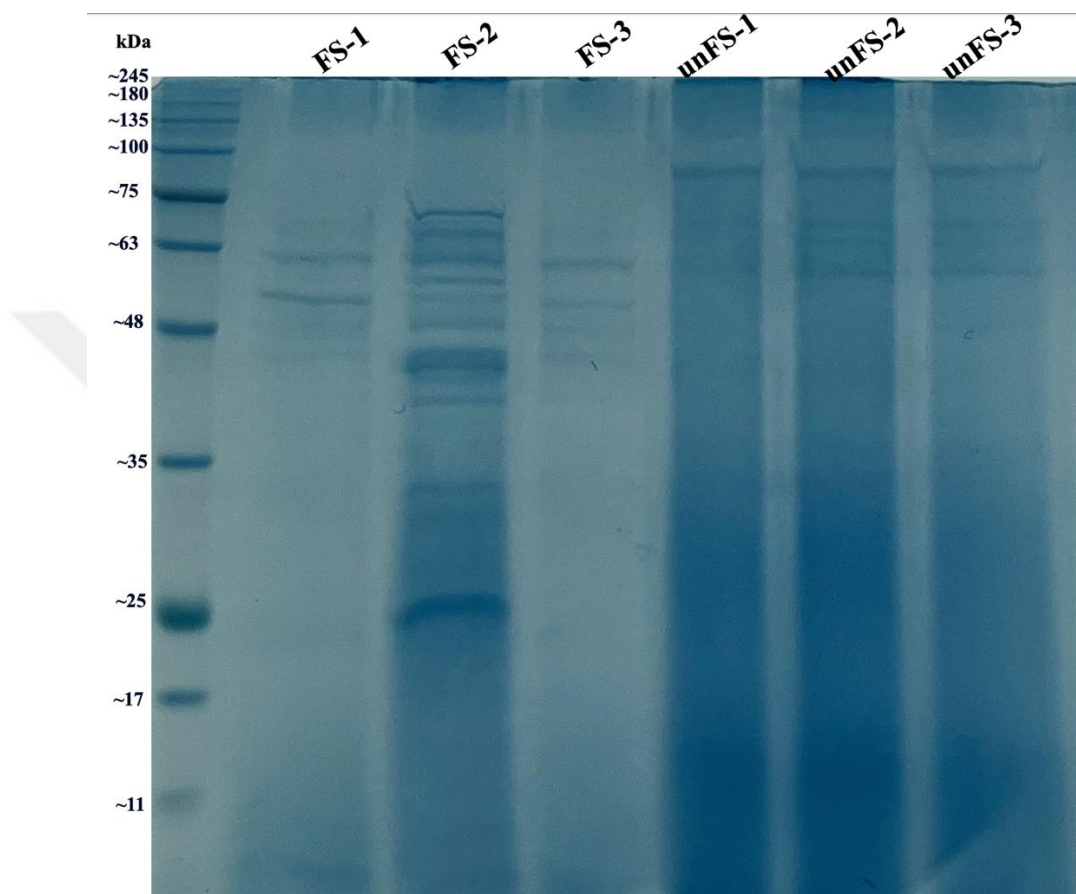


Figure 3.12: *S.platensis* SDS-PAGE profile of protein fractions stained by Coomassie Brilliant Blue obtained after 72h fermentation of mediums (5 %, w.v⁻¹) by *B.subtilis* (Line M. Molecular marker (GangNam-STAIN), (M) Marker; (FS-1) Fermented *S.platensis* - 1st replicate ; (FS-2) Fermented *S.platensis* - 2nd replicate ; (FS-3) Fermented *S.platensis* - 3rd replicate; (unFS-1) unfermented *S.platensis* - 1st replicate (control) ; (unFS-2) unfermented *S.platensis* - 2nd replicate (control) ; (unFS-3) unfermented *S.platensis* - 3rd replicate (control).

SDS-Page provided us with information on the protein diversity and quantities of the control (unFS) and fermented (FS) forms of *S.platensis*. Stained protein fractions were

observed after 72 h fermentation of *S.platensis* medium with *B.subtilis*. The analysis was performed in 3 replicates for unFS and FS samples. In unFS, bands in the range of 11-48 kDa and 63-75 kDa were observed. SDS-Page studies of *S.platensis* in the literature were analysed. In SDS-Page analysis on *S.platensis* protein isolates, various bands in the range of 10-50 kDa were observed. It was suggested that these bands may correspond to proteins such as c-phyococyanin and allophycocyanin (Benelhadj et al., 2016). In addition, the enzyme Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RuBisCO), which plays a role in carbon fixation, may express the observed bands. The molecular weight of RubBisCo consists of two parts, large and small subunits, with the large subunit being approximately 55 kDa and the small subunit being approximately 14 kDa (**Ismail & Piercey-Normore, 2021**). When the FS samples were compared with unFS samples, the bands with a molecular weight of 35-75 kDa remained the same. In the unFS-2 sample, a smear image was obtained differently from the other samples with more than one band. The reason for this can be considered as incorrect loading, hand error or sample contamination. It was observed that low molecular weight protein bands disappeared after microbial hydrolysis. However, the same bands continued to be observed in unFS and FS samples. In order to observe the release of microalgae components supporting our study, SDS-Page analysis at the end of ultrasound-assisted extraction of *S.platensis* showed that the most intense bands were in the range of 35 to 55 kDa (**Aiello et al., 2019**).

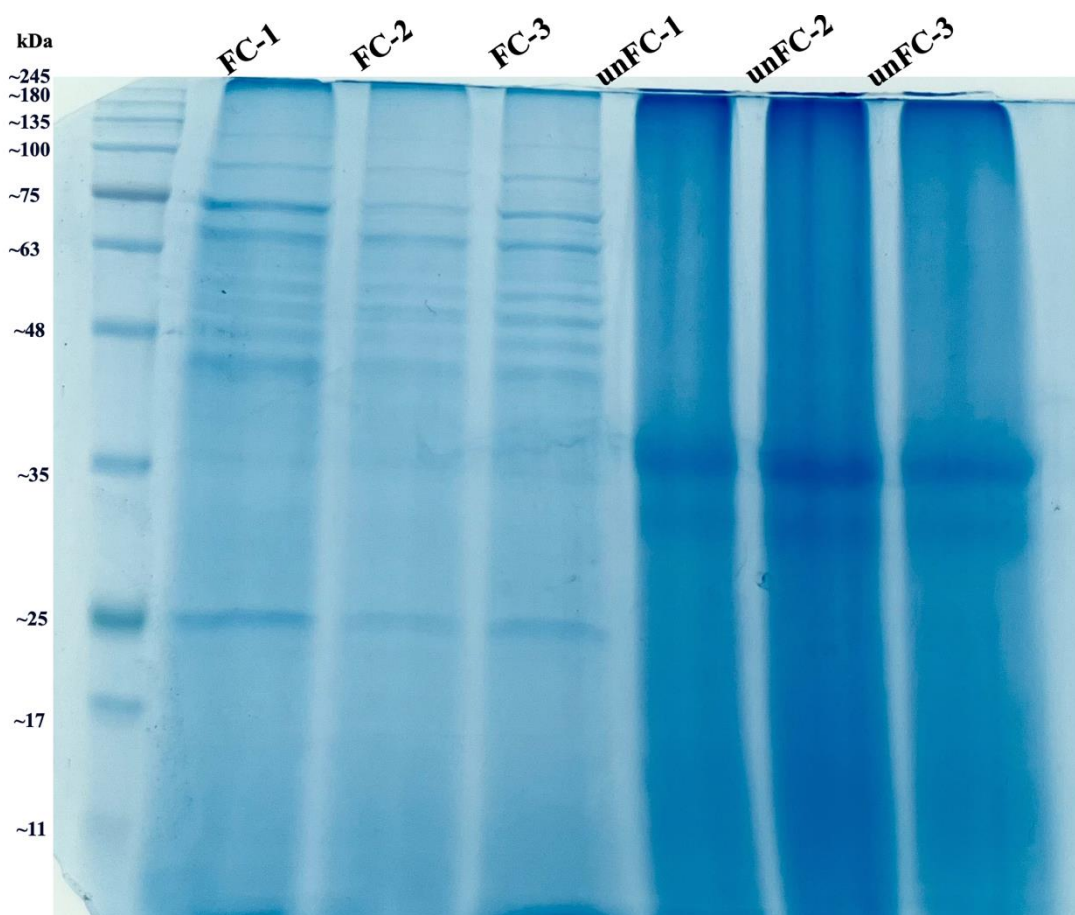


Figure 3.13: *A. protothecoides* SDS-PAGE profile of protein fractions stained by Coomassie Brilliant Blue obtained after 72h fermentation of mediums (5 %, w.v⁻¹) by *B.subtilis*. (Line M. Molecular marker (GangNam-STAIN), **(M)** Marker; **(FC-1)** Fermented *A. protothecoides* - 1st replicate ; **(FC-2)** Fermented *A. protothecoides* - 2nd replicate ; **(FC-3)** Fermented *A. protothecoides* - 3rd replicate ; **(unFC-1)** unfermented *A. protothecoides* - 1st replicate (control) ; **(unFC-2)** unfermented *A. protothecoides* - 2nd replicate (control) ; **(unFC-3)** unfermented *A. protothecoides* - 3rd replicate (control).

The stained fractions of 72 h fermentation of *A. protothecoides* (FC) and control (unFC) samples were observed in 3 replicates. Bands in the range of 25-135 kDa were observed in unFC. In a study on protein extraction of *C. vulgaris*, fractions of proteins larger than 50 kDa were observed (**Andreeva et al., 2021**). The bands observed between 25-32 kDa were interpreted to be related to LHC-II (light-harvesting complex of photosystem II) proteins in a similar study on *C. vulgaris* (**Andreeva et al., 2021**) For the bands between 48-63 kDa, it was thought that the RuBP carboxylase protein

of 55 KDa might be involved (**Sharma et al., n.d.**) In a study on the protein content of *C. sorokiniana* isolates, 109.02 kDa chloroplast rubisco activase protein was matched (**Tejano et al., 2019**). The band observed between 100-135 kDa in our study can be considered as the presence of a similar protein. In FC samples, an intense single band was observed in the range of 25-48 kDa. This situation can be interpreted as high molecular weight proteins may be degraded as a result of microbial hydrolysis.

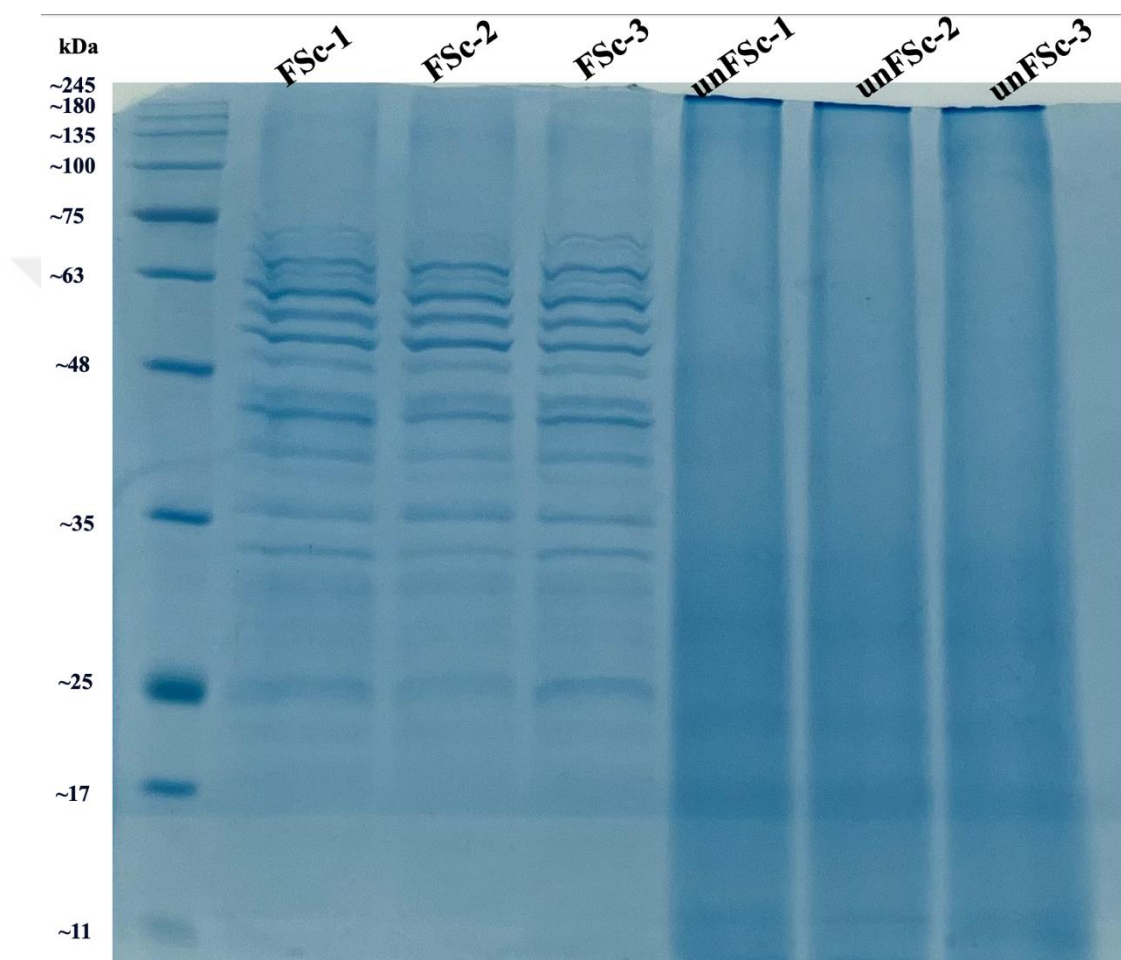


Figure 3.14: *S.limacium* SDS-PAGE profile of protein fractions stained by Coomassie Brilliant Blue obtained after 72h fermentation of mediums (5 %, w.v⁻¹) by *B.subtilis* (Line M. Molecular marker (GangNam-STAIN), (M) Marker; (FSc-1) Fermented *S.limacium* - 1st replicate ; (FSc-2) Fermented *S.limacium* - 2nd replicate ; (FSc-3) Fermented *S.limacium* - 3rd replicate ; (unFSc-1) unfermented *S.limacium* - 1st replicate (control) ; (unFSc-2) unfermented *S.limacium* - 2nd replicate (control) ; (unFSc-3) unfermented *S.limacium* - 3rd replicate (control).

Control (unFSc) and fermented (FSc) samples of *S.limacinum* were stained in 3 replicates and their fractions were observed by SDS-Page analysis. In unFSc, bands between 17-180 were observed. In a different study with *S.limacinum*, it was thought that bands with molecular weights ranging from 66 kDa to 14.2 kDa could be pepsin, trypsin and α -chymotrypsin enzymes (Moaveni et al., 2022b). A significant difference in band intensity and number was observed between unFSc and FS samples. The fermentation process changed the structure and amount of proteins in *S.limacinum*. Proteins of 75 kDa and above have a similar appearance to unFSc after fermentation, indicating that higher molecular weight proteins are less altered or more stable.

3.3.4.5. Determination of ACE Inhibitory Activity of Fermented *S.platensis*, *A.protothecoides* and *S.limacinum* Products

ACE-I activity of non-fermented and fermented products of biomasses were measured using HPLC. Statistical significance of the measurements was analyzed by two way ANOVA.

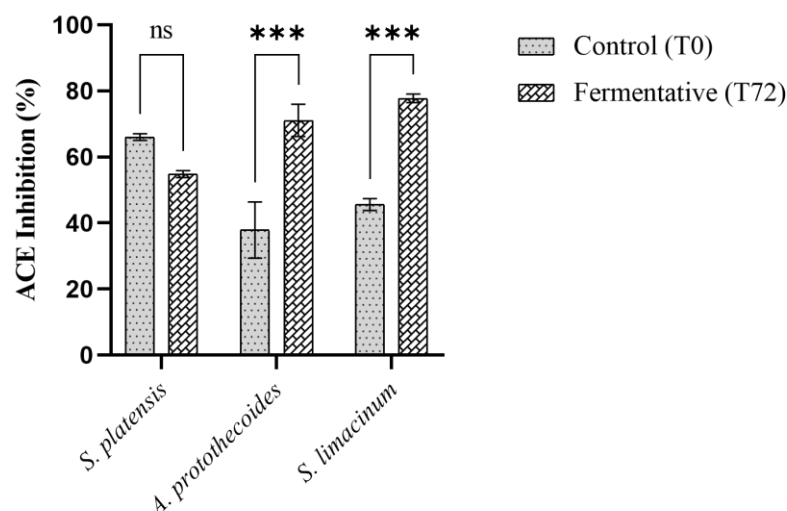


Figure 3.15: Determination of ACE Inhibition of of fermented microalgae (*S.platensis*, *A. protothecoides* and *S.limacinum* mediums (5 %, w/v)) products due to fermentation *B.subtilis* and nonfermented counterparts (control) for 72 h. (The values are presented as mean $\pm$ SD. *** indicates the significance level at $p < 0.0001$, compare with other varieties according to Bonferroni's multiple comparison test).

ACE Inhibition potential is a popular agenda in terms of potential health benefit, especially in hypertension issues. ACE inhibition activity is measured to assess the potential of substances to inhibit angiotensin-converting enzyme (ACE), which plays a crucial role in the regulation of blood pressure (Mullally et al., 1997). Although no statistically significant results were obtained in *S.platensis* biomass, a decrease in ACE Inhibition was observed as a result of the fermentation process. Fermented *S.platensis* showed 66.07% ACE inhibition activity, while unfermented *S.platensis* showed 54.87% activity. Fermentation increased ACE inhibition in *A.protothecoides* and *S.limacinum*. Fermented *A.protothecoides* (71.07%) showed approximately twice the ACE inhibition activity compared to unfermented (37.87%) samples. *Chlorella* ACE inhibition activity was examined in a study by adding supplements, L-Name with and without *Chlorella*, to the diets of mice. The findings indicated that *Chlorella* supplementation led to a delay in the onset of hypertension and cardiorenal remodelling. Additionally, it was observed that *Chlorella* inhibited ACE activity, lowered tissue tumour necrosis factor- α concentrations, and reduced renal CYP4A expression (S. C. Yang et al., 2013). The evaluation was statistically significant with

a p value of 0.0006. *S.limacinum* fermented by *B.subtilis* was the biomass showing the best ACE inhibition activity in this study. Fermented *S.limacinum* (77.74%) showed activity compared to unfermented *S.limacinum* (45.57%). Fermentation in *S.limacinum* media resulted in a statistically significant result with a increase in the product obtained. ($p=0.0007$). There are not many specific studies on ACE inhibition of *Schizochytrium* in the literature. However, high ACE activity due to its rich fatty acid, protein, antioxidant activity has been supported by studies. This is supported by the fact that it can produce healthy bioactive compounds (**Pekkoh et al., 2022**). Antioxidants have the ability to scavenge reactive oxygen species (ROS) or free radicals (**Verspreet et al., 2021**) ROS accumulation causes overexpression of ACE inhibiton enzyme (**Verspreet et al., 2021**). Therefore, antioxidants gain importance in this field. In another study, microalgae inhibited ACE-1 by 73.4-87.1% at physiologically relevant concentrations compared to a commercial control (**Vilakazi et al., 2021**) When the effect of fermentation on ACE activity was examined, it was found that *Lactobacillus helveticus* 130B4 contained in fermented milk contains an ACE inhibitory peptide, and ACE inhibitory activity is resistant to digestive proteases and heat (**Shuangquan et al., 2008**). As a result of the study, it was concluded that the fermented product may be effective against hypertension when taken orally (**Shuangquan et al., 2008**).

3.3.4.6. Determination of Free Amino Acids for nonfermented Microalgae (nonFM) and Fermented Microalgae (FM) Products

It is important to analyse the free amino acid profile after fermentation. This is because analysing the free amino acid profile of microalgae biomass after microbial hydrolysis provides insight into the biochemical changes that occur. It can provide information about the quality and potential health benefits of the product obtained. A heat map plot of the free amino acid profile can be seen in Figure 3.16. In this graph, the coding CH-I (*A.protothecoides*), SP-i (*S.platensis*) and SC-I (*S.limacinum*) refers to FM products. CH-C (*A.protothecoides*), SP-C (*S.platensis*) and SC-C (*S.limacinum*) refer to the

nonFM, i.e. control group. On the right side of the map, the marker expressing free amino acid concentration was used.



Figure 3.16: Determination of Free Amino Acid (FAA) of *B.subtilis* in *S.platensis*, *A. protothecoides* and *S.limacium* mediums (5 %, w.v⁻¹) and unfermented mediums (control) for a specified time courses (0 and 72 h) of fermentations. **(CH-C)** *A.protothecoides* Control ; **(CH-I)** Fermented *A.protothecoides* ; **(SP-C)** *S.platensis* Control ; **(SP-I)** Fermented *S.platensis* ; **(SC-C)** *S.limacium* Control and **(SC-I)** Fermented *S.limacium*

When the FM profiles were analysed respectively, a decrease in TFAA concentrations was observed in *A.protothecoides* after fermentation. This can be considered as the utilisation of amino acids for the growth of *B.subtilis*. Only Leucine (increased from 511 $\mu\text{M.L}^{-1}$ to 1207 $\mu\text{M.L}^{-1}$) and Tyrosine (increased from 203 $\mu\text{M.L}^{-1}$ to 1041 $\mu\text{M.L}^{-1}$) is increased as shown in heat map. The reason for this increase may be the breakdown of proteins in *A.protothecoides* during the fermentation process (Nakashima et al., 2014) , the breakdown of the cell wall and the release of amino acids into the fermentation medium (Rodrigues & Da Silva Bon, 2011) or the accumulation of free amino acids such as tyrosine and isoleucine in the medium due

to the use of organic carbon sources **(Xu et al., 2021)**. This may have led to the increase in FAA concentrations in all FM in the study. There are not many studies in the literature on the FAA profile of *Chlorella* fermentation with *B.subtilis*. However, leucine increase is noticed in *S.limacinum*. We can say that *S.limacinum* is the FM with the highest change in TFAA profile after fermentation. Increases in Glutamic Acid, Alanine, Valine, Methionine, Lysine and Proline were observed. However, the highest increase was observed in the amino acid Leucine (increased from 339 $\mu\text{M.L}^{-1}$ to 3427 $\mu\text{M.L}^{-1}$). High leucine concentration is an element that supports lipid production in microalgae **(Heredia-Arroyo et al., 2010)**. We have already mentioned that *S.limacinum* is a high lipid-producing microalgae species that is particularly effective in the production of multiple fatty acids such as DHA production. As a result of its fermentation with *B.subtilis*, the breakdown of *Schizochytrium* and the emergence of high leucine concentration has been supported by studies **(Kim et al., 2016)**. In addition, the high FAA diversity of *S.limacinum* actually provides reference to the antioxidant and ACE inhibition analyses performed before. *S.limacinum* is the FM species showing the highest potential in these studies. This was emphasised by the fact that the active peptides exhibit strong bioactivities and their beneficial effects are important in terms of antihypertensive and antioxidant **(Moaveni et al., 2022a)**. With the TFAA analysis, *S.limacinum* has been proved to be effective in these areas with its amino acid profiles. As a result of fermentation of *S.platensis*, a general decrease in amino acid concentrations was observed. The highest concentration decrease was observed in Glutamic Acid (decreased from 1180 $\mu\text{M.L}^{-1}$ to 283 $\mu\text{M.L}^{-1}$). In a study specific to *S.platensis* and *B.subtilis* fermentation **(Kurt et al., 2023)**, it was noted that the amount of glutamic acid was higher in FM samples. The reasons for a different result in this case may be interpreted as the use of extra enzymes in extraction period or the use of different proteolysis mechanisms by *B.subtilis* in microbial hydrolysis. In addition, parameters such as the strain used, fermentation conditions and inoculation volume may have caused different results **(Aledo et al., 2015; Ogbonda et al., 2007)**. An increase in methionine and phenylalanine was observed. The increase in methionine and phenylalanine as a result of fermentation shows an important result in terms of flavour and aroma. Because these two amino acids are important in the formation of flavour compounds (Xiao et al., 2022). This increase allows us to approach the sensory properties of fermented microalgae products. In many respects, TFAA analysis supported the biochemical activities performed and allowed us to make

a comparative analysis in terms of the potential benefits of FM products. However, since there are not many studies in the literature on the FM species used, this study is important.

3.3.4.7. Artificial Intelligence Based Prediction Model Development for Post-Fermentation Soluble Protein Concentrations

After fermentation, a data set was created from the data obtained by Lowry analysis of the Soluble Protein Amounts of the biomass fermented and not fermented. The data obtained were categorized as Duration and Inoculation Yes/No. The machine learning based regression methods used for evaluation at this stage were Random Forest (RF), Decision Tree (DT), Generalized Additive Model Regression (GAM), Support Vector Machine (SVM) and Gaussian processes (GP).

The data set was evaluated with RF, SVM, DT, GP and GAM machine learning based regression methods. As a result of this evaluation, models were created. Root mean square error (RMSE), normalized-root mean square error (N-RMSE) coefficient of determination (R^2), adjusted- R^2 and Mean Squared Logarithmic Error (MSLE) were considered. The best prediction models were obtained by comparing statistical regression methods.

Table 3.7: Performance evaluation of machine learning algorithms for Soluble Protein Amount responses in fermented and non-fermented biomasses depending on Inoculation Present/No Inoculation and Time factors.

Models	R^2	A- R^2	RMSE	N-RMSE	MSLE
RF	0,9464	0,9435	0,3116	0,0637	0,0005
DT	0,9574	0,9551	0,2777	0,0575	0,0004
GAM	0,9265	0,9226	0,3649	0,0768	0,0007
SVM	0,2653	0,2259	1,1537	0,2777	0,0080
GP	0,9843	0,9835	0,1685	0,0325	0,0001

When the table and model graphs are analysed, $R^2=0.98$ and $a-R^2=0.96$ values of the GP model are the highest. MSLE=0.0001, which measures percentage errors, has the second lowest value. In this way, we can say that the model with the best performance

in modelling is the GP model. The DT model showed a performance close to the GP model in R^2 and $a-R^2$ values. We can say that this model also explains the data set well. However, the fact that it has a higher margin of error than GP (MSLE=0.0004) causes us to evaluate it as the second best performing model. RF and GAM models showed moderate performance. SVM model is the lowest performing model among the models. R^2 and $a-R^2$ values are very low. The RMSE and normalised-RMSE values, which give the square root of the mean square difference between the predicted values and the actual values, are very high. We expect these values to be low in the models. In line with the results given by the model, SVM showed a noticeably lower performance. GP Model is considered as the most appropriate model for this data set.



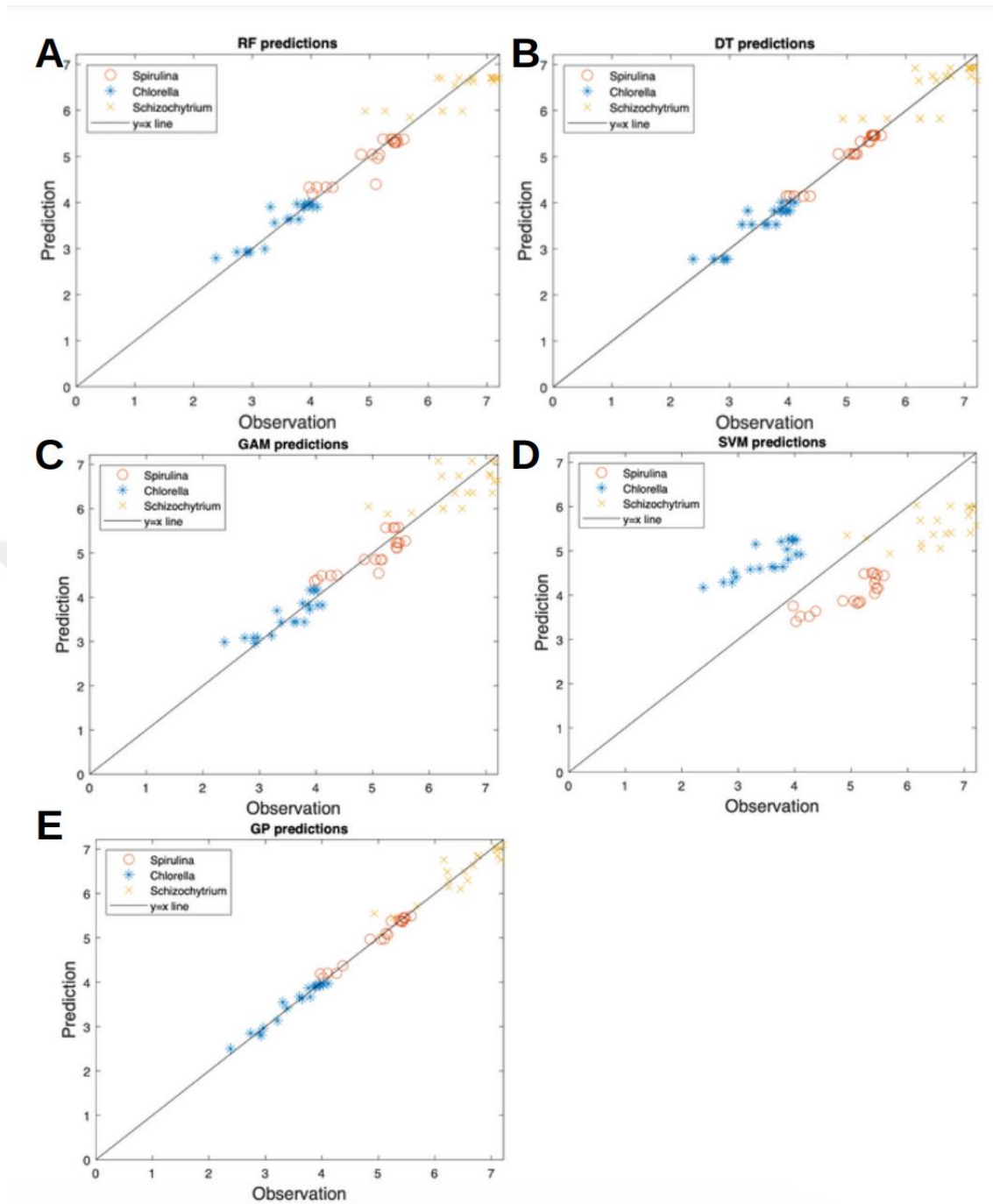


Figure 3.17: AI-Based Protein Content Prediction Analysis after fermentation of *S.platensis*, *A. protothecoides* and *S.limacium* biomasses and unFM samples. A) Random Forest Model ($R^2=0.94$) B) Decision Tree Model ($R^2=0.95$) C) Generalized Additive Model ($R^2=0.92$) D) Support Vector Model ($R^2=0.26$) E) Gaussian Model ($R^2=0.98$)

In the modelling studies, a joint study was carried out for all three biomasses. Protein amounts of samples of FM and nonFM products taken at different times were measured. A common data set was created as a result of the measurements. We can clearly see one of the biggest benefits of AI Models here. We can evaluate different microalgae species fermented with the same bacterial species on the same model. At the same time, we can see the information about the protein content of the unfermented form of these microalgae on the same model. Thanks to this study, this model will provide a high prediction for future studies on the fermentation of *S.platensis*, *A.protothecoides* and *S.limacinum* biomasses with *B.subtilis*. It will also allow approximate predictions for the use of similar biomasses and bacteria. AI-based approaches in bioprocesses are gradually included in our lives in the current process. The use of AI technology, especially in costly, time-consuming and industrial scalable studies, provides great advantages to research. You can test your experiment on the model with an already created dataset related to the experiment you have designed and thus you can create an approximate prediction before performing your experiment. For example, an AI-based hybrid AI-model (CNN) was studied for the production of biomass and bioactive phycobiliproteins (PBPs) in *Nostoc sp.* A dataset was created for testing and then it was observed that the model successfully predicted a combination of growth environment parameters (**Saini et al., 2021**). This highlighted the potential and importance of the modelling strategy for optimising the suitable conditions for cyanobacteria for the commercial production of biofuels (**Saini et al., 2021**). In a different study, AI approaches were used to optimise the parameters for converting sugar from palm sap to ethanol by yeast fermentation (**Ezzatzadegan et al., 2021**). ANN and ANFIS were used for process modelling. The results showed that the ANFIS model accurately predicted the bioethanol concentration in the fermentation process. In this way, it is mentioned that it provides an approach that sheds light on the future in terms of optimisation of relevant parameters and result prediction (**Ezzatzadegan et al., 2021**). Furthermore, in a different study on bioethanol production optimisation using ANN model, a sugar parameter dependent modelling was studied (**Chouaibi et al., 2020**). The ANN model obtained a successful prediction result and it was mentioned that it showed a promising approach for bioethanol production from agricultural wastes (**Chouaibi et al., 2020**) As a result, the importance of AI-based approaches in industrial bioprocesses is increasing. With the studies carried out, the development of automated systems, real-time parameter

estimations, easy diagnosis of deviations and minimum human intervention are important (**Mondal et al., 2023**). Such uses of artificial intelligence have been supported by this study and the importance of potential studies in this field has been revealed.



4. CONCLUSION

This study provided the opportunity to approach the efficient protein extraction of *S.platensis*, *A.protothecoides* and *S.limacinum* biomass from different perspectives. Microalgae are microscopic organisms with significant potential for a wide range of industrial applications due to their high-value compounds. Due to their high protein content, these algae have attracted attention in recent years as an alternative protein source and bioactive peptides for food, health and feed industries. However, efficient protein extraction from algal biomass depends on several factors such as microalgae cell type, method used for cell disruption, extraction time.

The aim of the study was to combine RSM and AI algorithms to achieve efficient protein extraction from algal biomass. RSM is a powerful statistical tool for optimising complex processes such as protein extraction by evaluating multiple factors and determining their effects on the response. There are many studies in the literature using RSM in this field. In addition, AI algorithms, which are a new type of approach, are aimed to present an up-to-date approach in this study. AI algorithms allow us to efficiently handle complex, non-linear relationships between extraction parameters and protein yield. The joint evaluation of multiple parameters at the same time allowed us to reveal the prominent aspects of AI algorithms in the comparison of RSM and AI algorithms in this study. In this way, the models created with AI algorithms have contributed to the literature for better efficiency and cost-effective experimental designs and industrial applications. In this way, AI algorithms will provide a comprehensive approach in future studies, enabling simultaneous improvement of multiple valuable components, increasing sustainability and maximising the economic potential of bioprocesses.

The second part of the study showed that *S.platensis*, *S.limacinum* and *A.protothecoides* biomasses can be a suitable substrate. The fermentation of *S.limacinum* biomass showed the highest performance in the analyses due to the high value compounds it contains. Moreover, the fact that it has been previously processed as de-oiled biomass demonstrated its importance from a sustainability and economic point of view. The analyses performed on the microalgal biomasses elucidated their nutritional value, bioactive compound content and overall suitability as fermentation substrates. The use of different microalgal biomasses as fermentation substrates will

contribute to future studies on the potential of microalgae for comprehensive physicochemical analysis, advancing the understanding of their nutritional and functional properties, facilitating the assessment of biomass quality and improved fermentation performance.

In the last stage of the study, a new dataset of total soluble protein content of FM and nonFMs was created. The dataset was applied with 5 different artificial intelligence based models. In this case, the GP model gave the best prediction results with $r^2=0.98$. The data obtained by microbial growth were also tried to be taught to AI-based models, but since nothing is known about the real-time intracellular metabolic state, the model approaches were sceptical. In general, the study was interpreted that *B. subtilis* can utilise proteinaceous materials from microalgal biomass for growth and metabolism. The study also revealed a potential approach to the applicability of existing technologies to bioprocesses based on the predictions of artificial intelligence algorithms. Since the data were generated in the experimental environment, limitations were encountered in the study. However, the more data we have in studies using artificial intelligence algorithms, the more accurate the modelling obtained will be. The use of Artificial Intelligence Based Algorithms offers promising approaches for making sense of non-linear and complex data, model prediction in experiments and acceleration of laboratory studies.

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PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

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APPENDICES

Appendix A: Total Soluble Protein Concentration In Supernatants Was Determined According To Lowry Et Al. (Lowry, Rosebrough, & Randall, 1951) With Bovine Serum Albumin (Bsa) As A Standard.

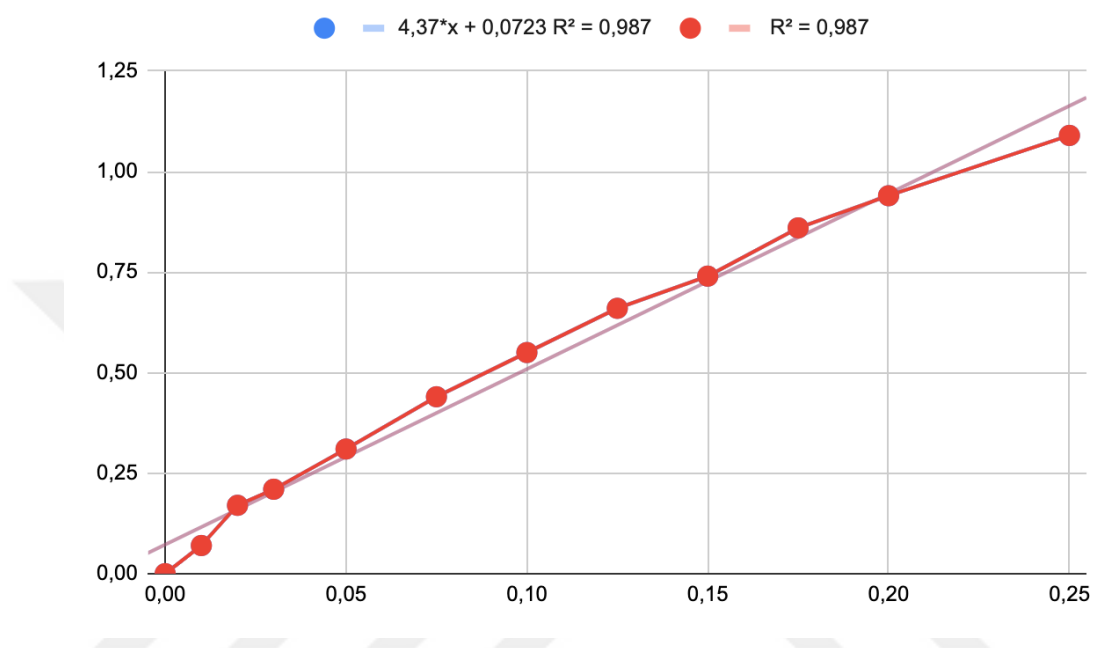


Figure A.1: BSA standard curve for the determination of total amount of protein by Lowry method.

Appendix B: Total Phenolic Contents Concentration In Supernatants As Milligrams Of Gallic Acid Equivalent (Mg Gae.L⁻¹). (Lamuela-Raventós, 2017)

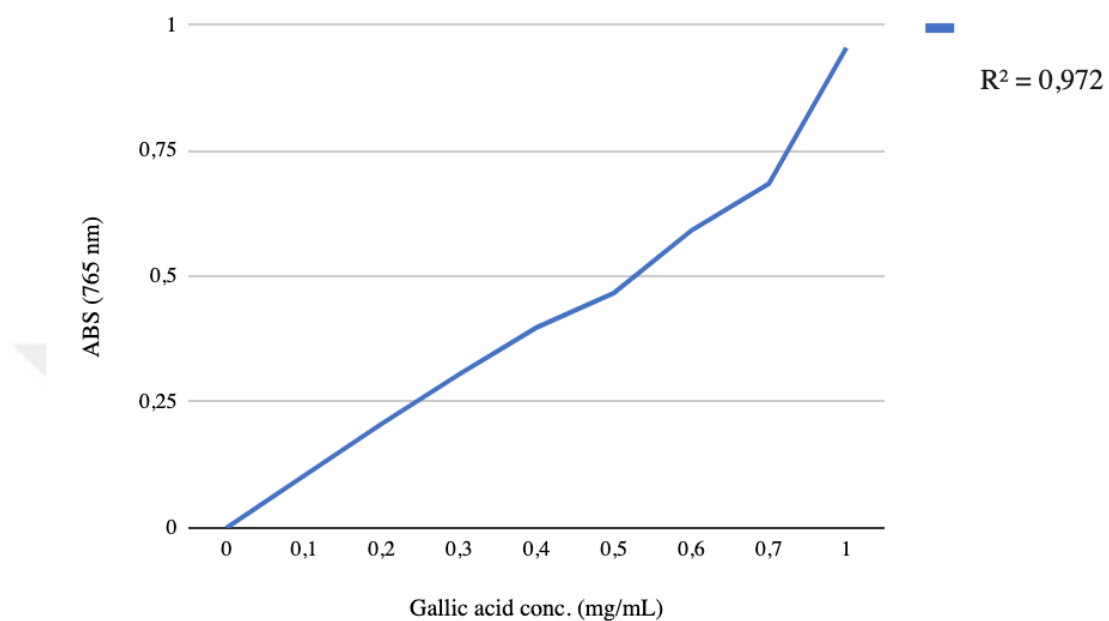


Figure B.1: Standard calibration curve of TPC at concentrations of 0, 200, 200, 300, 400, 500, 600 700 and 1000 mg/mL

Appendix C: Trolox Calibration Graph. The Calibration Curve Prepared Using Trolox Used For Teac Measurement

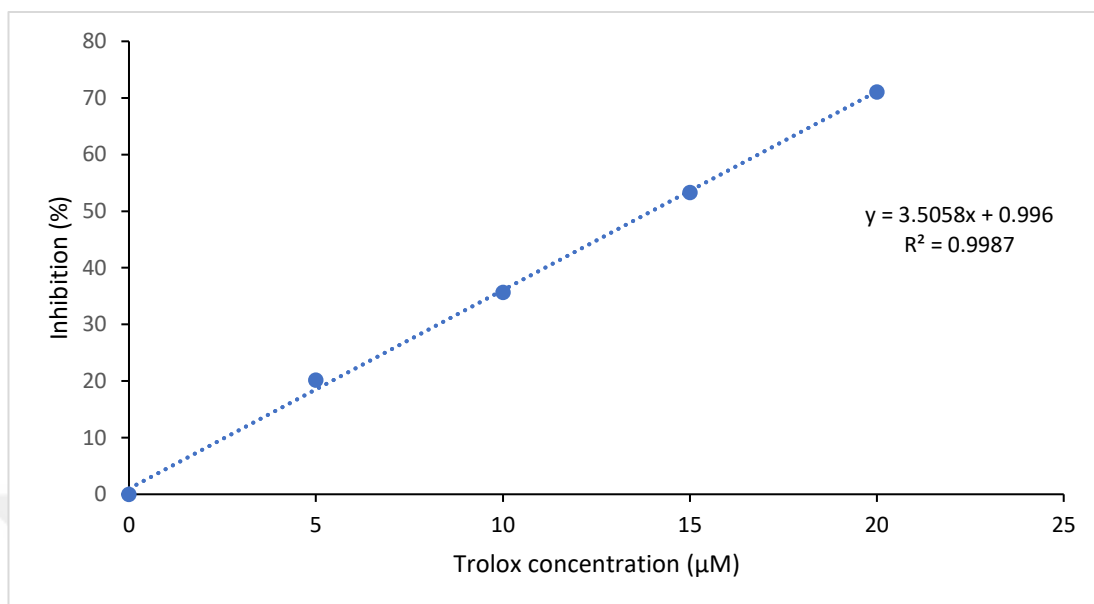


Figure C.1: Standard calibration curve of TEAC at concentrations of 5, 10, 15, 20 and 25 μM.