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Master Thesis

EFFECT OF CULTIVATION PARAMETERS ON IN-VITRO ANTI-INFLAMMATORY ACTIVITY OF MICROALGAE /

MİKROALGLERİN İN-VİTRO ANTİ-İNFLAMTUVAR AKTİVİTESİ ÜZERİNE ÜRETİM PARAMETRELERİNİN ETKİSİ

Alper Baran Sözmen

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Sunuş Tarihi: 09.08.2017

Bornova-İzmir

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Ege Üniversitesi Fen Bilimleri Enstitüsü

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Alper Baran Sözmen tarafından Yüksek Lisans tezi olarak sunulan "Effect of Cultivation Parameters on In-Vitro Anti-Inflammatory Activity of Microalgae / Mikroalglerin İn-Vitro Anti-İnflamatuvar Aktivitesi Üzerine Üretim Parametrelerinin Etkisi" başlıklı bu çalışma EÜ Lisansüstü Eğitim ve Öğretim Yönetmeliği ile EÜ Fen Bilimleri Enstitüsü Eğitim ve Öğretim Yönergesi'nin ilgili hükümleri uyarınca tarafımızdan değerlendirilerek savunmaya değer bulunmuş ve 03.08.2017 tarihinde yapılan tez savunma sınavında aday oybirliği/~~oyçokluğu~~ ile başarılı bulunmuştur.

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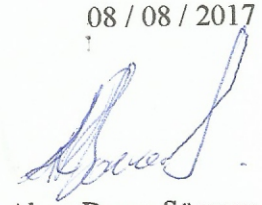

Alper Baran Sözmen

ÖZET

MİKROALGLERİN İN-VİTRO ANTİ-İNFLAMATUVAR AKTİVİTESİ ÜZERİNE ÜRETİM PARAMETRELERİNİN ETKİSİ

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Bu çalışmada bir mikro alg türü olan *Chlorella miniata*'nın baloncuk kolon tipi fotobiyoreaktörler içinde, BRISTOL ortamında ve seçilen ışık şiddetini veren beyaz floresan aydınlatma şartlarında, inkübasyon dolabında sabit şartlarda büyümesi incelenmiştir. *Chlorella miniata* ile 6 litrelik steril ortamlarında, (22°C, 245 μmol $m^{-2}s^{-1}$) sürekli bir stok biyokütle üretimi yapılmıştır. Üretim süreçleri 21 günlük bir büyüme evresi reaktör ortamından alınan günlük örneklerin analiz edilmesiyle kontrollü olarak tamamlanmıştır. Büyüme şartlarına bağlı olarak büyüme hızı sıcaklık, ışık şiddeti ve her ikisine de bağlı çeşitli modellerle incelenmiştir. Matematik modeller yardımıyla, uygun büyüme koşulları 21,38 ^oC sıcaklık ve 291,5 μmol foton m^{-2} s^{-1} olarak hesaplanmıştır. Biyokütleden Soxhlet ekstraksiyonu ile ekstraktlar elde edilmiş LC MS/MS çalışmaları sonucunda ekstraktların içeriğinde yüksek miktarlarda salisilik, kafeik vanilik, t-sinamik asit ve siyanidin ve mirisetin'e rastlanmıştır. Çalışma diğer bir amacı *Chlorella miniata* türünün olası antioksidan ve anti-inflamatuar kapasitesinin araştırılması ve bu olası kapasite ile yetiştirilme koşulları arasında bağlantılar aranmasıdır. Bu amaçla, trolox eşleniği olarak antioksidan kapasitenin ölçümü (TEAC), toplam antioksidan kapasitenin ölçümü (TAO), ferrik indirgen antioksidan kapasitenin ölçümü (FRAP), ksantin oksidaz inhibisyonu ve hiyaluronidaz inhibisyonu çalışmaları yetiştirilmiş mikro algler için yapılmıştır. Bu çalışmaların sonuçları, 22,91 mg/g alg TEAC, 776,70 mg/g alg gallik asit eşleniği FRAP ve 37,07% ksantin oksidaz inhibisyonu, 65,39% hiyaluronidaz inhibisyonu olarak bulunmuştur. **Anahtar sözcükler**: Chlorella miniata, matematik modelleme, antioksidan kapasite, anti inflamatuar etki, fenolik maddeler

ABSTRACT
EFFECT OF CULTIVATION PARAMETERS ON IN-VITRO ANTI-INFLAMMATORY ACTIVITY OF MICROALGAE

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The aim of the study was to acquire three findings regarding microalgae. The microalgae species *Chlorella miniata* was used for the study. The first step was to determine the specific growth rate changes of the microalgae with cultivation parameters, the experiments were carried out using varying cultivation conditions and they were performed using bubble column photobioreactors with a volume of 6 liters, and 1 vvm aeration, illuminated using white fluorescent light. Using mathematical modelling, the optimum temperature and light intensity for the maximum specific growth rate were calculated to be 21,38 ^{o}C and 291,5 μmol photon m^{-2} s^{-1} respectively. The last two parts of the study was to see if the microalgae species *Chlorella miniata* possess an antioxidant and anti-inflammatory potential and to see if there is a correlation between the cultivation conditions and these potentials. For this purpose the assays of Trolox equivalent antioxidant capacity (TEAC), ferric reducing antioxidant capacity (FARP), total antioxidant capacity (TAO), xanthine oxidase inhibition, and hyaluronidase inhibition were studied using extracts of microalgae obtained from the different cultivation parameters. It's important to mention that this was the first study using these assays on species of *Chlorella miniata* and the first that utilized the cultivation parameters on microalgae. The results were, 22,91 mg/g algae TEAC, 776,70 mg/g algae gallic acid equivalent of FRAP and 37,07% xanthine oxidase inhibition with a concentration of 10,5 mg algae per ml ethanol, 65,39% of hyaluronidase inhibition with a concentration of 0,9 mg algae per ml ethanol. The effects of the cultivation parameters on these assays were also a part of the study.
Keywords: *Chlorella miniata*, mathematical modelling, antioxidant capacity, anti-inflammatory potential and phenolic compounds

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1. Introduction

The industrial interest in producing and processing algae species had increased in the last decades greatly, mostly because of their easy and cheap cultivation and harvest. Also being not in competition in terms of fertile soil with agricultural products in a time of food shortages in world increased the feasibility of algae production. The algae were cultivated by lots of different methods, in different growth mediums and under various conditions during the studies until today and due to these studies it can be said that, the microalgae species, each of them need a distinct method, condition or medium for a higher yield of production whereas it becomes crucial to determine those in the name of a higher productivity. Hence the first step of this study investigated the nature of growth of the species, *Chlorella Miniata*, a green microalgae which belong to a genus known for high growth rates and lipid content.

Although it was important to determine a higher productivity range in terms of temperature and light intensity during cultivation, the main purpose of this study was to find the antioxidant and anti-inflammatory potential of the extract of the species, the phenolic compounds which are likely to cause these potential and their dependence on growth conditions. This study was one of the very first studies which investigated the anti-inflammatory effect of green microalgae and also the originality of the study lies in the search for relationships between growth conditions and the accumulation of phenolic compounds, antioxidant activity and anti-inflammatory potential of the algal extracts. It is safe to say, sources of phenolic compounds which are quite costly to acquire due to their easily denaturable nature, by temperature and light, and impossibility of synthetic production for the same reason are always relevant in industry. That’s why the aim of this study holds importance, concerning pharmaceutical and cosmetics industries.

2. Literature Survey

2.1 Microalgae and Chlorella

Algae are eukaryotic and primarily aquatic, be it fresh water or marine, photosynthetic organisms. They range in size from 1 micrometer to 60 meters. Many algae consist of only one cell, and also the largest algae have millions of cells. Microalgae are microscopic algae, typically found living both in the water column and sediment. They are unicellular species which exist individually, or in chains or groups.

Like plants on land algae are also the base of the food chain, and also like plants in addition to making organic molecules, algae produce oxygen as a by-product of photosynthesis. Algae are consumed both as processed and unprocessed food, used as alternative fuel sources and their extracts have value in the pharmaceutical industry (Rogers, 2011).

Green algae, or the chlorophyta; are known of their contents of both chlorophyll a and b. They also differ from the rest of the algae in forming the storage product in chloroplast instead of in cytoplasm. Their ability to accumulate carotenoids, high cell proliferation rates and lipid content promoted the industrial production of the green algae (Lee, 2008).

2.2 Photo-Bioreactors and Cultivation Modes

Algae are grown either in open culture systems or closed systems which utilizing a light source, also known as photo bio-reactors. Photo bio-reactors can use artificial light sources or solar light sources or both. Naturally illuminated systems generally include large illumination surfaces while artificial illumination is usually used in laboratories. Furthermore, some photo bio-reactors can be tempered by simply placing the reactor in a constant temperature room or cabin; the process is limited by compactness of the reactor, also several commercial reactors are readily tempered (Ugwu et al. 2007).

Bio-reactors can be classified as; bubble column reactors, airlift reactors (internal and external loop reactors), stirred tank reactors, fluidized bed reactors and packed bed reactors. The bubble column reactors are widely used in algal production systems mostly because of their simple design; O_2 transfer, mixing and other performance factors are influenced mainly by gas flow rate and rheological properties of the fluid (Pulz, 2001).

The operation modes are classified as batch, semi-batch, fed-batch and continuous modes due to the flow rates in and out of the system. The batch operation is the oldest and most widely used operation. The advantages of using a batch bioreactor are lower installation and operational costs, flexibility, and utilization simplicity. The biomass yields of different microalgae with different operation modes from some recent studies can be seen in Table 2.1. (Kadic and Heindel, 2013).

Table 2.1. Biomass Yield of Various Microalgae with Different Operation Modes.

Microalgae	**Operation Mode**	**Biomass Yield (g/L)**	**Reference**
Porphyridium purpureum	Batch	0.97	Fuentes-Grünewald et al. 2015
	Semi-batch	1.04	
Isochrysis galbana	Batch	0.25 ± 0.02	Picardo et al. 2013
	Continuous	0.42 ± 0.02	
Arthrospira platensis	Batch	4.82 ± 0.05	Xie et al. 2015
	Fed-batch (5mM Nitrate)	6.18 ± 0.04	
	Fed-batch (Medium)	6.78 ± 0.07	
	Batch	3.40	
Phaeodactylum tricornutum	Fed-batch	5.00	Cerón-García et al. 2013
	Batch	1.09 ± 0.05	
Chlorella sp.	Semi-batch	2.2 ± 0.12	He et al. 2016
	Batch	0.84 ± 0.02	
M. Dybowskii	Semi-batch	2.37 ± 0.03	

Mixotrophy is a trophic culture method in which microalgae can drive both photoautotrophy and heterotrophy utilizing both inorganic and organic carbon sources. It is suggested that the specific growth rate of microalgae under mixotrophic cultivation is approximately the sum of the growth rates under photoautotrophic and heterotrophic modes. However it is not proven so and also, it is believed that the two metabolic processes affect each other, and is not a simple combination. (Salati et al. 2017) Some recent studies on the matter are gathered in Table 2.2 which suggests: mixothropic culture cultivation has a higher yield than a photoautotrophic or a heterotrophic culture at lowest.

Table 2.2. Biomass Yield of Various Species of *Chlorella* Cultured with Different Trophic Culture Cultivation.

Microalgae	**Biomass yield**			**Reference**
	Autotrophic	**Mixothropic**	**Heterotrophic**	
Chlorella sp. Y8	0.22	0.45	0.17	Lin and Wu, 2015
Chlorella Vulgaris	0,31	1.70	N/a	Liang et al. 2009
Chlorella Protothecoides	1,00	4.07	4,00	Heredia-Arroyo et al. 2010
Chlorella sp. BTA 9031	0.93	1.55	N/a	Mondal et al. 2016
Chlorella Vulgaris ESP-31	0.80	3,00	0.20	Yeh and Chang, 2012
Chlorella sp.	0.60	1.2	0.38	Cheirsilp and Torpee, 2012
Chlorella Sorokiniana	0.68	5.08	4.23	Li et al. 2014

2.3 Effect of Light Intensity on Cultivation and Modelling

There exist a lot of different factors which affect the growth of the algae among those, light is generally at an improper level. In laboratory cultures the light intensity is too low to permit logarithmic growth and in nature the intensity is above saturation most of the day and may be high enough to cause inhibition. A study by Sorokin and Krauss (1958) on species of *Chlorella pyrenoidosa*, the light intensity saturation point differs from strain to strain and increases with temperature. It is crucial to understand that each species and even each strain might have a different saturation point. Table 2.3 shows different species of microalgae, light intensities utilized for cultivation and growth rate. Also Singh and Singh, 2015, concluded that the microalgae grow better in high light intensities as 200 μmol photon m^{-2} s^{-1}.

Table 2.3. Effect of Light Intensity on Microalgal Growth.

Microalgae	Light Intensity (mmol m^{-2} s^{-1})	Temperature (^oC)	Growth Rate (d^{-1})	Reference
C. Zofingiensis	460	25	2,304	Imaizumi et al. 2016
C. Protothecoide	35	26	2,04	Krzeminska et al . 2015
	130		2,184	
	420		2,16	
Chlorococcum Littorale	30	22	1,224	Ota et al. 2015
	65		1,848	
	100		2,184	
	170		2,64	
C. Vulgaris	70	27	0,864	Chang et al. 2016
	120		1,872	
	180		1,296	
C. minutissima	100	30	0,500-0,670	Aleya et al. 2011
C. zofingiensis	90	28	0,480	Del Campo et al. 2004
	460		0,720	

Microalgae	Light Intensity (mmol m^{-2} s^{-1})	Temperature (^oC)	Growth Rate (d^{-1})	Reference
Chlorella sp.	82	28	0,670	Chen et al. 2010
	260		0,740	
	368		0,700	
	590		0,650	

As seen in Table 2.3, it is not possible to define the effect of light intensity with one equation, since the model will not be able to cover each specie, reactor type, operation mode and culture medium. The ability to model algal productivity under various light intensities is the key to foresee the feasibility and sustainability of industrial-scale algae cultivation. However, a tremendous amount of studies on modeling approaches aim to describe the interactions between light intensities and growth rates in specific conditions.

In Table 1.4 various mathematical expressions are given which have been used to determine relationship of light intensity during cultivation and the growth of the microalgae *Chlorella miniata*. In the equations: μ stands for the specific growth rate in d^{-1} of the algae at the light intensity I in the unit of µmol photon m^{-2} s^{-1}, μ_{max} stands for the maximum possible theoretic specific growth rate at the studied conditions. K_I and I_k are both symbolizing the same term, the half-light saturation point also in the unit of µmol photon m^{-2} s^{-1}. K is the inhibition growth constant, describing the light inhibition in the related models. I_{opt} is the theoretical amount of light intensity needed for optimum algal growth and I_c the critical light intensity for onset of light inhibition. X is the cell concentration in the reactor in g/l and V is the reactor working volume in liters. I_m maintenance rate in mmol g^{-1} d^{-1} and V_F is the illuminated volume fraction of the reactor. Lastly I_{abs} is standing for the total light energy absorbed in the reactor by day in the unit of mmol/d.

Table 2.4. Growth Rate - Light Intensity Models.

Name of the Equation	Equation	Reference
Monod Equation (1)	$\mu = \mu_{max} \frac{I}{K_I + I}$	Cornet et al. 1995
Poisson Model (2)	$\mu = \mu_{max}(1 - \exp(-\frac{I}{I_k})$	Geider et al. 1998
Hyperbolic Tangent Model (3)	$\mu = \mu_{max}\, tan\, h\left(\frac{I}{I_k}\right)$	Chalker et al. 1980
Extended Expression of Hyperbolic Tangent Equation (4)	$\mu = \mu_{max} \frac{\mathrm{I}}{I_{opt}} \exp(1 - \frac{I}{I_{opt}})$	Steele, 1962
Aiba Model (5)	$\mu = \frac{\mu_{max} I}{k_s + I + \frac{I^2}{k_i}}$	Dechatiwongse et al. 2014
Linear model (6)	$\mu = \frac{\ln(\frac{I}{I_c})}{EL}$	Ogbonna et al. 1995
Modified Hyperbolic Tangent Model (7)	$\mu = \mu_m \frac{\tanh(\frac{I}{I_k})}{1 + K(I - I_{c)}}$	Pahl-Wostl and Imboden, 1990

The first equation on the table is proposed by Cornet et al. 1995, it is a mono-dimensional, monod type equation. It was used to model the growth rate of *Spirulina platensis* The kinetic laws for photosynthesis saturation curves vs available light energy leads to series of complex differential equations, modeling of light transfer and kinetics and the assessment of radiant energy absorbed in photo bio-reactors require at the very least two parameters for light absorption and scattering in the culture medium but a simple model based on the simplified, mono-dimensional equation is discussed in this article. This approach provides a much simpler way to determine a relationship between the illumination and growth rate. The model equation is suitable for indoor use and for any reactor geometry.

The second equation on Table 2.4 is based on the assumption that photosynthesis is a Poisson function of irradiance. (Geider et al. 1998) A study by Pahlow (2005) proposed that a series of models which included the poisson model are well suited to simulate growth under a wide range of conditions and to describe a number of unexplained phenomena, such as the nutrient and energy resources among competing metabolic requirements for nutrient uptake, light-harvesting, and growth.

A study by Kurano and Miyachi, (2005) investigated three of the mathematical representations on Table 2.4; they were compared for the specific growth rate-light response curve: Steele's exponential function, a Poisson function and a hyperbolic tangent function. For this purpose, cultivation of *Chlorococcum littorale* in batch cultures of 4 liter was carried out. However previous reviews of the equations for modeling (Jassby and Platt, 1976) the light saturation curves of photosynthesis concluded that for natural populations of marine phytoplankton that the most consistently useful mathematical representation was the hyperbolic tangent function. Extended information criterion taken into account the hyperbolic tangent function gave the best fit as evaluated by Kurano and Miyachi (2005).

Although the values of parameters μ_{max} and I_k estimated by both Steele's exponential function and the hyperbolic tangent functions were identical to six significant figures.

It was suggested by Pahl-Wostl and Imboden (1990) that due to data obtained various algal cultures and natural algae, that photosynthesis, growth rate and light intensity have a dynamic relationship. That's why static curves relating the relationships are not desirable. The Dynamic model for the photosynthetic rate of algae (DYPHORA) models the response of cell growth rate to light intensity by two characteristics. The fundamental of the model is in the dynamic response of the photosynthesis rate to light. It was said that photosynthesis, hence growth rate, and light intensity relations can't be described by models of static nature. In the study of Pahl-Wostl and Imboden (1990) the DYPHORA was used to fit a variety of experimental data and reproduce them, it was taken a strong argument that the model related the actual growth rate to light intensity. Although successful the study also stated that it had no intention of describing any physiological or biochemical processes which take place during cultivation.

Dechatiwongse et al. (2014) proposed the Aiba model to describe the increment of specific growth rate of the algae, with the increasing light intensity and after the point of light saturation, where the maximum possible specific growth rate was achieved, and the inhibition effects on the growth of microalgae. The equation includes a light saturation term and a light-inhibition term for this purpose.

Ogbonna (1995) investigated the effect of light intensity on specific growth rate during the stationary and exponential growth phases of the algae independently. Batch cultivation of *Chlorella pyrenoidosa* and *Spirulina platensis* has was carried out to monitor the effects of light intensity on growth rates of the microalgae. The relationships between the specific, linear growth rates and emitted light were visualized mathematically. Good correlations between the linear growth rates and the final cell concentrations were found for any type and size of photo bio-reactor, for both *Chlorella* and *Spirulina* cells. Various growth phases during light-limited batch cultivation of photosynthetic cells were predicted by a simple mathematical model, naming the linear model in the Table 2.4.

Bechet et al. (2013) prepared a review on desirability, applicability, sustainability and relevancy of various models which relates light intensity with growth rate and/or photosynthesis rate. The study categorized the models based on their abilities to predict light intensity growth relations considering, light gradients, light cycles, reactor type and geometry and light source. The models were investigated under three categories or types. First type describing the light intensity and photosynthesis rate relation of an entire culture, second type models are for calculating the productivity of the culture medium without considering light cycles and third type models were the models that took light gradient and light cycles into account, which are more applicable to outdoor cultivation. The study discusses over 40 models by the told considerations, noting that most of the models were applicable only for indoor cultivation. It states that for the photosynthesis rate calculation type two models do not have universality and also proposes the utilization of type two models, for the purposes of practicability and accuracy in predicting net productivity and engineering applications.

2.4 Effect of Temperature on Cultivation and Modelling

Temperature is an important element for growing algae. It strongly influences cellular chemical composition, the uptake of nutrients, carbon dioxide fixation, and the growth rates. It is know that the growth rate will increase with the increase in temperature up to its optimum and once it reaches its optimum, growth rate will decrease drastically with the increase in temperature (Juneja et al, 2013). Table 2.5 contains studies made to investigate the effects of temperature on algal proliferation and growth rate. Also Singh and Singh,. 2015 suggested in their report that microalgae favor temperatures between 22 and 35 ^{o}C.

Table 2.5. Effect of Temperature on Specific Growth Rate of Various Microalgae.

Microalgae	Temperature (^oC)	Light Intensity (mmol m^{-2} s^{-1})	Growth Rate (d^{-1})	Reference
Haematococcus Pluvialis	20	70	0.61	Giannelli et al. 2015
	23.5		0.70	
	27		0.76	
	30.5		0.80	
C. Wailesii	9	208	0.53	Nishikawa and Yamaguchi, 2008
	12.5		0.63	
	15		0.83	
	20		1.04	
C. Granii	9	297	0.43	Nishikawa and Yamaguchi, 2008
	12.5		0.64	
	15		0.74	
	20		1.15	
E. Zodiacus	8	273	0.89	Nishikawa and Yamaguchi, 2006
	9		0.93	
	12.5		1.11	
	15		1.49	
	25		2.01	
A. Catenella	10	59	0.18	Navarro et al. 2006
	12		0.11	
	14		0.30	
	16		0.10	
Cyanothece Sp.	25	320	0.55	Dechatiwongse et al. 2014
	30		0.76	
	32		0.88	
	35		0.86	
	37		1.12	
	40		1.56	

Microalgae	Temperature (^oC)	Light Intensity (mmol m^{-2} s^{-1})	Growth Rate (d^{-1})	Reference
C. Vulgaris	25	70	0.14	Converti et al. 2009
	30		0.14	
	35		0.12	
	38		0.00	

Table 2.5 shows that, algal growth is monitored in a temperature range between 15-35 ^oC and the maximum specific growth rate is strongly affected by temperature change. Modelling the temperature-specific growth rate relations requires an equation that can predict the optimum point of growth and the declines in the extreme points. Although there have been various studies on modelling the algal growth, there isn't much investigation on determining the most suitable model for different conditions. The contents of Table 2.6 are the temperature-specific growth rate models which were highly utilized.

Table 2.6. Growth Rate- Temperature Models.

Name of the Equation	Equation	Reference
Skewed Normal Distribution Model (8)	$\mu = \mu_{max} e^{\frac{-(T-T_{opt})^2}{2\sigma^2}}$	Dauta et al. 1990
Square Root Model (9)	$\mu = (b_1(T - T_{min}))^2$	Ratkowsky et al. 1983
Expanded Square Root Model (10)	$\mu = (b_2(T - T_{min})[1 - e^{c_2(T-T_{max})}])^2$	
Parker Equation (11)	$y = \gamma_{max}(\frac{T}{\Theta_{opt}} w^u)^z$	Talbot et al. 1991
Sinclair Equation (12)	$\mu = Ae^{\frac{-E_a}{RT}} - Be^{\frac{-E_b}{RT}}$	Zwietering et al 1991

Table 2.6 shows the mathematical models which were used to investigate the relation between the growth temperature and the specific growth rate of the algae *Chlorella miniata*. In the equations μ stands for the specific growth in d^{-1} rate at the temperature T in ^{o}C. T_{opt} is the calculated optimum temperature for the maximum specific growth rate and μ_{max} stands for the maximum possible theoretic specific growth rate at the studied conditions. σ is the standard deviation associated to the optimal temperature in ^{o}C. b_1, b_2, and c_2 are Ratkowsky parameters in the units of $^{o}C^{-1}$ $h^{-0.5}$ for b_1 and b_2, and $^{o}C^{-1}$ for c_2. T_{min} and T_{max} are the minimum and the maximum temperatures that growth is observed both in ^{o}C. In the Parker equation, w and u are both defined as functions of temperature as follows.

$$\boldsymbol{w = \frac{\Theta_0 - T}{\Theta_0 - \Theta_{opt}}} \quad \textbf{Equation 13}$$

$$\boldsymbol{u = \frac{\Theta_0 - \Theta_{opt}}{\Theta_{opt}}} \quad \textbf{Equation 14}$$

y in the equation represents the growth rate, γ_{max} is its maximum value and $\Theta_{opt}$is the temperature where y is maximum while $\Theta_0$is the temperature where y becomes 0. z is a power. Lastly in the Sinclair equation, A and B are the frequency factors of the respective activation energies of cell growth and cell death, E_a and E_b. The fit of the data were made with the method used in light intensity models.

A skewed normal distribution of specific growth rate due to temperature is assumed in the first equation in the table. Where T is the temperature, T_{opt} is the optimum (or calculated optimum) temperature for microalgal growth and σ is the standard deviation associated to the optimal temperature. The optimum temperature was 25 ^{o}C in the aforesaid study and the model proposed to has better fit within the range of those used in the study (Gonçalves et al. 2016).

The Square root model is actually not based on a biological study but an empirical approach to the linear increment of specific growth rate with temperature at temperatures lower than the optimum growth temperature. Where b is called the ratkowsky parameter and T_{min} is the minimum temperature where growth is observed. Hence ratkowsky et al. expanded the equation and reached utilized expanded square root model, where the model more successfully describes the reaction of specific growth rate to temperature, between the minimum and maximum temperatures where growth is observed. Although this model is only applicable in the range it has been defined it had consistent results in that interval (Zwietering et al. 1991).

Parker's equation has a different approach to the modelling of specific growth rate and temperature relations from the models discussed so far. It assumes maximum specific growth rate and optimum light intensity for growth are dependent of the temperature, and y represents those values is their maximum value and terms u and w are also functions of temperature. In the study of Talbot et al. Parker's equation was appropriately described the behavior of the investigated species. However the model gives underestimations for one of the studied species of maximum specific growth rate and optimum light intensity at lower temperatures, below 10 ^{o}C. (Talbot et al. 1991)

A study done by Perez et al. suggested a kinetic model to simulate the temperature effect on the specific growth rate and utilized a modified Arrhenius equation to describe the model. The study concluded that the suggested model was more suitable for cultivations closer to the lower growth limit temperatures. (Perez et al. 2008)

2.5 Modelling the Combined Effects of Light Intensity and Temperature

The approach so far is to model specific growth rate and temperature effects and specific growth rate and light intensity effects separately, but the combined effect should also be modelled to fit the experimental data consistently. A study by Ota et al. investigated the dependence to temperature of light intensity model parameters and defined the dependent parameter with a temperature model. Equation 1 is a derived Monod equation including an Arrhenius type equation to predict the maximum specific growth rate. This mathematical model takes account of the Arrhenius activation/deactivation energies expressed the temperature-dependent promotion and inhibition of the cell growth rate and treats the light intensity affects independently (Ota et al. 2015).

$$\mu = [A_0 e^{\frac{-E_a}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)} - B_0 e^{\frac{-E_b}{R}\left(\frac{1}{T}-\frac{1}{T_0}\right)}]\frac{I}{K_I+I} \qquad \textbf{Equation 15}$$

Bernard and Remond (2012) also studied the effects of light intensity and temperature on specific growth rate and they suggested defining the specific growth rate as a product of two equations, one function of light intensity and other function of temperature. The model proposed in this study is based on the cardinal temperature model with inflexion and platt model and the main assumption here is that cardinal temperatures are marginally affected by light, and thus temperature effect does not depend on light intensity. Equation 2 shows the equation proposed by Bernard and Remond (2012).

$$\mu(T,I) = \mu_{max}\frac{I}{I+\frac{\mu_{max}}{\alpha}\left(\frac{I}{I_{opt}}-1\right)^2} \qquad \textbf{Equation 16}$$

$$[\frac{(T-T_{max})(T-T_{min})^2}{(T_{opt}-T_{min})(T_{opt}-T_{min})(T-T_{opt})-(T_{opt}-T_{max})(T_{opt}-T_{min}-2T}]$$

Where T_{min} is the temperature lower than which there is no growth and T_{max} is the temperature above which there is no growth. The maximal growth rate l_{opt} occurs at temperature T_{opt} and α is the initial slope of the light response curve; I_{opt} is the light intensity at the point where maximum growth rate is achieved.

Another approach on the matter is to account for the combined effect of light intensity and temperature on specific growth rate of the algae. As Bechet et al. named "coupled" models aim to predict the response of the algae to temperature and light intensity with a single equation. As an example Equation 3 can be scrutinized for a clearer understanding.

$$\mu = 2\mu_{max}T(1+\beta_I)\frac{I/I_{opt}(T)}{1+2\beta_I I/I_{opt}(T)+(\frac{I}{I_{opt}(T)})^2} \qquad \textbf{Equation 17}$$

Coupled models theoretically demonstrate the effects of temperature on specific growth rate better than the uncoupled models, as long as the limiting factor of the growth is temperature or temperature dependent. Also they require a larger number of experimental data to process because of greater number of parameters in the models. For instance to utilize equation 3, at least 9 different experimental data is needed. This issue may cause over fitting and can change the consistency of the fit (Bechet et al. 2013).

2.6 Added-Value Compounds in Microalgae: Extraction and Identification

2.6.1 Extraction

Hot Continuous Extraction or soxhlet extraction method was introduced in 1879 for the first time; it's the most commonly used semi-continuous extraction method in small scale. The principle of the method is to use the same solvent to wash the algae, which is placed in a extraction tube or chamber in a porous cartridge, repeatedly. The solvent; that is going to be used for extraction is poured into a flask then vaporized and condensed in to the extraction tube or chamber until the liquid level reaches the top of the tube or chamber, then the contents are siphoned back in to the flask and the process is repeated. The process is carried out in a continuous manner until the desired material is completely extracted then the process is terminated, this method has the advantage of extracting large amounts of material with otherwise impossible amounts of solvent. In larger scales this provides great amounts of solvent to be saved. (Handa et al. 2008)

Kamarudin et al. made a comparison between conventional solvent extraction and soxhlet extraction in 2016, regarding extraction yield of compounds with antioxidant activities and phenolic groups. The study was made on three different species, resulting in the favor of soxhlet extraction which had a yield almost twice of the conventional extraction at most. The study also showed up that solvents that include ethanol tend to have a higher extraction yield of investigated compounds in the study.

Boeing et al. (2014) studied the effect of different solvents on the yield of extraction of added value compounds on microalgae and plants. Solvent combination of methanol, ethanol, acetone and water are the most commonly used substances; are studied on three species, and stated that different solvents give different results for different added-value compound however the specie doesn't seem to have a large impact on the results.

2.6.2 Phenolic Compounds

Over the last decades the interest on phenolic compounds were growing. The main causes were their antioxidant activities, and their appearances in the food and pharmaceutical products in our everyday lives. The activity of phenols are mostly reasons of ability to scavenge radical groups by hydrogen or electron transfer which are more rapid reactions than radical attacks on organic substances. (Rappoport, 2003)

Table 2.7 gives the results of previous studies on various species of algae.

Table 2.7. Previous studies on algae, results of total phenolic compounds in Gallic Acid equivalents.

Species	Total Phenolic Content (mg Gallic Acid Equivalent	Reference
Heterogenous Culture	134	Rehul et al, 2016
Eisenia bicyclis	193	Machu et al, 2014
Ulva clathrata	5,08	Farasat et al, 2014
Sargassum tenerrimum	2,13	Movahedinia and Heydari, 2012
Gracilaria corticat	2,42	
Kai Algae	1066,96	Pornpimol et al, 2015
Acanthophora spicifera	40,583	Zakaria et al, 2011
S. filipendula	12,87	Bambang et al, 2013
Padina sp	10,16	
S. binderi	9,09	
Sargassum swartzii	11,05	Sadati et al, 2011

Hua-Bin Li et al. investigated the phenolic content of 23 species of microalgae utilizing the Folin- Ciocalteu method; the species included eight *Chlorealla* species. The phenolic content of different species found to be quiet variable, from 2.12 to 39.87 mg GAE/g. It has been shown that the species *Nostoc ellipsosporum*, *Chlorella protothecoides* #7,and *Chlorella pyrenoidosa* #3 had the highest amount of phenolic contents. (Li et al. 2007)

Brown (*Laminaria japonica, Eisenia bicyclis, Hizikia fusiformis, Undaria pinnatifida*) and red (*Porphyra tenera, Palmaria palmata*) seaweed, green freshwater algae (*Chlorella pyrenoidosa*), and cyanobacteria (*Spirulina platensis*) were investigated in a recent study for the contents of phenolic compounds and epicatechin was found to be the most dominant among them. From the data obtained by spectrophotometry and antioxidant capacity of the water soluble compounds (ACW) determination it was evident a linear relationship existed between ACW and phenolic contents. Some algal products have been suggested to be promising functional foods rich in polyphenols. (Machu et al 2015)

In the study by Gomez et al. 2016, the total phenol contents of four species of microalgae were quantified by spectrophotometry. The studied species were *Chaetoceros muelleri, Thalassiosira weissflogii, Dunaliella tertiolecta,* and *Tetraselmis* chuii, with different irradiances and containers. The highest amount of phenolic compounds was found *in D. tertiolecta* and *C. muelleri*. The conclusion of the study was that the production of phenolic compounds was higher in green microalgae, without any solid connection to irradiance or the type of the containers. (Gomez et al 2016)

2.6.3 Antioxidants

Antioxidants occur naturally in food and natural health products or are added to them intentionally to extend their shelf life, or are used as supplements to improve health status. The oxidation of food, mainly its lipid components, leads to off-flavor development and spoilage. Thus, control of oxidative processes is of interest to scientists, manufacturers, and consumers. In human body, oxidants are by-products of normal metabolism that, if not properly controlled, result in oxidation and eventual damage to DNA, proteins, lipids, and sugar molecules. Oxidation of these biomolecules in the body leads to a number of degenerative diseases such as cancer, cardiovascular disease, cataract, immune system decline, and brain dysfunction as well as the aging process. (Decker et al, 2015)

Table 2.8 includes the previous studies on various species of algae, which investigated the antioxidant capacity, by different antioxidant capacity determining assays.

Table2.8. Previous studies on algae, results of antioxidant capacities by various assays.

Species	Concentra-tion mg/ml	Trolox equivalent antioxidant capacity (µmol trolox equivalent g^{-1} dry weight(	Total Antioxidant Activity (% inhibition)	Ferric Reducing Aantioxidant Potentaial (µmol Gallic acid equivalent g^{-1} dry weight)	Reference
D. Dichotoma	1	33,4	-	-	Demirel et al, 2009
C. Sinuosa	1	29,9	-	-	
Spyridia fusiformis	0,1	-	6,37	-	Bhuvaneswari et al, 2013
Chondrococcus hornemanni	0,1	-	9,32	-	
Chlorella #1	3,33	51,03	-	64,65	Goiris et al, 2012
Chlorella #2	3,33	59,57	-	24,34	
Chlorella vulgaris	3,33	19,97	-	42	
Brown Algae	3,33	-	-	3,55	Kelman et al, 2012
Green Algae	3,33	-	-	2,29	
Red Algae	3,33	-	-	1,59	
C. linum	2	-	43,43	-	Farasat et al, 2013

Hua-Bin Li et al. also studied on the possible antioxidant activity of the 23 microalgae species, using Trolox equivalent antioxidant capacity assay. The correlation coefficients between the antioxidant capacity and the phenolic content were also investigated. It was determined that the coefficients between the antioxidant capacities and the phenolic contents were found to be very small, which suggests: phenolic compounds are not a major contributor to the antioxidant capacities of microalgae, unlike plants, vegetables and fruits leading to a foresight of microalgae containing different antioxidant compounds from plants. (Hua-Bin Li et al. 2007)

Geetha et al. were screened on reactive oxygen species scavenging capacity, total antioxidant capacity and lipid peroxidation inhibition potential in *Chlorella pyrenoidosa* also known as Sun Chlorella. The aqueous extracts of studied algae showed significant antioxidant potential by positively modulating the antioxidant activity in in vitro study sample. (Geetha et al 2010)

Simic et al. completed a study with the aim of examining in vitro antioxidant antimicrobial activities of the extracts of the green microalga, *Trentepohlia umbrina*. The methods of free radical scavenging, superoxide anion radical scavenging, reducing power, determination of total phenolic compounds and determination of total flavonoid content has been used to determine the antioxidant activity of the studied species. It has been concluded that the extract of the algae had high antioxidant potential in terms of reducing power and superoxide anion radical scavenging. (Simic et al. 2012)

2.6.4 Compounds Possessing Anti-inflammatory Potential

The anti-inflammatory effects of the compound Violaxanthin, has been proved long ago, Soontornchaiboon et al. studied the anti-inflammatory potential of *Chlorella ellipsoidea* with this inspiration, the study was carried out using the lipopolysaccharide (LPS)-stimulated RAW 264.7 mouse macrophage cells, and resulted violaxanthin successfully inhibiting the inflammatory pathways. (Soontornchaiboon et al. 2012)

A study by Bitencourt et al had examined the possible anti-inflammatory effects of *C. Mexicana* methanolic extract. Colitis was induced on mice cells by dextran sodium sulfate treatment. Afterwards, *C. mexicana* methanolic extract was given on the following days. The results showed that the application of the extract removed the symptoms and suggested that the algae may have a medical use. (Bitencourt et al 2015)

Sibi and Rabina studied the anti-inflammatory activities of *Chlorella vulgaris* extracts, using an in-vitro assembly, investigating the inhibition of pro-inhibitory mediators. The study concluded that the extracts of *C. vulgaris* promise an efficient anti-inflammatory agent and an alternative to commonly used anti-inflammatory drug. (Sibi and Rabina, 2016)

3. Present Study Flowsheet

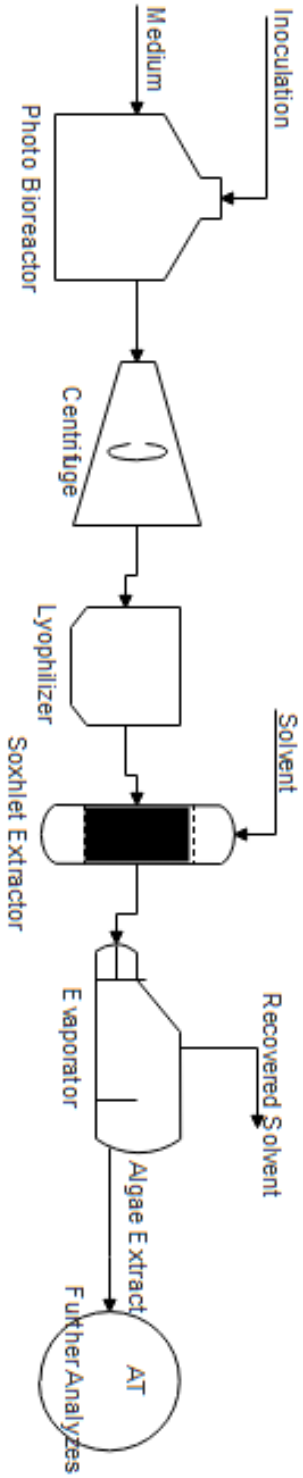


Figure 3.1. Flow sheet of the study.

4. Material and Method

4.1 Cultivation of Algae

The study started with the cultivation of the microalgae species in Ege University, Chemical Engineering Department, at Research Laboratory 8.

Figure 4.1. Research Lab 8, Chemical Engineering Dept./ Ege University

Figure 4.2 gives the outline of the flowsheet of this study which starts with the cultivation of algae *Chlorella Miniata* and ends with the analysis of the algae extract.

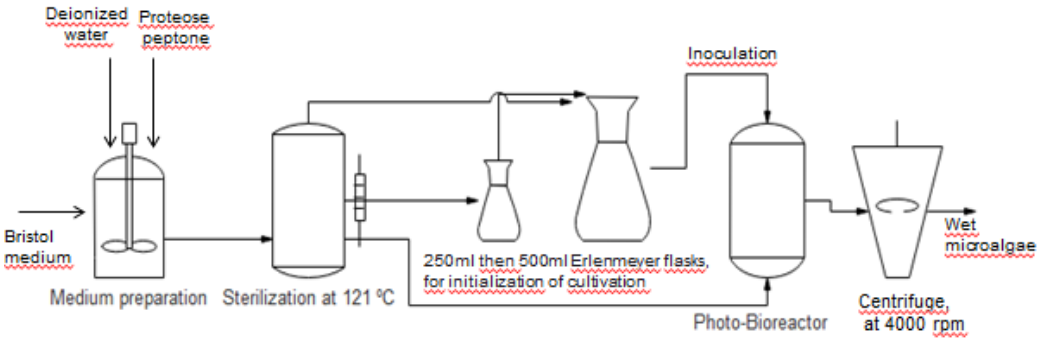


Figure 4.2. Flowsheet of the Algae Production Process also known as Cultivation.

The Microalgae which has been cultivated was obtained from the Culture Collection of Algae at the University of Texas (Austin, U.S.A) (UTEX) isolated in Delft, South Holland, by M.W. Beijerink (1892). *Chlorella miniata* was inoculated in Peptone Protease (ATCC medium: 847 Algal protease broth) medium given in Table 4.1.

Figure 4.3. Stock solutions for the culture medium.

Table 4.1. ATCC medium: 847 Algal protease broth.

	Compound	**Concentration in Stock Solution**	**Stock Solution / Medium Concentration**
Bristol Medium	$NaNO_3$ (Merck)	10 g/400ml	10 ml/l
	$CaCl_2 \cdot 2H_2O$ (UPARC)	1 g/400ml	10 ml/l
	$MgSO_4 \cdot 7H_2O$ (Merck)	3 g/400ml	10 ml/l
	K_2HPO_4 (Carlo Erba)	3 g/400ml	10 ml/l
	KH_2PO_4 (J. T. Baker)	7 g/400ml	10 ml/l
	NaCl (Merck)	1 g/400ml	10 ml/l
Protease Peptone	Protease Peptone (Merck)	1g /l	

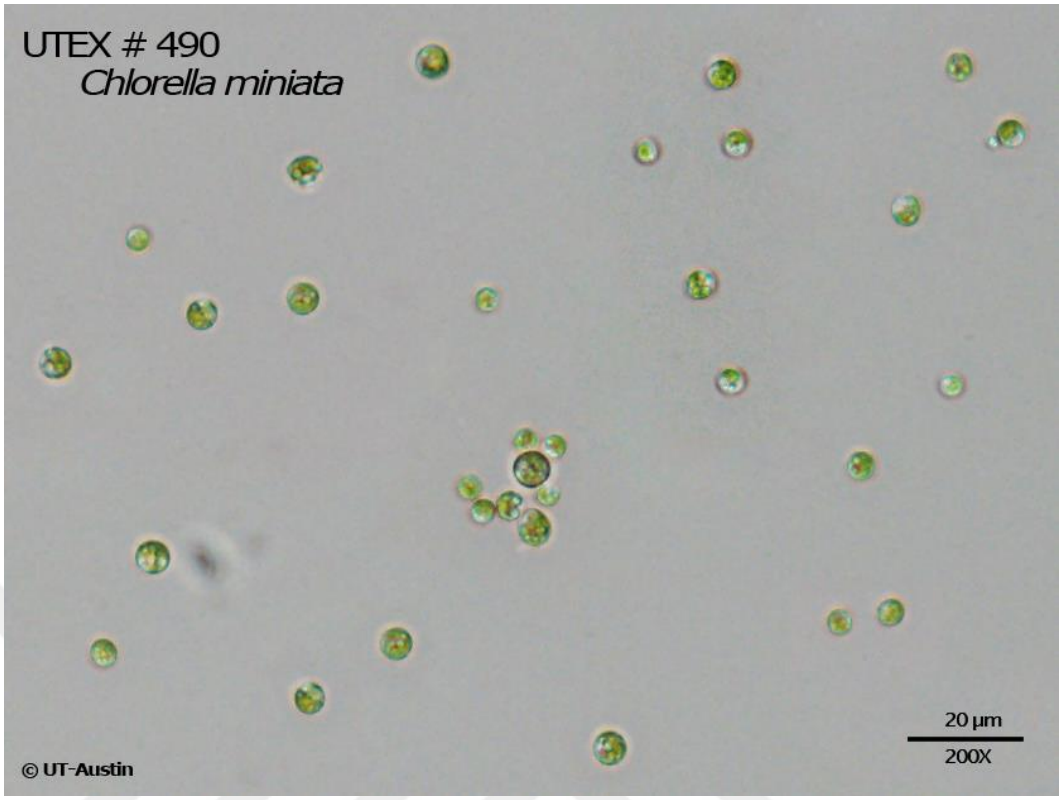


Figure 4.4. Chlorella Miniata under Microscopy from University of Texas.

Stocks were prepared for one use only, to ensure the very same content of medium at each run. 10 liter photo bioreactors were used for cultivation and utilized at 60% capacity. The aeration was provided as an airlift bioreactor. First step of the growth was the preparation of medium, afterwards the prepared medium was sealed and sterilized (at 121°C for 20 min) and stored at refrigerator temperature (+4 ^{o}C) for further use

Figure 4.5. Algae from University of Texas (*Chlorella miniata*, UTEX 490).

Figure 4.6. Culture mediums readied for inoculations.

Initialization of the culture was made in 250 ml Erlenmeyer flask than the algae were transferred to 500 ml Erlenmeyer flasks to prepare first inoculums. The inoculation; which is 5% by volume was completed in a sterilized cabin. The sterilization was done by exposing the cabinet to UV light and afterwards contacting the surface that is going to be worked on with 70% Ethanol. After the inoculation, reactors were transferred to the thermal cabinets where the cultivation took place for 21 days. The stock culture was held under constant light intensity, temperature and aeration rate conditions to obtain stock culture which were 174

Figure 4.7. The cabinet that has been used during the study.

µmol photon m^{-2} s^{-1}, 22 ^{o}C and 1 vvm respectively. The further inoculations were made using the stock culture. The parametric cultivation study was carried out in thermal cabinets at different light intensity and temperature conditions seen on Table 4.2 for 21 days of cultivation period. The aeration rate (1 vvm) and pH (6.7 before autoclave) held constant for all parameters. The cultivated microalgae cells were then harvested by a centrifuge (*rotofix 32 A*) at 4000 rpm for 7 minutes and the medium was removed with deionized water. After washing the algae with distilled water for purpose complete removal of medium, the harvested cells were stored at -20°C and until lyophilized at -52°C and 0.030 mbar, and stored at -15 ^{o}C.

Figure 4.8. The Autoclave (*HMC HM 50-L*) which was used for sterilization during the study.

Figure 4.9. The centrifuge (*rotofix 32 A*) **which was used throughout the study.**

Table 4.2. The Studied Light Intensity and Temperature Parameters.

Temperature (^{o}C)	Light intensity (µmol photon m^{-2} s^{-1})
22	66
22	112
22	174
22	236
22	275
22	310
22	385
24	236
24	275
14	174
18	174
24	174
26	174
28	174
30	174
18	310
20	310
24	310

Figure 4.10. The Inoculated Medium in the Thermal Cabinet.

Temperature of the cabinets were controlled daily by thermometers and the light intensity was calculated by the equation 4 in the unit of lux, also measurements were made by a device consisting of 6 gy-30 sensors, an ATmega 328 micro-contoller, which works at 16 MHz. The device measured the light intensity in the thermal cabinet in lux, which was later converted to μmol photon m^{-2} s^{-1}. The light was supplied by cool white fluorescent lights of 8, 18 and 24W. The conditions of the points experimented on were chosen considering the further utilization of the obtained data for modelling purposes, constant light intensity and constant temperature were maintained to monitor the sole effect of the one another. Also taking into account the information provide by previous studies done on green microalgae and other species of Chlorella by various scholars. The 18 chosen experimentation points can also be seen on Figure 4.11 below.

$$E_v = \Phi / A \qquad \textbf{equation 18}$$

Where E_v stands for the light intensity or illuminance, Φ stands for luminous flux in lumens A is the surface are in m^2. The lux value was converted to the unit of μmol photon m^{-2} s^{-1} for the sake of further calculations.

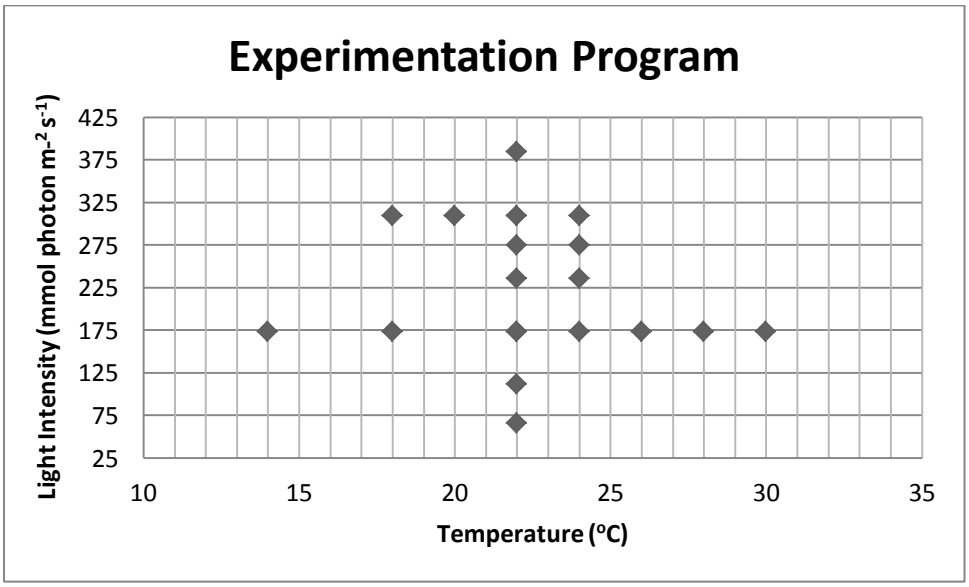


Figure 4.11. The Diagram of Studied Temperature and Light Intensity Parameters

4.1.1 Controlling the Growth of Microalgae:

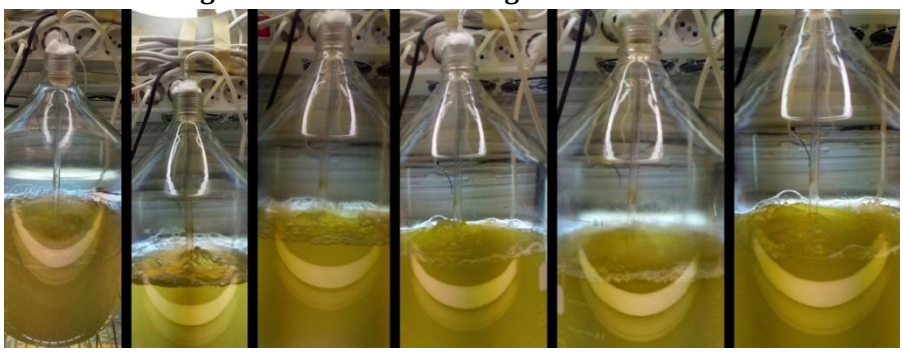


Figure 4.12. Stock growth (174 µmol photon m^{-2} s^{-1} and 22°C)on days 1, 3, 5, 10, 15 and 21.

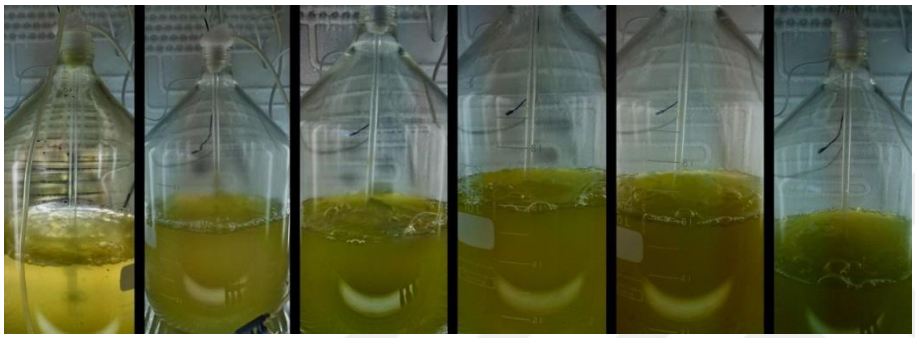


Figure 4.13. Growth at 257 µmol photon m^{-2} s^{-1} and 24°C which had one of the highest biomass yields among the studied parameters, on days 1, 3, 5, 10, 15 and 21.

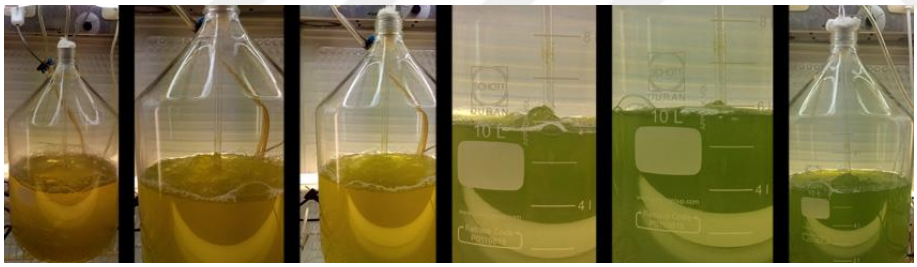


Figure 4.14. Growth at 385 µmol photon m^{-2} s^{-1} and 22°C which had one of the highest biomass yields among the studied parameters, on days 1, 3, 5, 10, 15 and 21.

Figure 4.15. Growth at 112 µmol photon m^{-2} s^{-1} and 22°C parameter 2 which had one of the lowest biomass yields among the studied parameters, on days 1, 3, 5, 10, 15 and 21.

The growth of the microalgae was controlled by means of dry cell weight, optical density and chlorophyll content. For this aim, throughout the cultivation period, 10 mL of samples had been taken from the medium under aseptic conditions and the samples were stored in refrigerator at amber conditions (Becker, 1994) until the related analyzes were done.

Figure 4.16. The Spectrophotometer (*T80+ UV/VIS Spectrophotometer*) Used for the Analyzes During Cultivation.

4.2 Control and Modelling of the Algal Growth

Cell Density/Turbidity: The samples taken from the reactor were put in to quartz vials and read in spectrophotometer at 680 and 665 nm. The results were then converted to cell density by calibration curves.

Dry Weight: 5 ml of sample was passed through a vacuum filter using dry filter papers which were weighted beforehand. Afterwards the papers let dry in the oven over night. Weighted again and the difference between the last weight and weight of the filter paper was calculated.

Chlorophyll a and b determination: 5 ml of sample was centrifuged for 10 minuntes at 4000 rpm and the medium was removed, then 5 ml methanol was added and let rest in a water bath at 100^{o}C, for an hour. Afterwards it was centrifuged at 4000rpm, for 5 minutes and spectrophotometer measurements at 665, 630, and 645 nm were applied on the supernatant. Then the following equations were used to calculate the amount of chlorophyll.

$$\text{Chlorophyll a} = 11{,}6\ D_{665} - 0{,}14\ D_{630} - 1{,}31\ D_{645}$$

$$\text{Chlorophyll b} = \text{chlorophyll b} = 20{,}7\ D645 - 4{,}34\ D665 - 4{,}42\ D630$$

$$\mu g\ \text{Chlorophyll (a,b)} / L = \text{chlorophyll (a,b)}\ v / V$$

Where v stands for the sample volume, I the path length in *cm*, V the medium volume; and D_{665}, D_{630}, and D_{645} were the measured absorbance values at the corresponding wavelengths.

Figure 4.17. Lyophilized Algae before extraction.

4.2.1 Modelling the Algal Growth

MATLAB R2015a was used to process the data obtained by the experiments with the aid of the curve fitting tool which utilizes the method of nonlinear least squares, the parameters were calculated for each equation separately.

The average relative error was calculated for each model to determine the most suitable fit. Equation 5 was used for this purpose. Where N is the total number of data, $x_{i,exp.}$ are the experimental and $x_{i,calc.}$ are the calculated values of specific growth rates.

$$Avarage\ Relative\ Error = \frac{1}{N}\sum_i \left|\frac{x_{i,exp.}-x_{i,calc.}}{x_{i,exp.}}\right| x100$$

Equation 19

4.3 Determination of Value Added Compounds in Algal Extracts

4.3.1 Extraction

The harvested and lyophilized microalgae were extracted by soxhlet extraction method. The extraction conditions were kept constant by means of solid-liquid ratio, extraction time and solvent type under boiling point of the solvent studied.

Figure 4.18. Varying temperature (Increasing from left to right, 14, 18, 24,26, 28, and 30 ^{o}C respectively.

Figure 4.19. Varying light intensity (Increasing from left to right: 112, 174, 236, 275, 310, and 385 µmol photon m^{-2} s^{-1} respectively.

Figure 4.20. The Lyophilized Algae

A solid-liquid ratio of 1:600 was used, the chosen solvent was pure ethanol and each extraction took 8 hours. The reason of selection of the solvent was preliminary studies performed in the laboratory. After the extraction period, the solvent was removed from the extract by using Hei-VAP series Heidolph rotary evaporator. The final product was solved in 15 ml of ms grade ethanol and stored at -15 ^{o}C until analyzes were done.

Figure 4.21. The Extraction of the Algae.

4.3.2 Evaporation of the Solvent

The excess solvent of the obtained extracts then had been evaporated using a rotary evaporator, the dry extracts were dissolved in 15 ml of MS grade ethanol for further analyses, ending up with a concentration of 1g algae / 30 ml ethanol. The concentrated extracts were stored in black bottles at -20 ^{o}C

Figure 4.22. The utilized rotary evaporator (Hei-VAP series, Heidolph).

4.3.3 Determination of Phenolic Compound Content

Phenolic compound content of the extracts had been analyzed by LC-MS, MS system, since the best approach for qualitative identification of phenolic compounds was considered to be LC-MS because it has had been successfully used to identify different kinds of phenolic in different biomass samples, for the well-known phenolic compounds by using the external standard method, the qualitative identification of phenolic compounds of the algal extracts were performed by using LC-MS MS system. The standards were prepared at 0,1mg/ml concentration, and standard mix including the standards given in Table 4.3 was prepared using a dilution of 1/20 and internal standard of DMAE Caffeate with same concentration, which was also added to each algae extract to determine the loss of phenolic compounds due to matrix effect. The standard curves were prepared suitable to internal standard method, response of compound to response of internal standard vs. concentration of compound as seen in Figure 4.23 below for salicyclic acid. Algae samples were diluted by 9/20 with methanol and standard mixture. A linear gradient method had been used for MetOH/Water mobile phase at a flow rate of 0.3 mL/min with Hypersil BDS C18 (250 x 4.6 mm, 5µm) column at 40 °C. The method took 5 minutes, starting with 100% water and a 1 minute wash, and then gradually increasing the amount of MetOH and continued with 100% MetOH for 3 minutes. The target phenolic compounds that were investigated were Cafeic Acid, Kaempferol, Ferulic Acid, Epicatechin, Quarcetin, Myricetin, Rutin, 4-Hydroxy Benzoic Acid, Salicyclic Acid, Trans-Cinamic Acid, Gentisic Acid, Protocatechuic Acid, Para-Coumaric Acid, Vanilic Acid , Gallic Acid, Syringic Acid, Ethyl Ferulate, CAPE, Catechin, Chlorogenic Acid, Ellagic Acid, Cyanidin, Naringenin, Delphinidin , and β-Carotene.

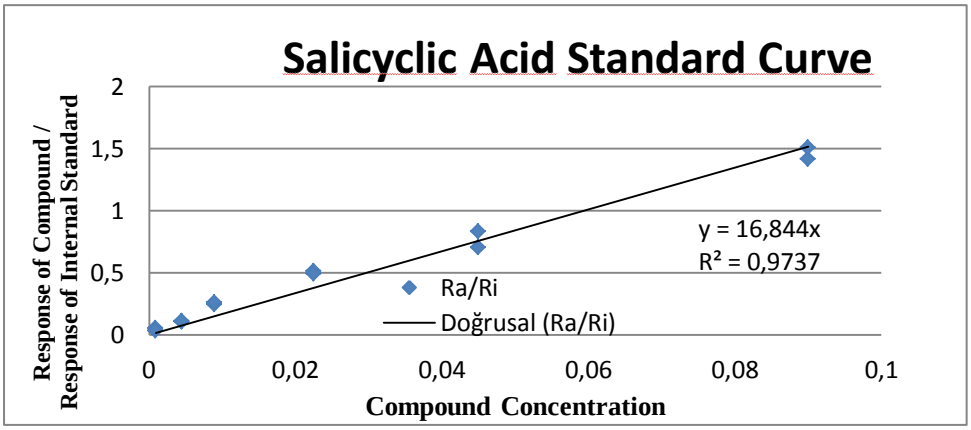


Figure 4.23. The standard curve of salicyclic acid prepared for internal standard method.

Figure 4.24. The Waters Acquity UPLC MS/MS.

Table 4.3. MRM and SIR values of phenolic compounds.

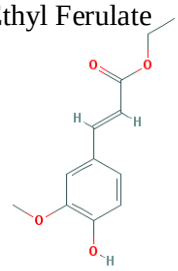
Compound	Ion Mode	Mass	Daughter	Collision	Cone Vol.
B Carotene* (Sigma 22040)	ES+	537			40
Catechol (Sigma C9510)	ES+	111	65,9	11	30
			30	11	
Caffeic Acid (Fluka 60020)	ES+	181,05	83,03	27	30
			135,09	19	
Ferrulic Acid	ES+	195	177	14	22
Kaempherol (Fluka 96353)	ES+	287	153	33	55
			165	33	40
Cyanidin (Sigma 94099)	ES+	287,1	137	20	55

Compound	Ion Mode	Mass	Daughter	Collision	Cone Vol.
Epicatechin (Fluka 68097)	ES+	291	123,5	15	30
			139,05		
			165,1		
Quercetin (Sigma Q4951	ES+	303,1	153,2	31	55
			229,2		43
Ellagic Acid (Sigma 14668)	ES+	303,1	229	30	65
Delphinidin (Sigma 43725)	ES+	303,1	229	20	55
Myrisetine (Sigma 72576)	ES+	537	321	33	46
Rutin (Merck 5.00017.0100)	ES+	611,2	303,1	20	35
			465	10	30
Trans-Cinamic Acid (Fluka 97013)	ES-	147	103	12	26
Trans-Cinamic Acid*	ES-	147			26
Gallic Acid (Sigma 91215)	ES-	168,9	124,7	20	40
Gallic Acid*	ES-	168,9			23
Myristic Acid*(Sigma 70079)	ES-	227,3			39
Maleic Acid (Sigma M0375)	ES-	115,1	71,1	10	24
Salicyclic Acid (Fluka 52341)	ES-	137,1	65	25	33
4-Hydroxy Benzoic Acid (Sigma PHR1048)	ES-	137,1	93	10	36
Gentisic Acid (Sigma 149357)	ES-	152,9	109,1	12	37
Procatechuic Acid (Fluka 03930590)	ES-	153	109	12	37
Para-Coumaric Acid (Sigma C9008)	ES-	163,1	119,1	11	30
Vanilic Acid (Fluka 68654)	ES-	167	123	10	30
			152,1	15	30
Syringic Acid (Fluka 63627)	ES-	197	167	20	30
Ethylferulate (Sigma 320617)	ES-	221,1	133,1	24	29
			206,1	17	29
1,1-DMAE Caffeat (Sigma 40785)	ES-	247	133	40	40
Naringenin (Sigma 52168)	ES-	271,1	151,1	18	32
CAPE (Sigma C8221)	ES-	283,5	134,9	27	40
			180	24	
Catechin (Sigma 43412)	ES-	289,1	245,2	16	34
Chlorogenic Acid (Sigma C3878)	ES-	353,1	191,2	22	27

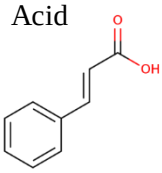
**SIR analyses were utilized instead of MRM.*

The molecular structures of the encountered phenolic compounds can be seen below. The structure itself, the quantity and position of hydroxyl groups are highly related to the antioxidant and anti-inflammatory capacity of the phenolic compounds hence the structure of the molecules have a great importance.

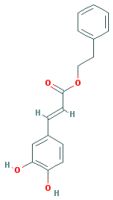
Ethyl Ferulate



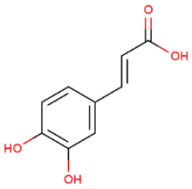
Trans-Cinnamic Acid



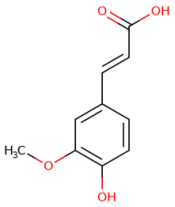
CAPE



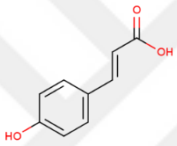
Caffeic Acid



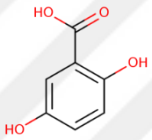
Ferulic Acid



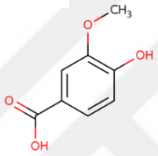
p-Coumaric Acid



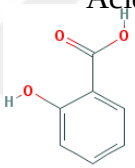
Gentisic Acid



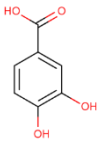
Vanillic Acid



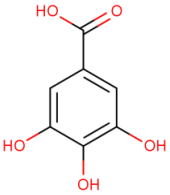
Salicyclic Acid



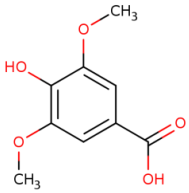
Protocatechuic Acid



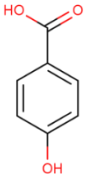
Gallic Acid



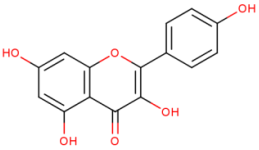
Syringic Acid



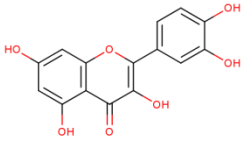
4-Hydroxy Benzoic Acid



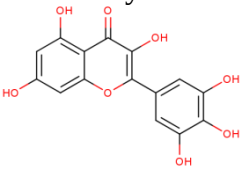
Kaempferol



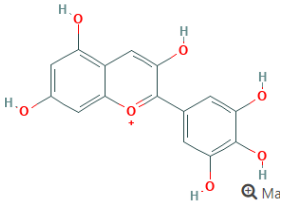
Quercetin



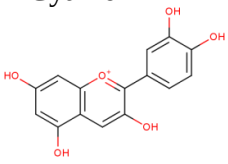
Myricetin



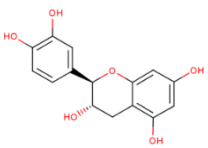
Delphinidin



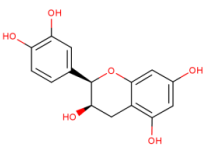
Cyanidin



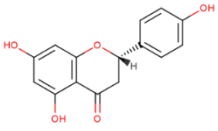
Catechin



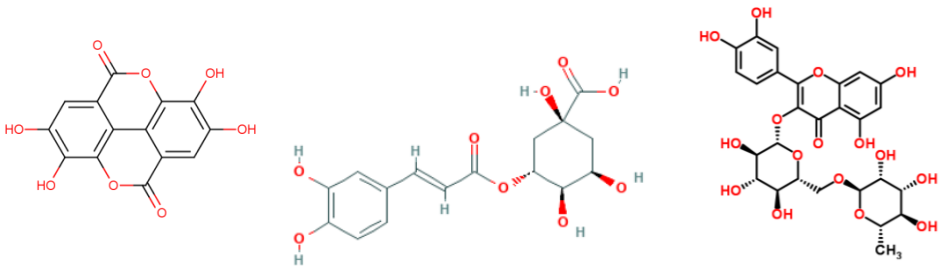
Epicatechin



Naringenin



Ellagic Acid Chlorogenic Acid Rutin



4.3.4 Determination of Antioxidant Capacity

Antioxidant activity of the extracts have been determined by using FRAP (Ferric Reducing Antioxidant Potential), TEAC (Trollox Equivalent Antioxidant Capacity) and DPPH (DiPhenyl PicrylHydrazyl) methods.

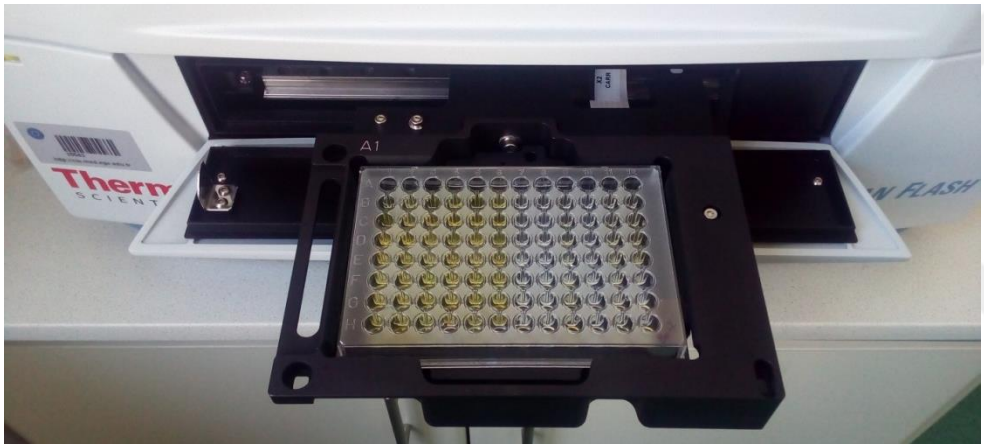


Figure 4.25. The microplate reader (Thermo Fischer, Varioskan Flash).

Trolox Equivalent Antioxidant Capacity (TEAC): A reactive which consists ABTS (Sigma Aldirch) (2,2'-azinobis3-ethylbenzthiazolinesulfonate)and potassium persulphate (Sigma Aldirch) has been diluted by phosphate buffer (Bio Basic Canada) to 7mmol/l and 4.95 mmol/l respectively and 190 µl of it was mixed with 10 µl algae extract and absorbance was read against blind samples in 734 nm wavelength in a microplate reader to determine the antioxidant potential of the extract in terms of trolox equivalent, reduction in the blue-green color had been monitored (Re et al, 1999). The steps of the assay can be tracked in Figure 4.26.

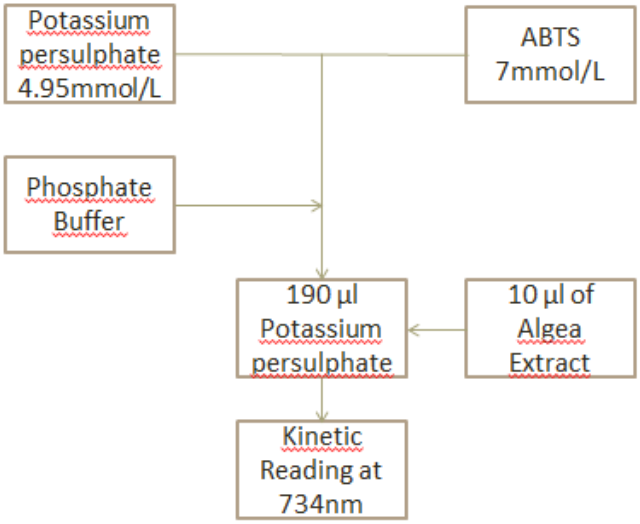


Figure 4.26 Trolox Equivalent Antioxidant Capacity assay.

Figure 4.27. The Algae Extracts before and after TEAC assay.

Total AntiOxidant (TAO): 190 µl solution of 0.1mM DPPH (Sigma Aldirch) (1,1– diphenyl-2-pikrylhydrazin) has been rapidly mixed well with 10 µl algae extract. The decline in absorbance was recorded at 550nm against blinds over a period of 20 min in 5 minute intervals in microplate reader. The decreases of absorbance or color purple corresponded to inhibiton of oxidation (Yıldırım et al, 2005). The steps of the assay can be tracked in Figure 4.28.

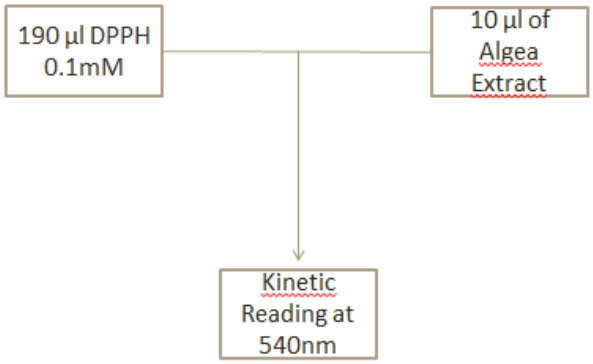


Figure 4.28 Total AntiOxidant capacity assay.

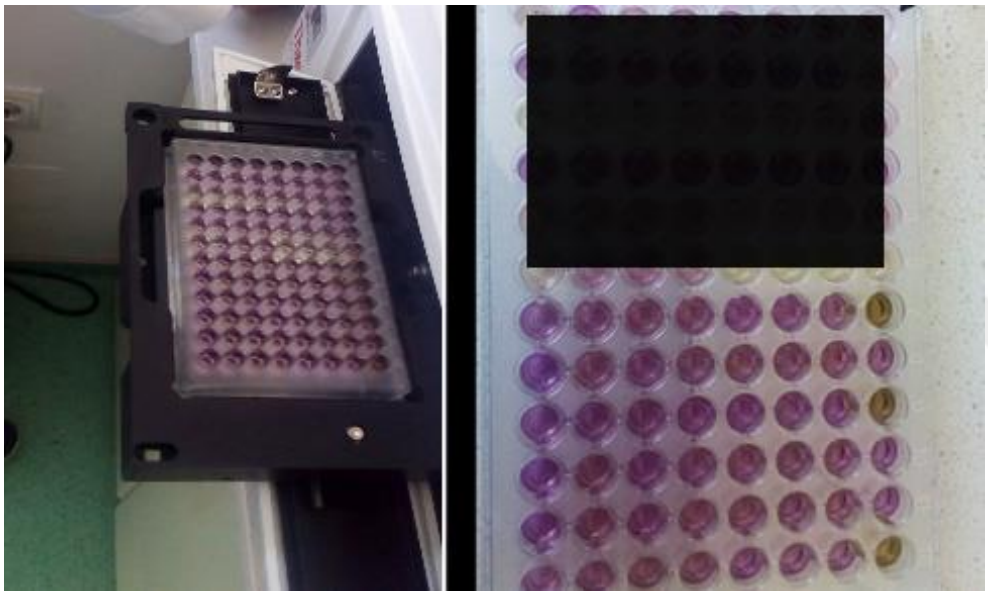


Figure 4.29. The Algae Extracts before and after TAO assay.

Ferric Reducing Antioxidant Potential (FRAP): 190 µl solution (10:1:1, v/v/v) of acetate buffer (Bio Basic Canada) (10 mM, pH=3.6), TPTZ (Sigma Aldirch) (2,4,6 tripyridyl-s- triazine) (10mM) and $FeCl_3$ (Sigma Aldirch) (20mM) has been added into 10 µl of algae extract. The mixture then was incubated at room temperature for 30 minutes results were expressed as gallic acid equivalents at 620 nm in a microplate reader and navy blue color showed oxidant activity with comparison to gallic acid standards and blinds (Pulido et al, 2000). The steps of the assay can be tracked in Figure 4.30.

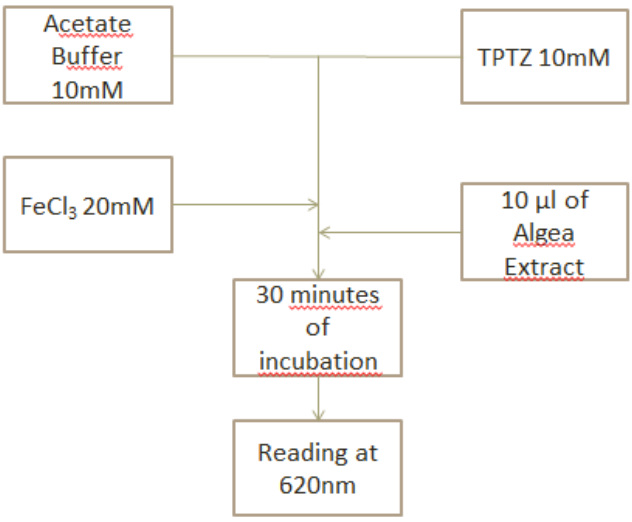


Figure 4.30. Ferric Reducing Antioxidant Potential assay.

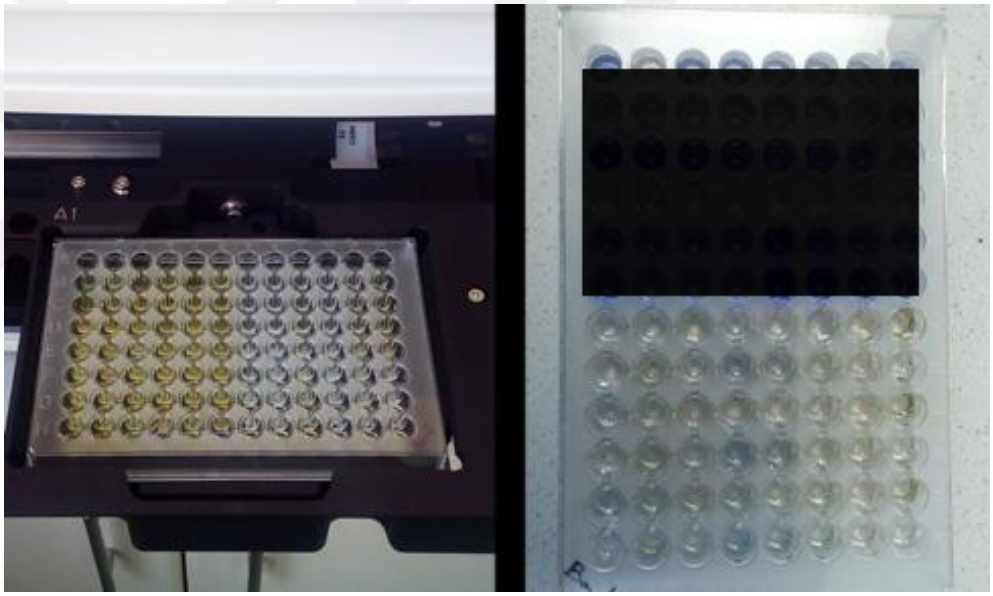


Figure 4.31. The Algae Extracts before and after FRAP method.

4.3.5 Determination of Anti-inflammatory Activity

The inhibition of hyaluronidase activity was determined using the modified method described by Silva et al. (2011). A phosphate buffer was prepared as the first step of the assay; for this purpose, Sodium phosphate (Bio Basic Canada) 0,2M, Sodium formate (Sigma Aldirch) 0,1M, Bovineserum albumin Sigma (Aldirch) 2mg/10ml, was mixed and pH was set to 6.8-7.2. The reaction mixture had been constituted by 20 µL of extract, 50 µL of phosphate buffer , 20 µL (750 units/ml) of Hyaluronidase

enzyme (Type IV-S: bovine testes, Sigma Aldirch) and 50 µL of Hyaluronic Acid (Calbiochem) 10mg/ml which was incubated at 37 °C for 30 min. (blinds without enzyme and extracts were both used.) After, incubation period, 0.1 mL of alkali borate (Alfa Aesar) 0.8 M was added. The mixture had been placed in a water bath at 100 °C for 5 minutes and 0.5 mL of p-dimethylaminebenzaldehyde (Alfa Aesar) 0,2M was added and the absorbance was measured at 580 nm using water as control (Silva et al, 2011). The appearance of violet color indicates the enzyme activity hence the decrease in coloring shows inhibition. The steps of the assay can be tracked in Figure 4.32.

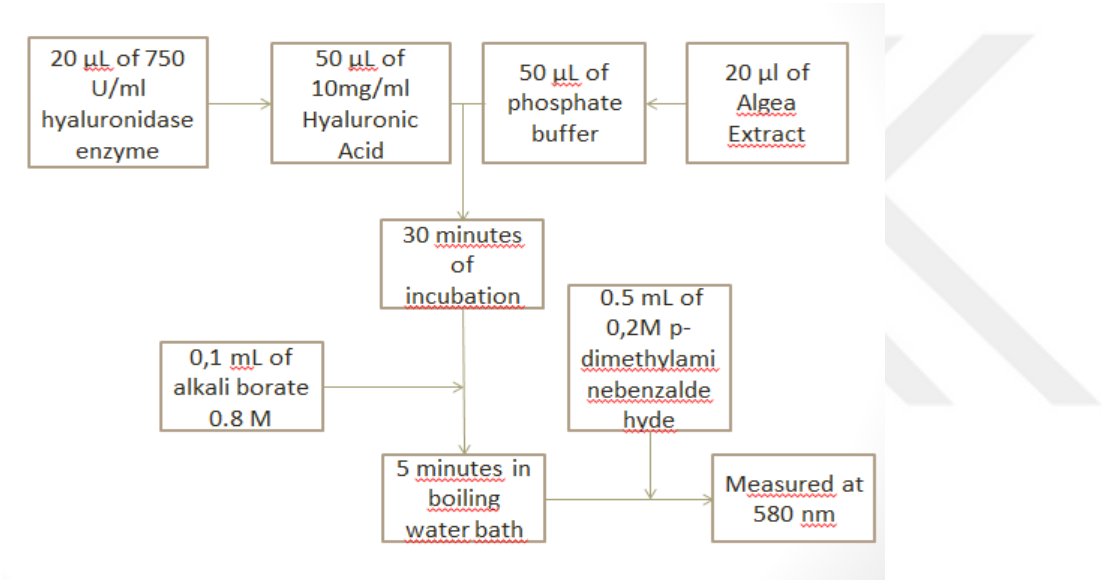


Figure 4.32. The inhibition of hyaluronidase assay.

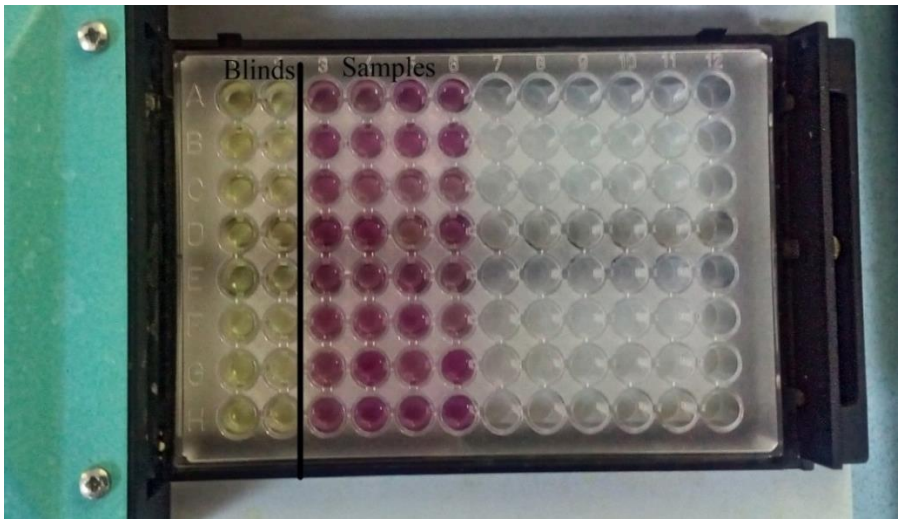


Figure 4.33. The Algae Extracts before and after Hyaluronidase Inhibition method.

A modified xanthine/xanthine oxidase method introduced in the study by Tan et al (2000) also was utilized. Xanthine (Sigma Aldirch) was dissolved in 0.05 M NaOH and adjusted to 10 mM. After the addition of Algae extract (20 40 or 60 µL dosage) and 80 µL of 500 µM WST-1 (trc Canada) solution, (blinds without enzyme and extracts were both used.) it's left for incubation for 15 min at 37°C. Then 10 µL of 0,05 U/ml xanthine oxidase (Sigma Aldirch) was added and the measurements were carried out at 450nm (Tan et al, 2000). The decrease in absorbance indicated the inhibition. The steps of the assay can be tracked in Figure 4.34.

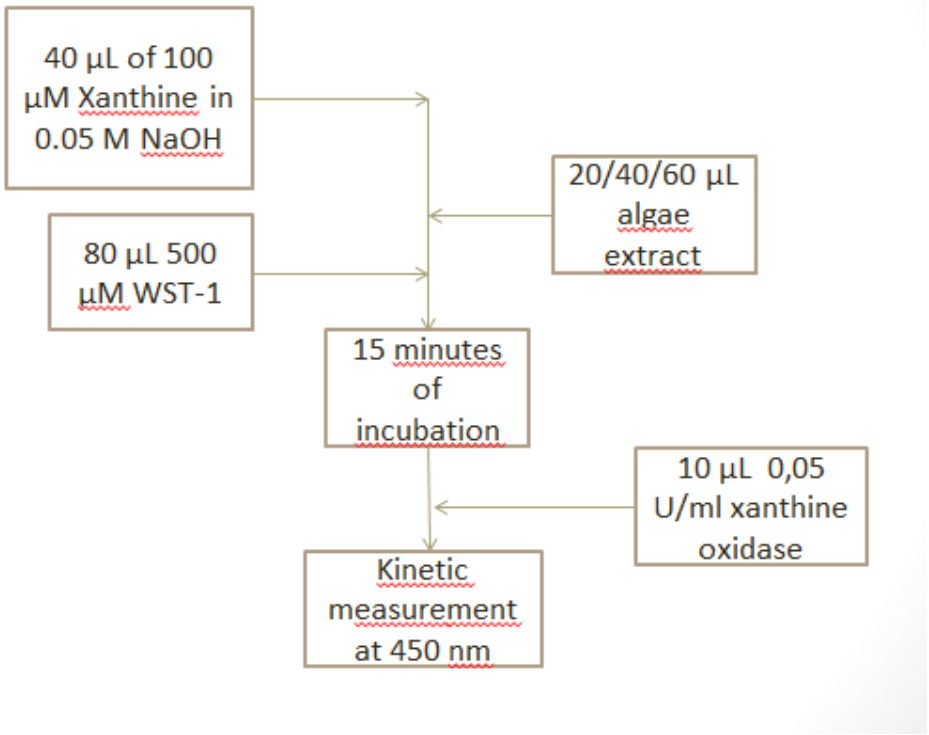


Figure 4.34. Xanthine/xanthine oxidase inhibition assay.

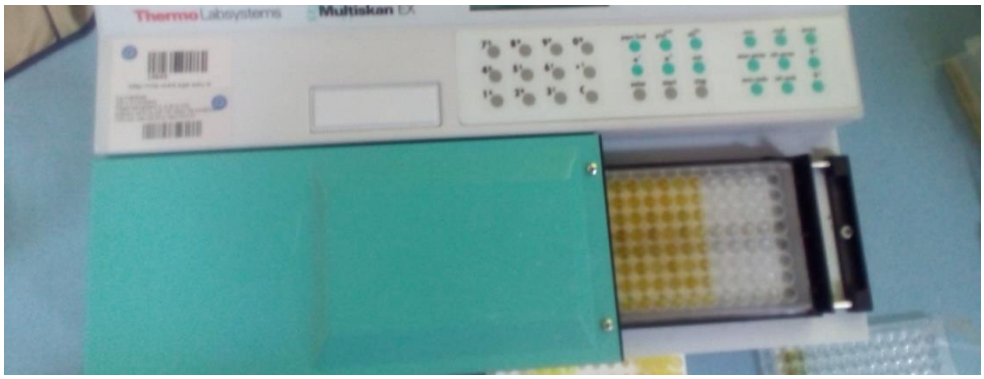


Figure 4.35. The Algae Extracts during Xanthine/xanthine oxidase Inhibition method. (Read in Thermo Labsystems, Multiskan EX)

5. Results and Discussion

5.1 Cultivation

Cultivation was the first step of this study. The result can be seen on figures 5.1 and 5.2. The percentage standard deviation of the final dry biomasses had been calculated to be 2.19%.

The highest specific growth rate found to be 0,1392 under the conditions of 24 ^{o}C temperature and 310 µmol photon m^{-2} s^{-1} light intensity. Compared to other algae species studied by various scientists (Giannelli et al. 2015, Nishikawa and Yamaguchi, 2008, Nishikawa and Yamaguchi, 2008, Nishikawa and Yamaguchi, 2006, Navarro et al. 2006, Dechatiwongse et al. 2014, Converti et al. 2009, Imaizumi et al. 2016, Krzeminska et al . 2015, Ota et al. 2015, Chang et al. 2016, Aleya et al. 2011,D el Campo et al. 2004, Chen et al. 2010) *Chlorella Miniata* exhibited a higher specific growth rate than most at its peak only lower than *C. Vulgaris*, *C. Zofingiensis*, and *C. Protothecoide.*

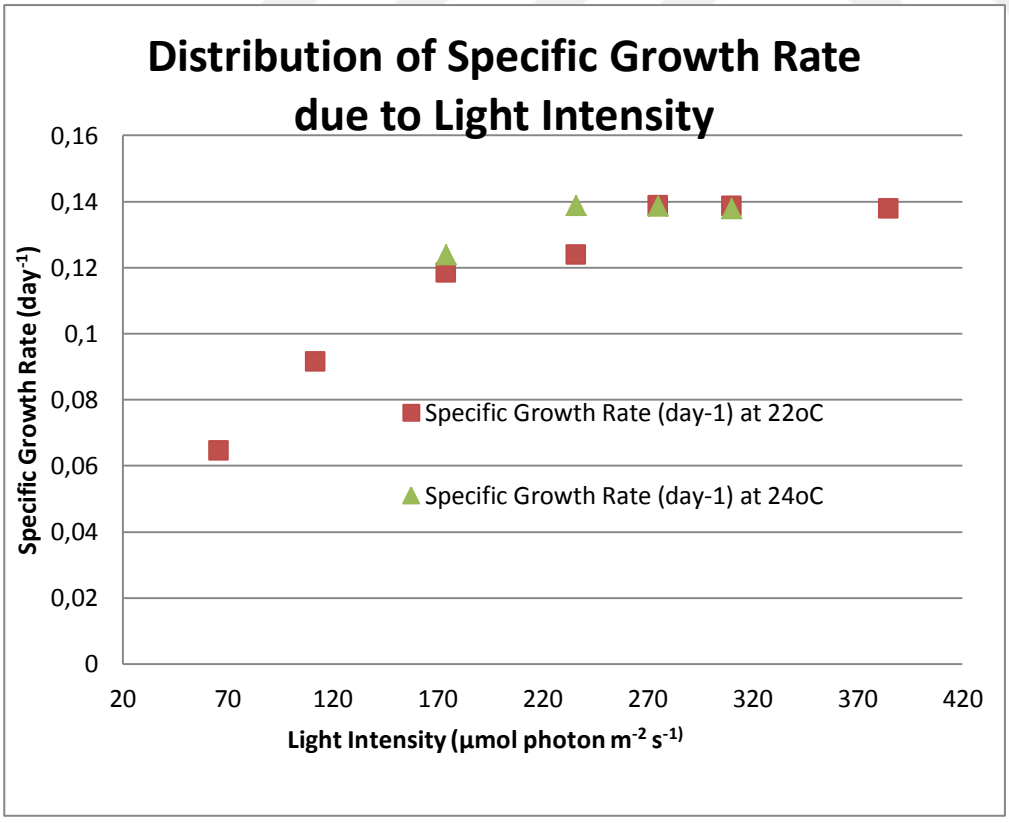


Figure 5.1. Effect of light intensity on algae growth.

The experiments run under varying light intensities showed that the Algae favors light intensities higher than 275 µmol photon m^{-2} s^{-1}. Also there weren't any indications of photo-inhibition in the studied light intensity range. Although after the light saturation point at 275 µmol photon m^{-2} s^{-1}, increasing light intensity has none to very little influence on specific growth rate. The algae growth decreased dramatically with decreasing light intensities and growth of the algae at the lowest light intensity value found to be less than half of the growth rate at light saturation zone. The study done by Chen et al. (2010) also stated that the *Chlorella sp.* had a similar light saturation point while lower light saturation points were also encountered before (Krzeminska et al . 2015 and Chang et al. 2016)

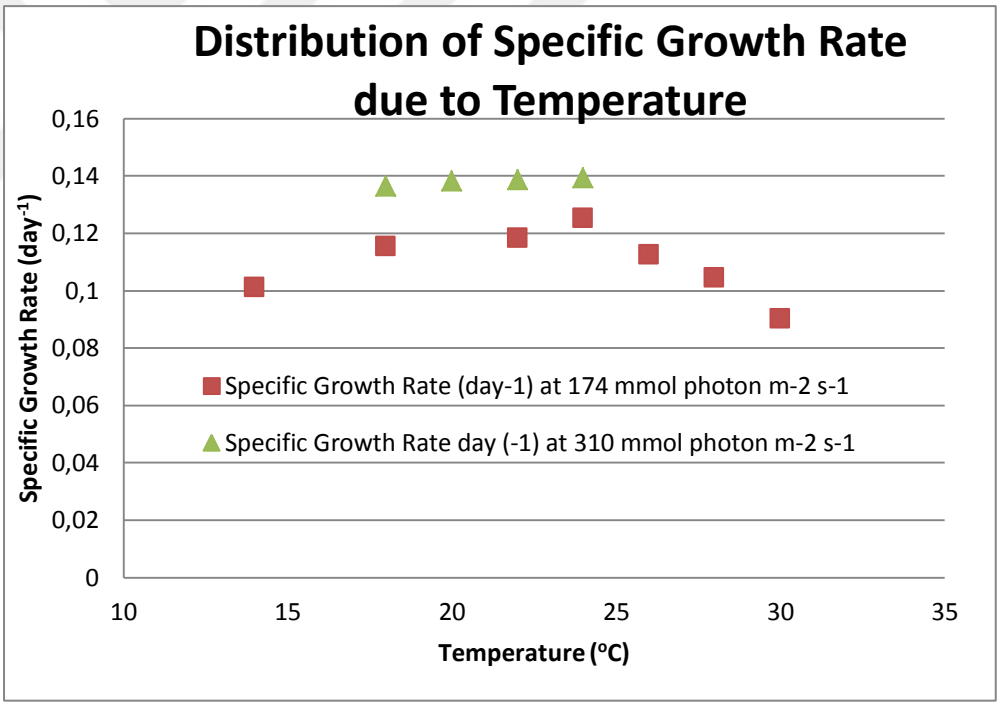


Figure 5.2. Effect of temperature on algae growth.

The experiments run under varying temperatures showed that the Algae favors temperatures between 22-26 ^{o}C. The highest specific growth rate was calculated at 24 ^{o}C and the growth rate decreases with increasing and decreasing temperatures, drastically. Converti et al. (2009) also stated in a study on *C. Vulgaris*, that the strain tends to have a higher growth rate

between temperatures of 25-30 ^{o}C. Increasing the light intensity had an effect on the growth rate which decreased the amount of influence of temperature on specific growth rate, temperature still affected the growth however the change in the growth rate was minimal in the 18-24 ^{o}C temperature range.

On Table 5.1 the productivities belonging to different *Chlorella* species obtained by various studies, are listed. The productivity of algae are in between 0,008 and 0,113 g l^{-1} d^{-1}, calculating a mean value of 0,05 g l^{-1} d^{-1}. The highest productivity of *Chlorella miniata* in range of this study was found to be 0,016 g l^{-1} d^{-1}, which was lower than the average productivity value of the listed species' productivities.

Table 5.1. Comparison of productivities of various *Chlorella* species with this study. [a] *Not Mentioned.*

Species	**Temperature (^{o}C)**	**Light Intensity (µmol photon m^{-2} s^{-1})**	**Culture Mode**	**Productvity (g l^{-1} d^{-1})**	**Reference**
Chlorella prototheco ides	26	35	Batch	0,085	Krzeminska et al, 2015
Chlorella prototheco ides	26	130	Batch	0,091	
Chlorella prototheco ides	26	420	Batch	0,090	
Chlorella vulgaris	27	70	Continuous	0,008	Chang et al, 2016
Chlorella vulgaris	27	120	Continuous	0,021	
Chlorella vulgaris	27	180	Continuous	0,021	

Species	**Temperature (°C)**	**Light Intensity (µmol photon m^{-2} s^{-1})**	**Culture Mode**	**Productvity (g l^{-1} d^{-}1)**	**Reference**
Chlorella sp. Marine	30	42	Batch	0,017	Cheirsilp and Torpee, 2012
Chlorella sp. Fresh	30	42	Batch	0,012	
Chlorella vulgaris ESP-31	25	60	Batch	0,040	Ye and Chang, 2012
Chlorella vulgaris	Room	N.M.[a]	Batch	0,013	Liang et al, 2009
Chlorella sp.	25	70	Batch	0,020	
Chlorella sp. L1	Pond	Pond	Batch	0,073	He et al, 2016
Chlorella sorokiniana H2	Pond	Pond	Batch	0,051	
Chlorella sp. L1	Pond	Pond	Semi-continuous	0,088	
Chlorella sorokiniana	25	100	Batch	0,113	Li et al, 2014
Chlorella sorokiniana	37	100	Batch	0,045	
Chlorella vulgaris	30	70	Batch	0,100	Abreu et al, 2012
Chlorella minutissima	30	33,75	Continuous	0,100	Freitas et al, 2017
Chlorella minutissima	30	16,88	Continuous	0,040	
Chlorella minutissima	30	8,44	Continuous	0,030	
Chlorella miniata	24	310	Batch	0,015	**This Study**

5.2 Modelling of Algal Growth

The modelling of the algal growth was essential to be able to interpret the relation between cultivation temperature, light intensity and specific growth rate of the algae. In this manner a variety of mathematical models were used and the ones with the more accuracy and precision were chosen to describe a three dimensional mathematical model to represent the behavior of the algae species in varying temperature and light intensities during cultivation.

The goodness of the fit was measured by calculating the average relative error (avre < 0,05). R-squared which was a statistic that often accompanies regression output, was not included in this study because of its tendency to increase with every new explanatory variable. Ford stated that R-squared does not measure goodness of fit, especially when comparing mathematical models and the goodness of their fit. Also it was not possible to explain the interactions and predictive error with R-squared. (Ford, 2015) Spiess and Neumeyer evaluated the validity of R-squared on studies including nonlinear models in pharmacological and biochemical researches. The results were promoted Ford's statement, after intensive study the researcher concluded that R-squared performs underwhelming in terms as a basis for mathematical model fit goodness and stated that researchers should ideally remove it from scientific literature including nonlinear model fitting (Spiess and Neumeyer 2010).

5.2.1 Light Intensity Dependent Models

The models all represented decent fits to the experimental values but Aiba Model had the lowest average relative error (avre) among the studied mathematical models. This described the increase of growth rate with the increasing light intensity at the lower light intensity values than the light saturation point and also mirrored the static condition of growth after the point of light saturation. Since there was no encounter with photo-inhibition during any of the runs, it can be easily said that Aiba Mathematical Model was the most proper mathematical model for this study.

Table 5.3. The light intensity - specific growth rate mathematical model results.

	Equations	**μ_{max}** (d^{-1})	**K_i** (µmol photon m^{-2} s^{-1})			**% avre**
Monod	$\mu = \mu_{max} \frac{I}{K_I + I}$	0,1954	127,3			2,95
		μ_{max} (d^{-1})	**I_k** (µmol photon m^{-2} s^{-1})			
Poisson	$\mu = \mu_{max}(1 - \exp(-\frac{I}{I_k})$	0,1456	110,5			2,33
		μ_{max} (d^{-1})	**I_k**(µmol photon m^{-2} s^{-1})			
Hyperbolic Tangent	$\mu = \mu_{max} \tan h\left(\frac{I}{I_k}\right)$	0,1389	137,7			2,40
		μ_{max} (d^{-1})	**I_{opt}** (µmol photon m^{-2} s^{-1})			
Extended Hyperbolic Tangent	$\mu = \mu_{max} \frac{I}{I_{opt}} \exp(1 - \frac{I}{I_{opt}})$	0,1396	325,7			1,94
		μ_{max} (d^{-1})	**I_k** (µmol photon m^{-2} s^{-1})	**I_c** (µmol photon m^{-2} s^{-1})	**K**	
Modified Hyperbolic Tangent	$\mu = \mu_m \frac{\tanh(\frac{I}{I_k})}{1 + K(I - I_c)}$	0,1697	176,2	27,07	0,000559	2,52
		μ_{max} (d^{-1})	**k_s** (µmol photon m^{-2} s^{-1})	**k_i** (µmol photon m^{-2} s^{-1})		
Aiba	$\mu = \frac{\mu_{max} I}{k_s + I + \frac{I^2}{k_i}}$	0,3897	320,9	387,4		1,68
		E (day/m)	**I_c** (µmol photon m^{-2} s^{-1})	**L** (m)		
Linear	$\mu = \frac{\ln(\frac{I}{I_c})}{EL}$	0,2358	14,22	95,17		3,65

Collins and Boylen (1982) applied the Monod Model on *A. variabilis*, resulting a K_i value of 32,05 µmol photon m^{-2} s^{-1} and a µmax value of 0,098 d^{-1}, both not remote from the values calculated in this study. Although the maximum growth rate was encountered at 35°C and 564 µmol photon m^{-2} s^{-1} in their study which was a far larger value than the one obtained in this study and this maybe the cause of an inferior fit, especially at higher light intensities despite the low avre value of monod model, there were far better models in terms of describing the algal growth. (Collins and Boylen,1982). On the other hand Kurano and Miyachi, used the same mathematical model on a study on *C. littorale* and reached the values of 0,134 h^{-1} and 95,8 µmol photon m^{-2} s^{-1}, both approximate to the previous study, and again the study had higher values of light intensity as 1200 µmol photon m^{-2} s^{-1} (Kurano and Miyachi, 2005).

Kurano and Miyachi, using the Poisson model in their study along with other models, and modeling the growth of *C. littorale* found the coefficients of the model to be 0,116 h^{-1} and 114 µmol photon m^{-2} s^{-1}, μ_{max} and I_k. The results of light saturation were quite close to each other but the maximum specific growth rates differed greatly, this was the predictable consequence of the differences in average specific growth rates of two algae. It can be anticipated that this mathematical model would be more suitable for microorganisms that have a higher specific growth rate than the one which was cultivated in this study. (Kurano and Miyachi, 2005). Sakshaug et al. studying a mathematical model derived from Poisson model on *Thalassiosira nordenskioldii* and *Chaetoceros furcellatus* for growth periods of two days and attaining the results of I_k 31–36 vs 49–100 µmol m^{-2} s^{-1} at low and high light intensities respectively also endorses the prior anticipation (Sakshaug et al, 1991).

A study done by Ota et al. in 2015 also utilized the hyperbolic tangent mathematical model on growth of *C. littorale*, the coefficients of the model was found to be; μ_{max} of 0,106 h^{-1} I_k of 56,5 μmol m^{-2} s^{-1} and an avre value of 8,34% which was not the preferred fit in that study. (Ota et al, 2015). Kurano and Miyachi included the same model in their study in 2005, for the same species. Result were μ_{max} of 0,115 h^{-1} I_k of 150 μmol m^{-2} s^{-1}, the study also stated that the equation was mostly the best fit for photosynthesis (P) – light intensity (I) relationship but also the relation between μ-I expressions differ from common P-I expressions. (Kurano and Miyachi, 2005). Ota et al. also studied the Extended Hyperbolic Tangent model, which was an inferior fit compared to the Hyperbolic Tangent model in the same study, with coefficients of μ_{max} of 0,108 h^{-1} I_k of 140 μmol m^{-2} s^{-1}, but the models' (both extended and modified hyperbolic tangent models) inclination to reduce the calculated value of specific growth rate with increasing temperature especially above the value of twice the light saturation and the aim of this study not being to note the light inhibition point but to determine the point of maximum growth in terms of light intensity, made those models undesirable. While they may be better choices in cases of light inhibition, in this study they describe an unsought area by experiments.

Dechatiwongse et al. utilized the Aiba mathematical model for the modelling of Cyanothece sp growth. Resulted a μ_{max} value of 0,552 d^{-1} and a light saturation value of 347 μmol m^{-2} s^{-1}, although the light inhibition term of that study was found to be much higher than this study it can be tied up to the lower light intensity range studied in this study and due to the lower value of light inhibition term this model describes the light intensity – specific growth rate relationships more vaguely, which was desirable considering the lack of data on those points. (Dechatiwongse et al, 2014). The result calculated by the mathematical model and the experimental results can be tracked on Figure 5.3.

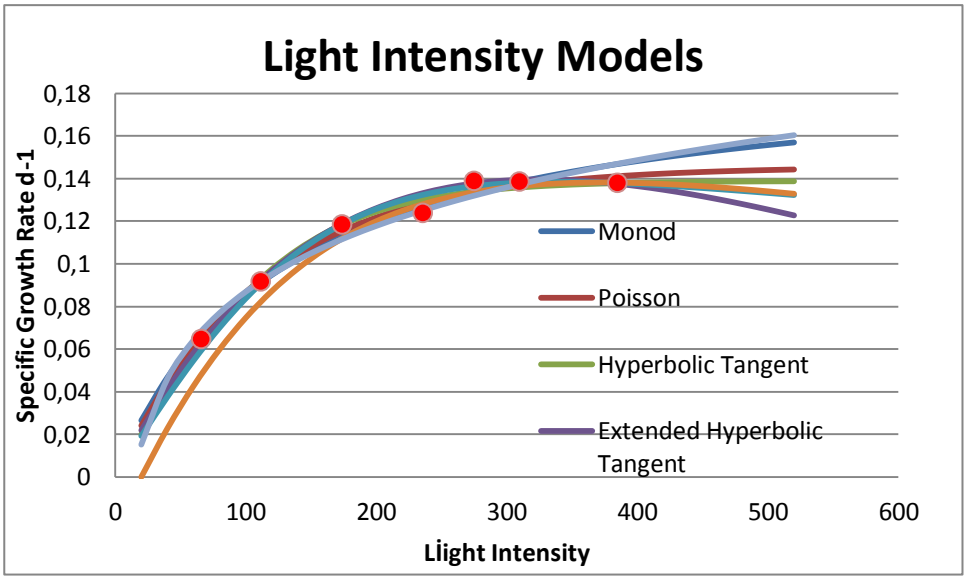


Figure 5.3. Results calculated by Light Intensity Mathematical Model in Comparison with Experimental Results.

Lastly the linear mathematical model, described the increase of specific growth rate due to the increase in light intensity well but after the point of light saturation the fit was poor, unlike the other mathematical models the linear mathematical model lacks the ability to portray the behavior of growth where the acceleration increment decreases and the change of specific growth rate approaches zero. Also it has been stated that the linear mathematical model differs in results with differences of geometry of the utilized photo-bioreactor. (Ogbonna et al. 1995)

5.2.2 Temperature Dependent Models

The Square Root Model didn't display a suitable equation, since it was more acceptable in result which have a more linear trend, or in other words in between the optimum growth temperature borders. Other than that each mathematical model represented a decent result. Sinclair Model had the lowest average relative error (avre) among the studied mathematical models.

Table 5.4. The temperature - specific growth rate mathematical model results.

	Equation	**μ_{max} (d^{-1})**	**T_{opt}(^oC)**	**σ**		**% avre**
Skewed Normal Distribution	$\mu = \mu_{max} e^{\frac{-(T-T_{opt})^2}{2\sigma^2}}$	0,1208	21,3600	0,0814		1,74
		b_2 ($^oC^{-1}$ $h^{-0.5}$)	**T_{min} (^oC)**	**c_2 ($^oC^{-1}$)**	**T_{max} (^oC)**	
Expanded Square Root Model	$\mu = (b_2(T - T_{min})[1 - e^{c_2(T-T_{max})}])^2$	0,8946	0,8775	0,0057	39,2400	7,41
		γ_{max} (day^{-1})	**θ_0 (^oC)**	**θ_{opt} (^oC)**	**z**	
Parker	$y = \gamma_{max}(\frac{T}{\theta_{opt}} w^u)^z$	2,978	0,0266	21,28	-4420	6,30
		E_a (kJ/mol)	**A** (h^{-1})	**E_b** (kJ/mol)	**B** (h^{-1})	
Sinclair	$\mu = Ae^{\frac{-E_a}{RT}} - Be^{\frac{-E_b}{RT}}$	52,110	5,19E+10	57,380	4,03E+11	1,44

A recent study by Gonçalves et al investigated the growth of *C. vulgaris*, where the skewed normal distribution mathematical model was used and the acquired model coefficients were μ_{max} 1,30 d^{-1}, T_{opt} 25,4 ^oC, and σ 7 ^oC. (Gonçalves et al, 2016)

Ratkowsky et al. determined a temperature interval of growth for a variety of species where T_{min} values were between -7 and 30 ^oC and T_{max} values were between 37 and 47 ^oC, both values were coherent with this study. (Ratkowsky et al, 1983) Another study by Zwietering et al, utilized the same model resulting coefficients; T_{min} of 2,82 ^oC, T_{max} of 44,9 ^oC, b_2 of 0,0377 $^oC^{-1}$ $h^{-0.5}$and c_2 of 0,250 $^oC^{-1}$, the temperature values were coherent with this study but the constants b_2 and c_2 calculated to be distant. (Zwietering et al, 1991) Overall the average relative error was greater than other mathematical models studied, and further utilization of this model wasn't desirable.

Another study that had been done in 1991 by Talbot et al investigated growth of three species and the relationship of growth with temperature. Applying the Parker mathematical model, the coefficients acquired were: for *Oscillatoria agardhii*, γ_{max} 0,5 day^{-1}, θ_{opt} 37 ^oC; for *Ankistrodesmus falcatus* γ_{max} 1,1 day^{-1}, θ_{opt} 37 ^oC,; and for *Phormidium bohneri* γ_{max} 1,7 day^{-1}, θ_{opt} 37 ^oC. (Talbot et al, 1991) The results obtained during the course of this study sustained the problem Talbot et al encountered; the higher number of coefficients which was the probable cause of the higher avre value of this mathematical model.

The Sinclair mathematical model resulted the smallest avre value compared to the other temperature related mathematical models, the study by Perez et al studied the temperature – gowth relations of the species *P. tricornutum*, and found the coefficients of the mathematical model; E_a to be 117,23 kJ/mol, A to be 0,26 h^{-1}, E_b to be 163.28 kJ/mol, and B to be 0,18 h^{-1} expending the model and addition of the term T_0 (assuming it to be 25 ^oC) resulted in much lower values of A and B in this study. (Perez et al, 2008). On the other hand Zwietering et al used the very same mathematical model as this study and estimated the coefficients of the mathematical model; E_a to be 107,2 kJ/mol, A to be 1,249E+21 h^{-1}, E_b to be 107,4 kJ/mol, and B to be 1,319E+21 h^{-1}. These values were in coherency with this study however the model stands not credible because of the close values of E_a and E_b resulting a subtraction of two large values to calculate the growth rate.

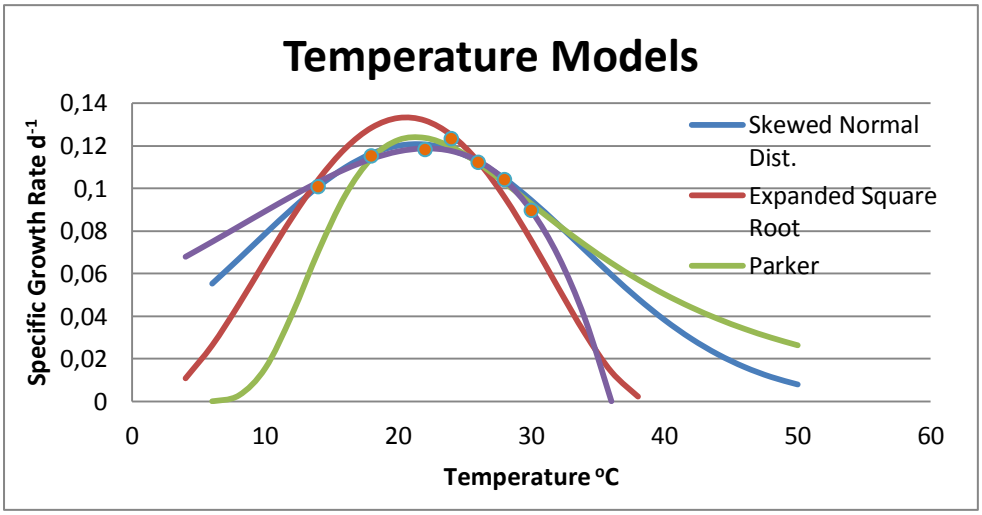


Figure 5.4. Results calculated by Temperature Mathematical Model in Comparison with Experimental Results.

5.2.3 Modelling the Combined Effect of Temperature and Light Intensity

Since Aiba Model and Skewed Normal Distribution Model were able to reach a lower avre value and reasonable coefficient values, coupling them to describe the specific growth rate as a function of temperature and light intensity unearthed a mathematical model that mimics the growth of the studied species with a very low average relative error. The two variable models also returned a relatively low avre as expected. The coefficients can be tracked on Table 5.5.

$$\mu = \mu_{max} e^{\frac{-(T-T_{opt})^2}{2\sigma^2}} \frac{I}{k_s + I + \frac{I^2}{k_i}} \qquad \textbf{Equation 20}$$

Table 5.5. The results of Aiba and Skewed Normal Distribution model.

T_{opt} (°C)	k_i (µmol photon m^{-2} s^{-1})	k_s (µmol photon m^{-2} s^{-1})	μ_{max} (d^{-1})	σ	%avre
21,38	291,5	390,1	0,46	0,0793	1,95

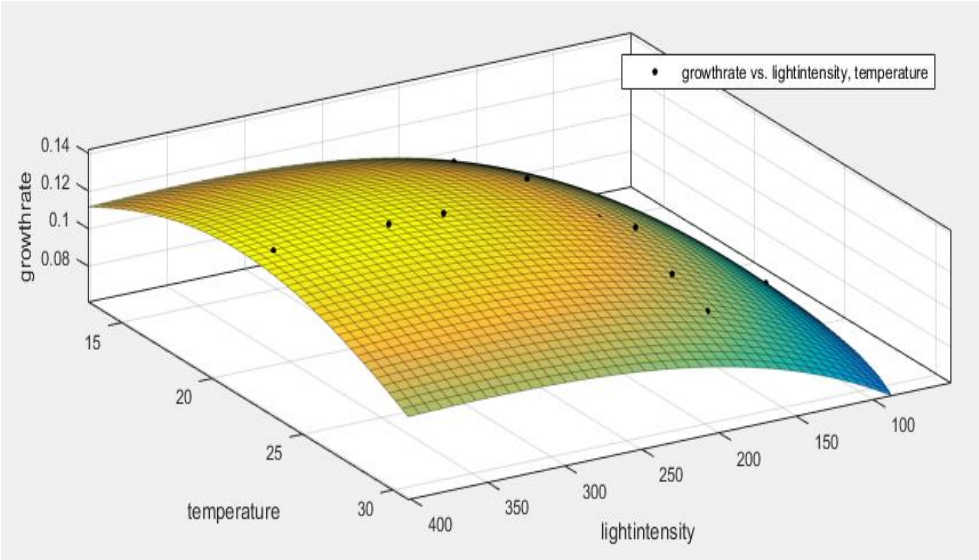


Figure 5.5. The 3-d fit of the Aiba and Skewed Normal Distribution model.

5.3 Economic Analyzes of the Algae

As Laurens stated in the State of Technology Review in 2017, dried *Chlorella* biomass has a mean market value of $20-40/kg. (Laurens, 2017) In this study a kg of dry Chlorella miniata biomass calculated to cost 15,26$ about 90% of this cost was the electricity used for illumination and heating/cooling. Assuming a $20 market price, the laboratory produced *C. miniata* will have a profit margin of, 31,15%, assuming the median market price the margin increases to 96,72%.

Table 5.6. Prices of the chemicals used in the growth medium.

Chemicals	**Price in $ per ton of Chemical**	**Company**
$NaNO_3$	350	Kemele Chem.
$CaCl_2 \cdot 2H_2O$	80	Fresice Chemical
$MgSO_4 \cdot 7H_2O$	75	R.S. Maxunite
K_2HPO_4	800	J. S. Kolod
KH_2PO_4	800	J. S. Kolod
NaCl	460	Crownsue Co., Ltd.
Protease Peptone	1300	Shandong Guanghao Biological Products Co.

5.4 Extraction Yield of the Algae

The extraction yields of the algae grown under different conditions can be seen in Table 5.6, the yield shows little change with growth conditions though a slight increase can be observed with increasing light intensity. Previous studies also suggest a total extraction yield of 12,25% - 15,86% by ethanol. (Rulong et al, 2012). Another study by Plaza et al, resulted a total extraction yields of 13,20% both studies appear to be coherent with this study (Plaza et al, 2010).

Table 5.7. The extraction yields of the algae.

Temperature (^{o}C)	**Light Intensity (**µmol photon m^{-2} s^{-1})	**Total Extraction Yield %**
14	174	14,81
18	174	13,44
22	174	13,6
24	174	14,59
26	174	14,60
28	174	13,30
30	174	14,36
22	66	13,04
22	112	13,29
22	174	13,61
22	236	14,14
22	275	15,67
22	310	16,34
22	385	16,96
Avarage extraction yield		14,41

5.5 Phenolic Compound Contents of the Algae Extracts

The Lc MS/MS method was studied for 29 phenolic compounds in total, and the compounds with significant amounts are given in the following tables. The method of internal standard were used for determination of the phenols, DMAE Caffeate was used as the internal standard.

Miranda et al. (2001) stated that phenolic compounds salicylic, trans-cinnamic, chlorogenic, and caffeic acids were found in the *Chlorella sp.* extract. The results of this study also confirms that *C. Miniata* also contains high amounts of Salicyclic, Trans-Cinnamic, and Caffeic Acid but the concentration of Chlorogenic Acid were found to be lower compared to other three.

A study by Mahcu et al. in 2014, revealed that *Chlorella Pyrenoidosa*, contains the phenols Gallic Acid and 4-Hydroxybenzoic Acid, amounts of 5 µg/g dry algae and 20,5 µg/g dry algae respectively. Although those phenols were proved to be included in *Chlorella Miniata* too, their amounts were lower compared to *Chlorella Pyrenoidosa*.

5.5.1 Phenolic Acids

Phenolic acids are formed of a benzene ring and a carboxylic acid group, the number and the position of the hydroxyl group in the molecule differ the structure of the Phenolic acids whereas the molecule structure is given below in Figure 5.6 (Goleniowski et al, 2013).

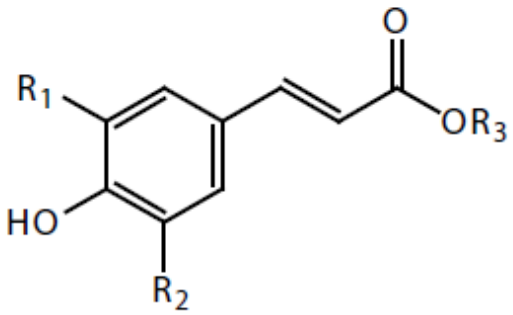


Figure 5.6. Phenolic Acid molecular structure.

5.5.1.1 Hydroxicinnamic Acid Derivatives

Because of the hydroxyl group in the Hydroxycinnamic Acid Derivatives, they are far better hydrogen donors compared to their Phenylacetic Acid relatives. The antioxidative influence of the Hydroxycinnamic Acids however are decreased by the very same reason that increases the influence of the latter, the dihydroxlitaion of 3,4 position. This effect almost equalizes both, for example: the antioxidant activity of Protocatechuic Acid is almost the same as Caffeic Acid but the conversion of Caffeic Acid th Ferulic Acid by substitution reaction increases the antioxidant capability of the compound, as a rule of thumb, it should be taken into account that Acrylic Acid group which appears in Hydroxycinnamic Acid derivatives, provides a higher stabilization ability than Benzoiz Acids. (Rice-Evans et al. 1995)

The hydroxycinnamic acid derivatives that had the highest response were T-Cinnamic, Caffeic and Ferulic acids, while Caffeic acid had its peak at low light intensities such as 66 and 112 μmol photon m^{-2} s^{-1} and temperatures between 14-22 ^{o}C, Ferulic acid results were higher around light intensity point and slightly higher temperatures. Although other compounds; Ethyl Ferulate, CAPE, and p-Coumaric acid were present their quantity were a lot lower than Caffeic acid and Ferulic acid. On the other hand T-Cinnamic Acid had higher contents at border temperatures and peaked at 310 μmol photon m^{-2} s^{-1} of light intensity. Although the extraction resulted in low levels of vanillic acid, its coherency with antioxidant activity made it relevant.

The highest yields of Hydroxycinnamic Acid Derivative results of the Lc MS/MS study can be tracked on Figure 5.7.

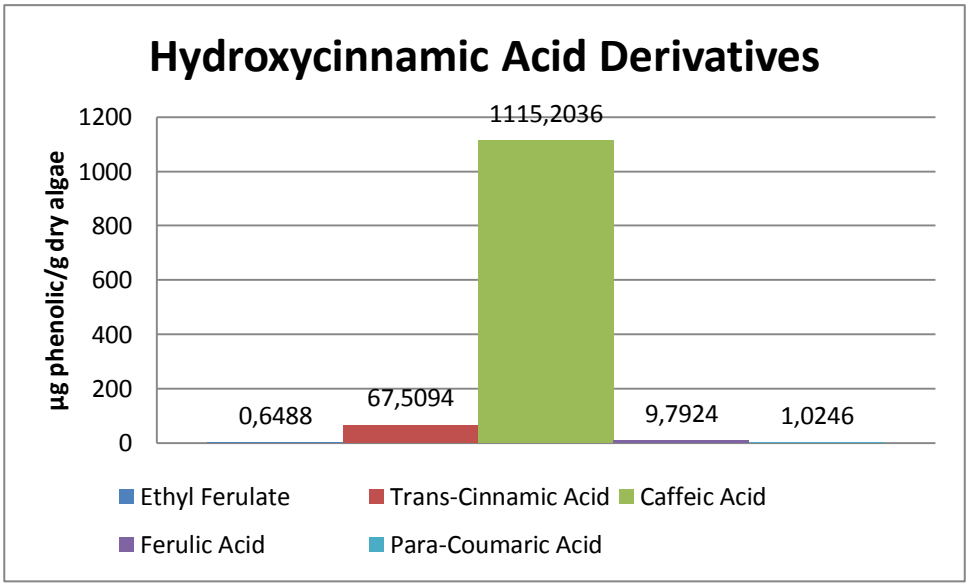


Figure 5.7. Hydroxycinnamic acid derivatives Lc MS/MS results.

5.5.1.2 Distribution of Hydroxycinnamic Acid Derivatives based on Temperature and Light Intensity

Figures 5.8 and 5.9 describe the relationship between hydroxycinnamic acid derivatives accumulation in algae and temperature and light intensity parameters.

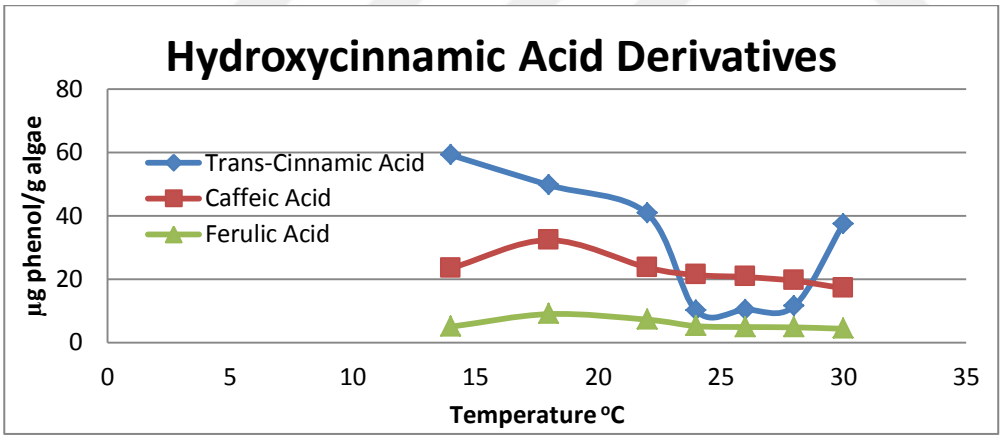


Figure 5.8. Effect of temperature on accumulation of Hydroxycinnamic acids.

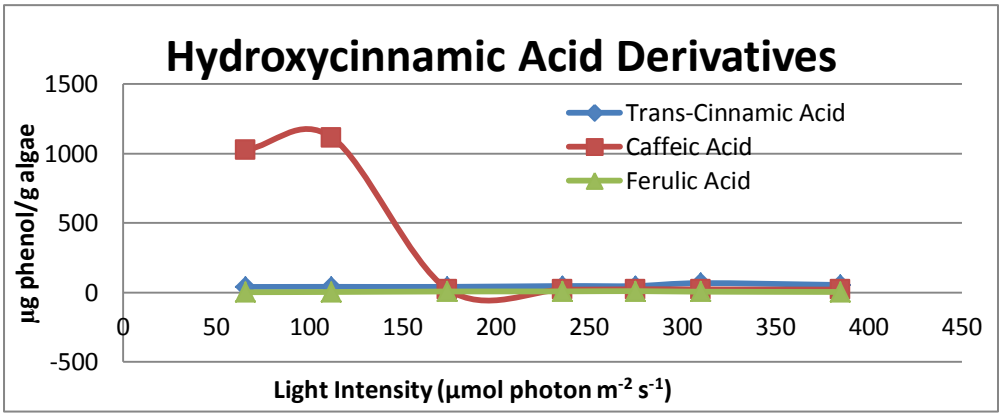


Figure 5.9. Effect of light intensity on accumulation of Hydroxycinnamic acid derivatives.

In table 5.8 the previous studies on T-Cinnamic acid extraction are listed, in comparison with this study.

Table 5.8. Comparison of studies, Trans-Cinnamic Acid.

Species	Chinese Cinnamon	Olive, green raw	Lingonberry	Olive, black raw	Strawberry	*C. Miniata*
Trans-Cinnamic Acid µg/g	200,1	144,3	41,2	7,7	2,2	67,51
Reference	He et al, 2005	Boskou et al, 2006	Ehala et al, 2005	Boskou et al, 2006	Ehala et al, 2005	This Study

Comparison of studies on Caffeic acid content of various studies to this study is given in table 5.9.

Table 5.9. Comparison of studies, Caffeic Acid.

Species	Vitex agnus castus	Black Chokeb-erry	Echin-acea	Com-mon Sage	Black cohosh	Spea-rmint	Cumin	*C. Miniata*
Caffeic Acid mg/g	2,70	1,41	0,63	0,21	0,25	0,25	0,17	1,12
Reference	Şarer and Gökbulut, 2008	Zheng and Wang, 2003	Lee, 2010	Kivilompolo et al, 2007	Kivilompolo et al, 2007	Kivilompolo et al, 2007	Shan et al, 2005	This Study

5.5.1.3 Benzoic Acids

The antioxidant activity of Benzoic Acids is highly dependent of the position of the hydroxyl group, the only and most effective position being the Meta position. As the number of hydroxyl groups increase the dependency decreases, although it doesn't always mean a higher number of hydroxyl groups will ensure a higher antioxidant capacity. (Rice-Evans et al. 1995) The highest amounts of benzoic acids encountered in algal extracts in course of this study are given on the Figure 5.10.

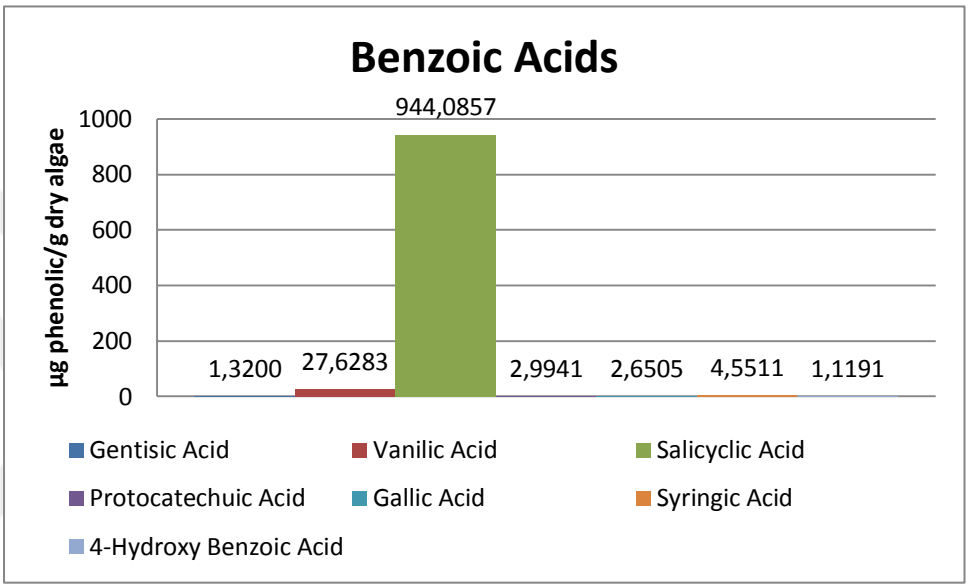


Figure 5.10. Lc MS/MS results of Benzoic acids.

Table 5.10 lists the previous studies which sought Vanillic acid content in various species.

Table 5.10. Comparison of studies, Vanillic Acid

Species	Common Sage	Rosemary	Olive, green raw	Olive, black raw	Grapefruit	*Chlorella Miniata*
Vanillic Acid µg/g	58,5	92	30	6,1	0,02	27,62
Reference	Kivilompolo et al, 2007	Kivilompolo et al, 2007	Boskou et al, 2006	Romani et al, 1999	Gorinstein et al, 2004	This study

Salicyclic acid; known for its anti-inflammatory effects, was encountered during the study and it was one of the compounds which had a dense presence in extract. It also had a trend like the results of Xanthine-Xanthine oxidase method, increasing with increasing temperature parameters and had its peak at light saturation point, likewise the Xanthine-Xanthine oxidase method results again. The comparison of various species' salicyclic acid with C. miniata is given in Table 5.11.

Table 5.11. Comparison of studies, Salicyclic Acid.

Species	Pap-rika	Th-yme	Dill	Gara m - masa la	Rose mary	Cumin	Cane l-la	Ani-seed	*C. Mini-ata*
Salycyc lic acid mg/g	2,03	1,83	0,94	0,67	0,68	0,45	0,43	0,23	0,94
Refer-ence	Swain et al, 1985								This study

It has been stated by a previous study by Masuoka and Kubo (2004) that salicyclic acid derivatives (e.g. Anacardic Acid) inhibit generation of superoxide radicals by xanthine oxidase. The study uncovered the mechanism of this inhibition to be; the binding of Anacardic Acid to allosteric sites near the xanthine-binding domain in xanthine oxidase. Another Benzoic acid with a higher content compared to others was Vanilic acid, its reaction to temperature was complete opposite of Salicyclic acid, it decreased with increasing temperature but again had a peak close to light saturation point.

Overall the study of Lc MS/MS the highest amount of phenolic compound that has been found was Salicyclic acid, only competed by Caffeic acid which had higher results only at light intensities of 66 and 112 µmol photon m^{-2} s^{-1}.

5.5.1.4 Distribution of Benzoic Acids based on Temperature and Light Intensity

The variance of salicyclic acid content in the algal extract dependent on temperature and light intensity are given in figures 5.11 and 5.12.

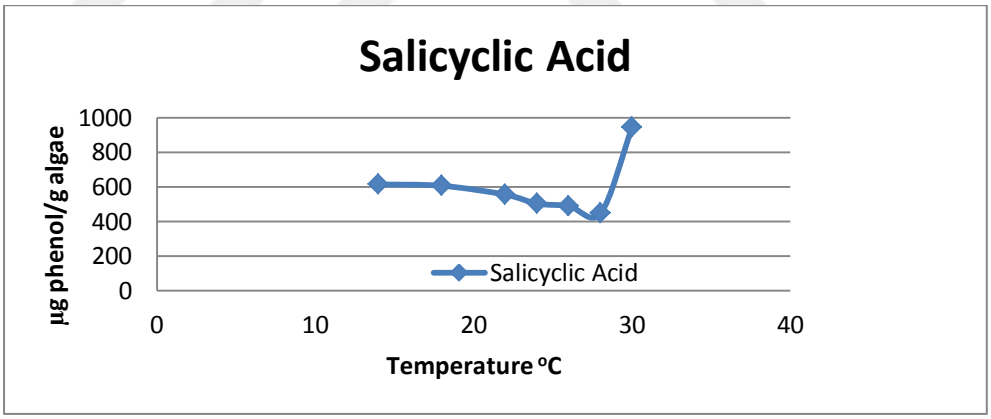


Figure 5.11. Effect of temperature on accumulation of Salicyclic Acids.

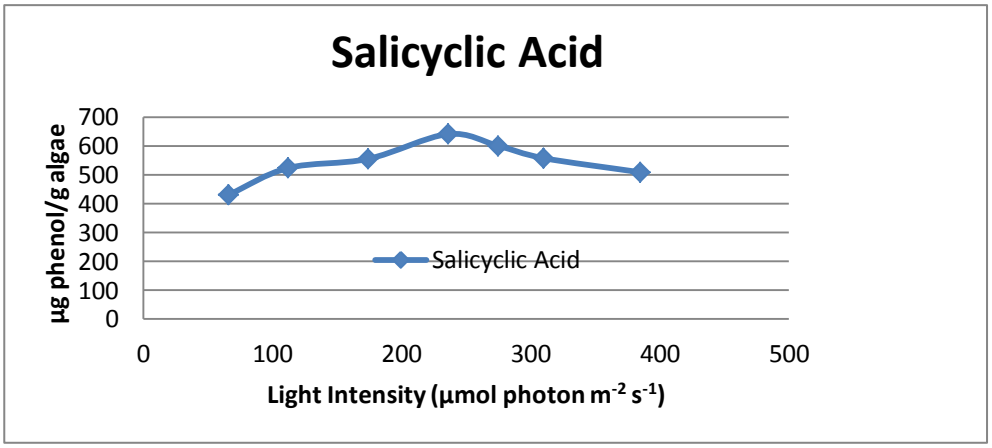


Figure 5.12. Effect of light intensity on accumulation of Salicyclic Acids.

5.5.2 Flavonoids

Flavonoids also possess an antioxidant capacity, their ability to inhibit oxidation leans on free radical scavenging activity and superoxide radical scavenging. Especially Catechin derivatives proved to provide lipid oxidation. Also cell culture experiments indicated that, flavonols had a higher antioxidant capacity compared to flavanols. (Rice-Evans et al. 1995)

The flavonoids are formed of two benzene rings, joined by a C3 chain (Goleniowski et al, 2013). The molecular structure of flavonoids is given in Figure 5.13 and the results of Lc MS/MS study of flavonoids are given in figure 5.14.

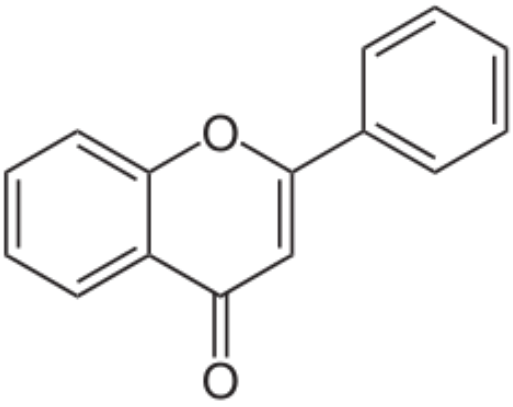


Figure 5.13. Molecular structure of Flavonoids.

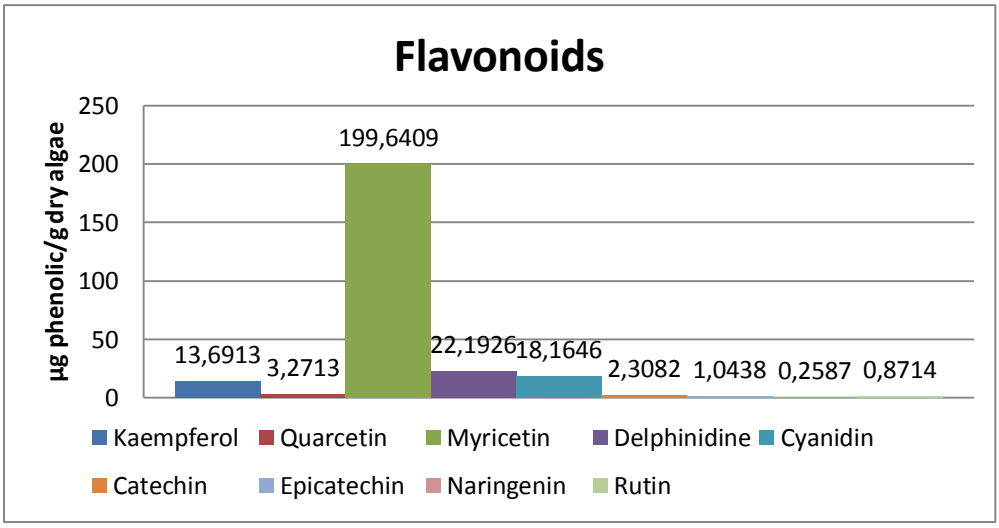


Figure 5.14. Lc MS/MS results of Flavonoids.

5.5.2.1 Flavonols

It is suggested that a high flavonol content in diet reduces the risk of vascular diseases, improvement of existing consequences of vascular diseases. Also improvements in diseases of heart, brain and memory, pancreatic cancer had been monitored by various studies (Sahelian, 2016).

The Flavonol with the highest content encountered was Myricetin, Quercetin on the other hand, although the content was lower, the coherency of it with the antioxidant capacity made it relevant with this study. The comparison of other studies' results of Quercetin and Myricetin with this study can be seen on tables 5.12 and 5.13 respectively.

Table 5.12. Comparison of studies, Quercetin.

Species	Chocolate, dark	Bilberry, raw	Onion, white	Almond	Red Raspberry	*C. Miniata*
Quarcetin µg/g	250,0	12,7	3,0	2,0	2,0	3,3
Reference	Counet et al, 2006	Ehala et al, 2005	Price and Rhodes, 1997	Milbury et al, 2006	Mullen et al, 2002	This Study

Table 5.13. Comparison of studies, Myricetin.

Species	Bird chili	Bell pepper	Sweet potato leaves	Parsley	Cranberry	Blueberry	*C. Miniata*
Myricetin µg/g	236	171,5	97,4	80,8	43,3	14,7	199,64
Reference	Colgan, 1993						This study

5.5.2.2 Distribution of Flavonols based on Temperature and Light Intensity

All of the studied Flavonols exhibited increments with increasing temperature until degree higher than 26-28 ^{o}C with the exception of Kaempferol which was encountered in higher amounts at border temperatures as 14 and 30 ^{o}C. Overall Flavanols were not present in the exracts in amount as high as previous compounds but Myricetin had a peak which can compete them in between the light saturation point, between the light intensity values of 236 and 275 µmol photon m^{-2} s^{-1}.

5.5.2.3 Anthocyanins

Recent studies proved that, anthocyanins provide protection against liver diseases, reduction of blood pressure, antimicrobial and anti-inflammatory effects. Also they possess antioxidant capacity due to their ability to scavenge free oxygen radicals however they might also act as prooxidants (Konczak and Zhang, 2004). Out of Anthocyanins, only significant encountered results were Delphinidine and Cyanidin both had similar amounts in the extracts. Table 5.14 compares the Cyanidin content of this study with contents found out by previously done studies.

Table 5.14. Comparison of studies, Cyanidin.

Species	Black bean	Red Raspberry	Red strawberry	*C. Miniata*
Cyanidin µg/g	16,3	5,3	5,0	18,16
Reference	Macz-Pop et al, 2006	Maatta-Riihinen et al, 2004		This study

5.5.2.4 Distribution of Anthocyanins based on Temperature and Light Intensity

Cyanidin is monitored to be higher at low light intensities and decrease with increasing light intensity, Delphinidine on the other hand, had the maximum values at 174 µmol photon m^{-2} s^{-1} and 22 ^{o}C. Increasing temperature also increased the amount of Cyanidin while decreasing Delphinidine content. The distribution is given in Figures 5.15 and 5.16.

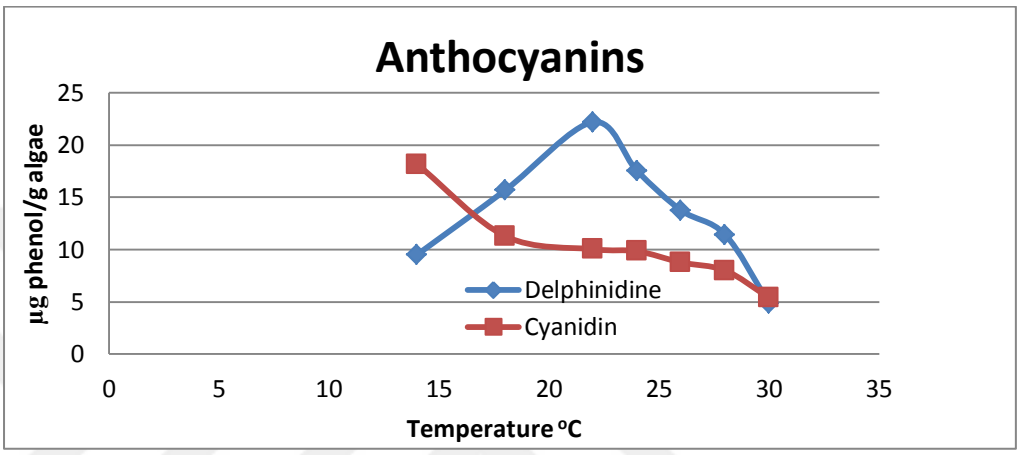


Figure 5.15. Effect of temperature on accumulation of Anthocyanins.

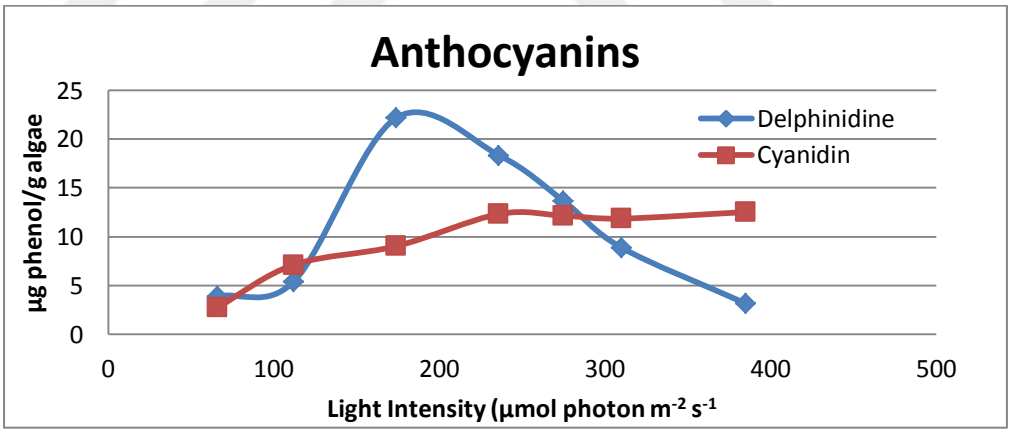


Figure 5.16. Effect of light intensity on accumulation of Anthocyanins.

5.5.2.5 Flavanols

Flavanols and nutrition including flavanols are often used and suggested against diseases of hypertension, heart diseases and dementia. The compounds are considered having cosmetic and pharmaceutical potential, especially because of their effects against aging process (Fischer et al, 2006).

5.5.2.6 Distribution of Flavanols based on Temperature and Light Intensity

The Flavanols; Catechin and Epicatechin were also returned noteworthy results, both chemicals had higher peaks around the temperatures and light intensities which are closer to the stock medium (22^{o}C and 174 µmol photon m^{-2} s^{-1}). Both flavanols sought during this study had given higher results of accumulation around stock medium conditions as seen in Figures 5.17 and 5.18.

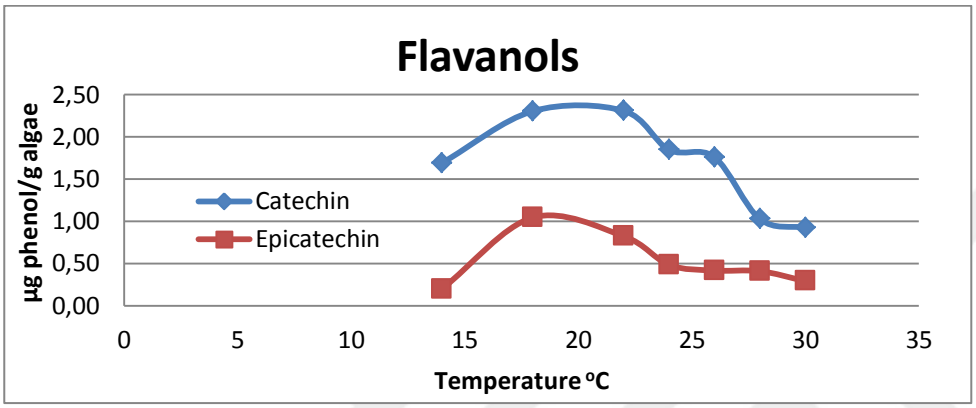


Figure 5.17. Effect of temperature on accumulation of Flavanols.

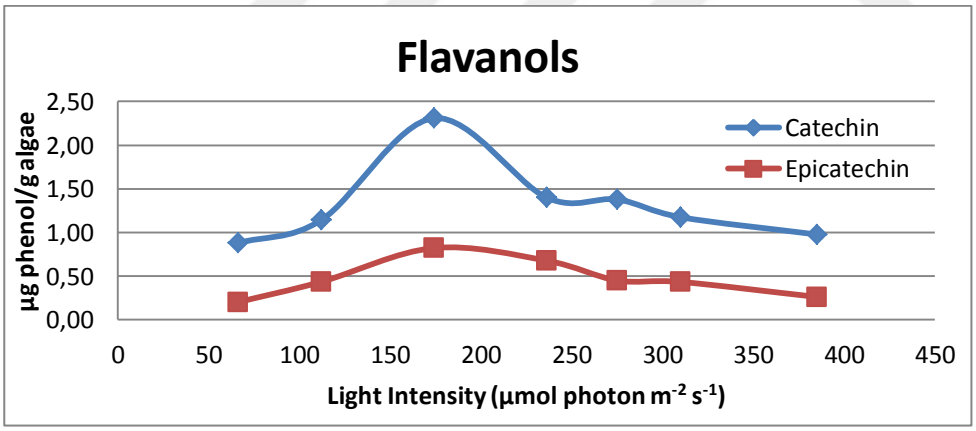


Figure 5.18. Effect of light intensity on accumulation of Flavanols.

5.5.2.7 Flavanones and Flavanonols

Other classes of Flavanoids which were studied were Flavanones and Flavanonols, they also returned fair results. Although their amounts and coherencies with antioxidant capacity and anti-inflammatory potential were inferior than the rest of the Flavanoids

5.5.3 Tannins

Tannins are polyphenols with proved antioxidant capacity, tannins molecules also have been showed to decrease mutagenic activity and antimicrobial effects (Chung et al, 1998). The molecular structure of tannins is given in Figure 5.19.

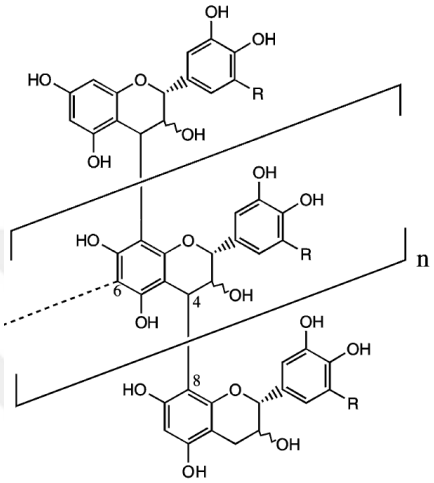


Figure 5.19. Molecular structure of Tannins.

Ellagic Acid being the most worthy of note, it had higher content at temperature of 26 ^{o}C and light intensity of 236 µmol photon m^{-2} s^{-1}. The results of various studies on search of Ellagic acid content in various species are given in Table 5.15.

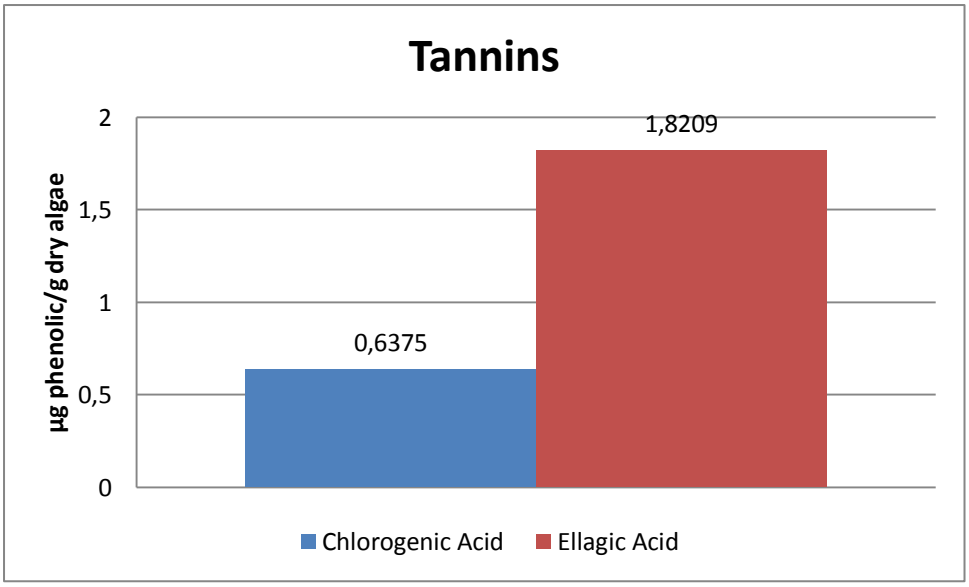


Figure 5.2023 Lc MS/MS results of Tannins.

Table 5.15. Comparison of studies, Ellagic Acid.

Species	**Walnut**	**Red raspberry**	**Muscadine grape**	***C. Miniata***
Ellagic Acid µg/g	59	21,2	9,0	1,82
Reference	Colaric et al, 2005	Wada and Boxin, 2002	Boyle and Hsu, 1990	This study

Ellagic acid, is known to be used as an antiproliferative and antioxidant agent. It's proven to inhibit binding of various carcinogens to DNA and is a chemoprotectan (Vattem and Shatty, 2005).

5.5.3.1 Distribution of Tannins based on Temperature and Light Intensity

The dependence of Ellagic acid content on cultivation temperature and light intensity is given in Figure 5.21. The highest Ellagic acid content was encountered at light saturation point and near optimum growth temperature.

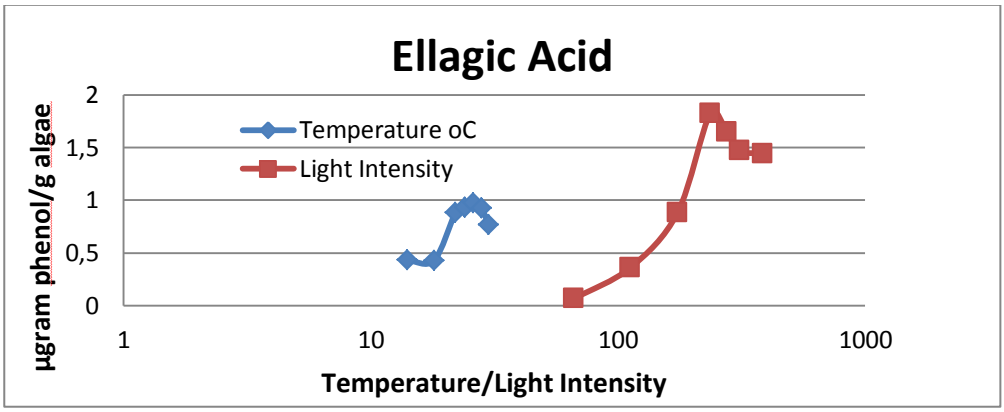


Figure 5.21. Ellagic acid accumulation depending on temperature and light intensity.

5.6 The Antioxidant Capacity of the Algae Extracts

The compounds which are claimed to have antioxidant has to has two basic properties, first is to be able to prevent or slow down radical mediated or auto-oxidation even at low concentration compared to the substrate and the second is after the prevention the produced molecule should be stable, and will not cause any further oxidation. (Catherine et al. 1995) The Antioxidant Capacity of the Algae Extracts were studied by the methods of TEAC, DPPH and FRAP.

5.6.1 Trolox Equivalent Antioxidant Capacity Assay

The studies on determination of antioxidant capacity of the algae resulted promising. Comparing the results of trolox equivalent antioxidant capacity to some fruits which are known to have high antioxidant activity, such as blackberry 5,0659 mg/g TEAC, olive (black) 3,6867 mg/g TEAC, and raspberry 4,2023 mg/g TEAC, (Pellegrini et al. 2003) the algae extracts had the mean value close to the fruits moreover at light saturation point, algae extract had much higher antioxidant capacity. The co.mparisons can be found in Table 5.16

Table 5.16. TEAC result of various studies.

Species	Amount	Reference
Blackberry	5,07 mg/g	(Pellegrini et al. 2003)
Olive (black)	3,69 mg/g	
Raspberry	4,20 mg/g	
Red wine	732 µg/ml	(Paixao et al, 2007)
White wine	439 µg/ml	
Tetraselmis sp.	17,37 mg/g	Goiris et al. (2009)
Neochloris oleoabundans	16,09 mg/g	
Chlorella sp.	14,91 mg/g	
Chlorella Miniata	22,91 mg/g	**This Study**

Trolox Equivalent Antioxidant Capacity did not display a major change due to varying temperature, but both increased with increased light intensities during growth and had a peak point at light saturation point. The distribution of Trolox Equivalent Antioxidant Capacity can be seen on Figure 5.22

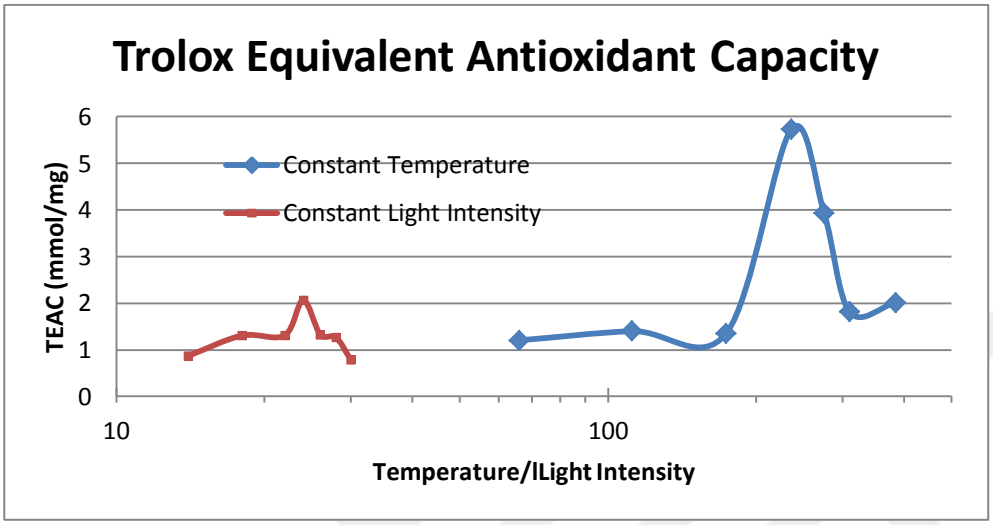


Figure 5.22. Trolox Equivalent Antioxidant Capacity (TEAC).

5.6.2 The Ferric Reducing Antioxidant Potential Assay

The Ferric Reducing Antioxidant Potential, found to be coherent with Trolox Equivalent Antioxidant Capacity in terms of effect of light intensity and temperature as seen on Figure 5.23, FRAP too was higher at light intensity point but the reaction to temperature appeared to differ slightly from TEAC.

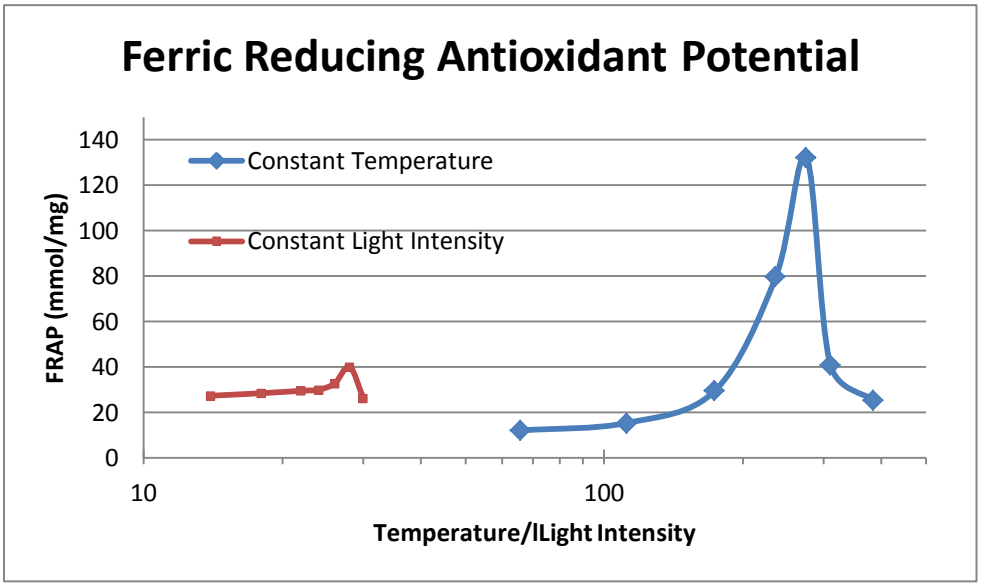


Figure 5.23. The Ferric Reducing Antioxidant of Potential (FRAP).

5.6.3 The Total Antioxidant Capacity Assay

Total antioxidant capacity of the algal extracts were found to be promising with a 13,50 % at its highest with used dosage though the inhibition is expected to increase with increasing dosage and concentration of extract. Table 5.17 compares the result of previous studies on different species, with this study.

Table 5.17. TAO result of various studies.

Species	% DPPH	Concentration	Reference
Ulva lactuca	30,20%	25g/100ml	Al-Amoudi et al, 2009
Digenea simplex	41,00%		
Sargassum Crassifolia	49,70%		
Chlorella Marina	23,08%	1g/20ml	Hemalatha et al, 2013
Dunaliella Salina	17,66%		
C. Vulgaris	74,00%	1g/10ml	Nailwal and Nailwal, 2013
in S. platensis	40,00%	1g/10ml	Arun et al, 2015
C. pyrenoidsa	18,85%		
N. muscorum	23,58		
C. vulgaris	8,17%	62 µg/ml	Jayshree et al, 2016
C. reinhardtii	6,25%		
C. vulgaris	92,57%	1 mg/ml	
C. reinhardtii	83,38%		
C. Miniata	13,50%	1,66 mg/ml	This Study

Total Antioxidant Capacity showed little change due to cultivation conditions compared to ferric reducing potential and trolox equivalent antioxidant capacity but also had a higher inhibition rate at light saturation point and temperatures between 22-26 ^{o}C. The distribution of Total Antioxidant Capacity can be seen on Figure 5.24

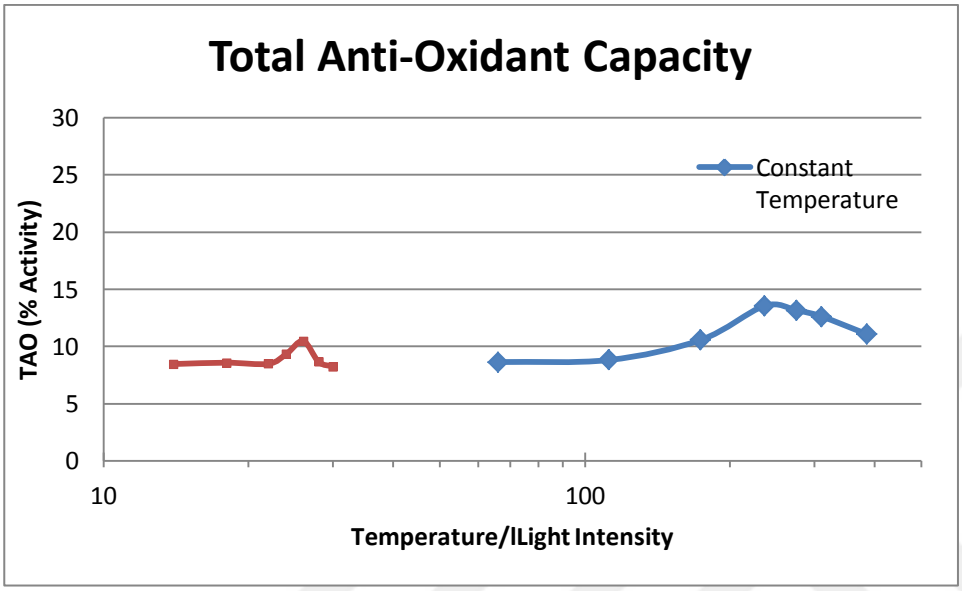


Figure 5.24. Total Antioxidant Capacity (TAO).

5.7 Anti-inflammatory Potential

Anti-inflammatory potential of the algal extracts were investigated by two methods; Xanthine – Xanthine Oxidase Inhibition and Hyaluronidase Inhibition. The algae extract had given reasonable result in both methods. The Xanthine Oxidase inhibition method was studied with 20 µl, 40 µl, and 60 µl of extracts, while there was no inhibition with the prior the other two doses had higher inhibition rates which can be seen in Figure 5.18. Anti-inflammatory effects tend to slightly increase with increasing temperature and light intensity during cultivation, although higher than the 310 µmol photon m^{-2} s^{-1}

5.7.1 Xanthine – Xanthine Oxidase Inhibition Assay

This study was the first one to determine the Xanthine oxidase inhibition potential of *Chlorella miniata* extract, yet there were no studies on other microalgae and their potential of xanthine oxidase inhibition potential, which rendered the comparison of *Chlorella miniata* with other microalgae impossible, however comparisons of *Chlorella miniata* with other studies on fruit, vegetable and various plants can be seen on Table 5.18. Though it is not one of the most potent species on inhibition in comparison with most of the list it stand to have a promising result of xanthine oxidase inhibition potential.

Table 5.18. Various studies of Xanthine Oxidase inhibition on different species.
[a] *Not Mentioned.*

Species	Studied Part	Concentration (dry biomass)	Inhibition %	Reference
B. racemosa	Infloresence Axis	1 mg/ml	59,54	Osman et al, 2016
T. stans	N.M.[a]	19,49 mg/ml	50,00	Govindappa et al, 2011
A.carambola	Leaves	100 mg/ml	23,61	Azmi et al, 2012
Carica papay	Leaves	100 mg/ml	78,38	
Dimocarpus longan malesianus	Leaves	100 mg/ml	46,88	
M. zapota	Leaves	100 mg/ml	70,81	
Salacca zalacca	Leaves	100 mg/ml	19,66	
S. Macrophylla	Seed	100 µg/ml (extract)	42,94	Wong et al, 2014
Punica Granatum	Seed	100 µg/ml (extract)	15,53	
P. zeylanica	Root	25 µg/ml	64,10	Nile et al, 2015

		(extract)		
Species	**Studied Part**	**Concentration (dry biomass)**	**Inhibition %**	**Reference**
Celery	N.M.[a]	100 mg/ml	73,89	El-Rahman and Abd–ELHak, 2015
Leek	N.M.[a]	100 mg/ml	43,71	
Parsley	N.M.[a]	100 mg/ml	82,57	
Molokhia	N.M.[a]	100 mg/ml	36,71	
Red onion	N.M.[a]	14,2 µg/ml (extract)	50,00	Nile and Park, 2014
White onion	N.M.[a]	17 µg/ml (extract)	50,00	
Tyellow onion	N.M.[a]	15,5 µg/ml (extract)	50,00	
Cnicus benedictus	Root	19.75 mg/ml	50,00	Can et al, 2017
M. talbotianium	Leaf	12.65 mg/ml	50,00	
Quercus infectoria	Galls	5mg/ml	92,00	Gholamhoseinian et al, 2017
Mentha longifolia	Aerial Parts	5mg/ml	75,00	
Fumaria parviflora	Aerial Parts	5mg/ml	35,00	
Cichorium intybus	Roots	5mg/ml	31,00	
Chlorella miniata		10,5 mg/ml, 1,64 mg/ml (extract)	37,07	**This study**

The light intensity and temperature parameters affected the Xanthine oxidase inhibition slightly; whereas it increased with temperature the light intensities remote from light saturation point decreased the inhibition percentages. Figure 5.25 plots the distribution of inhibition of xanthine oxidase with doses of 40 and 60 µl against growth temperature and light intensity.

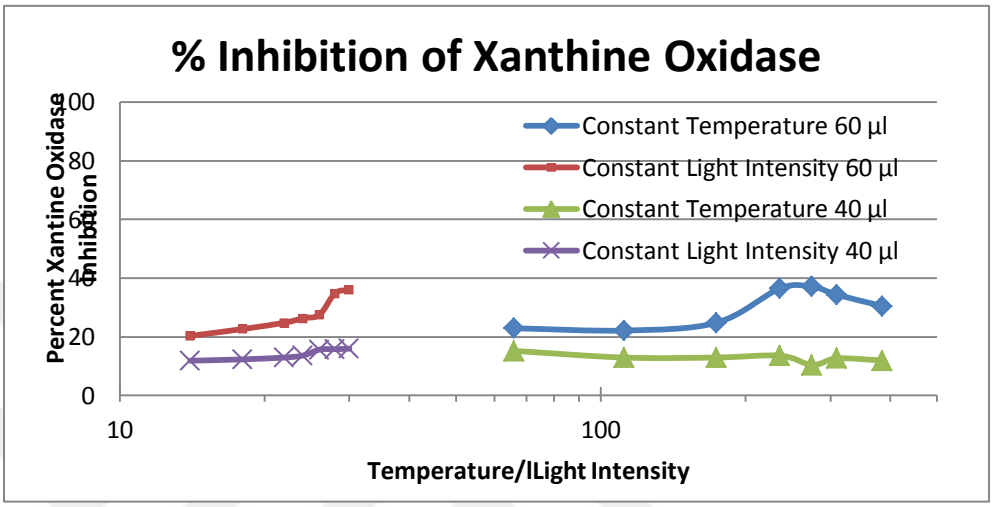


Figure 5.25. Xanthine – Xanthine oxidase inhibition method, results.

Sibi and Rabina (2016) investigated the anti-inflammatory activities of *Chlorella vulgaris* extracts in cell culture, the inhibition of pro-inhibitory mediators. The study concluded that the extracts of *C. vulgaris* promised an anti-inflammatory activity. In accordance with this study, we showed that *Chlorella Miniata*, also had anti-inflammatory effect in vitro. These effects were strongest for the extracts from the biomass cultivated under high temperature and light intensity conditions.

5.7.2 Inhibition of Hyaluronidase Assay

The highest potential of inhibition of hyaluronidase enzyme encountered in this study and results of various other studies can be seen on Table 5.19. This method was also applied on *Chlorella miniata* for the first time in this study also this was the first time that hyaluronidase enzyme inhibition was measured on a microalgae. This study revealed that *Chlorella miniata* had one of the highest inhibition potential of the studies referred in Table 5.19. Only potential competition of *Saccharum officinarum* with a lower inhibition potential with a lower dose of extract. Overall it can clearly be said that the extracts of *Chlorella miniata* have a high potential of usage as an anti-inflammatory agent.

Table 5.19. Various studies of Hyaluronidase inhibition on different species.
[a] *Not Mentioned.*

Species	Studied Part	Concentration (dry biomass)	Inhibition %	Reference
Onion	N.M.[a]	1,5 g/ml	11,70	Pena et al, 2013
G. procumbens	Leaf	100 µg/ml (extract)	21,83	Scotti et al, 2016
Oenothera biennis L.	Leaf	50 µg/ml (extract)	97,90	
Geopropolis from *Melipona orbignyi*	N.M.[a]	75 mg/ml	35,60	Santos et al, 2017
A. brevicaulis brevicaulis var. brevicaulis	Root	0,62 mg/ml	28,12	İlhan et al, 2016
A. baytopae	Seed	1,56 mg/ml	28,67	
A. cilicica	Root	1,35 mg/ml	49,49	
Caraway	N.M.[a]	336 µg/ml (extract)	50,00	Thippeswamy and Achur, 2014
Saccharum officinarum	Leaf	100 µg/ml (extract)	47,45	Ghiware et al, 2012
Cucumis sativus	Fruit	20,98 µg/ml (extract)	50,00	Nema et al, 2011
Gaultheria procumbens L.	Leaf	100 µg/ml (extract)	4,20	Michel et al, 2014

Species	Studied Part	Concentration (dry biomass)	Inhibition %	Reference
Triphala guggulu	N.M.[a]	4 mg/ml	84,60	Sumantran et al, 2007
Triphala shodith guggulu	N.M.[a]	4 mg/ml	100,00	
Chlorella Miniata		0,9 mg/ml 152,61 µg/ml (extract)	65,39	**This study**

Results of hyaluronidase inhibition method was parallel to xanthine – xanthine oxidase inhibition method, in terms of increase via increase of cultivation temperature and light intensity however; the increase slows down at a certain point, at 26 ^{o}C and 236 µmol photon m^{-2} s^{-1}. The highest percentage of inhibition of xanthine oxidase of 37,07% was found at the cultivation conditions of 275 µmol photon m^{-2} s^{-1} and 22 ^{o}C. The distribution can be seen on Figure 5.26. The inhibition potential of Hyaluronidase enzyme showed an increment with increasing light intensity and increasing temperature.

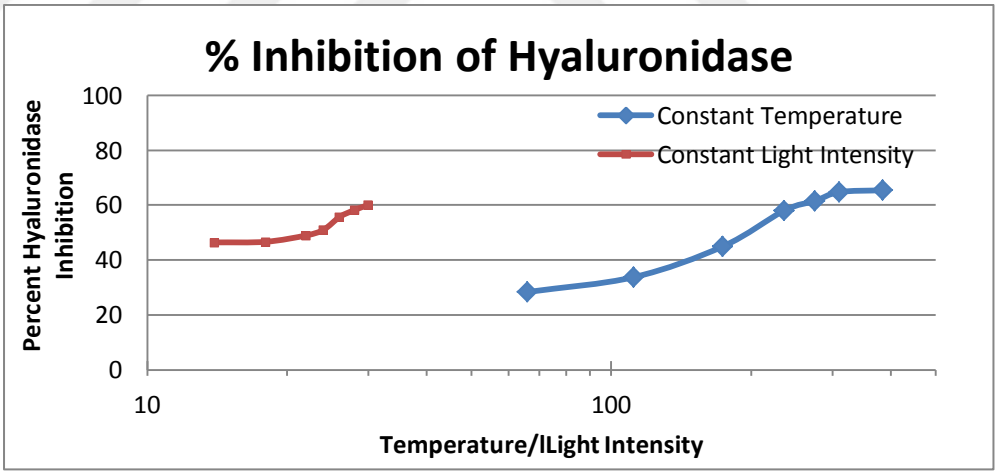


Figure 5.26. Hyaluronidase inhibition method, results.

6. Conclusion

This study was the first study on the growth conditions of the microalgae species *Chlorella miniata*, it was also the first which investigated how growth conditions affect the accumulation of phenolic compounds thus the anti-oxidant capacity and anti-inflammatory properties of the microalgae. Another aspect of the research was that it is the first among others that measured the potential of Xanthine oxidase inhibition and Hyaluronidase inhibition of the microalgae. This study also modeled the temperature and light intensity relationship with growth of the algae *Chlorella miniata*, traced the phenolic compound content of the extracts of the algae and proved the anti-oxidant capacity of it.

The first step of the study was cultivation of the algae and the mathematical modelling of the growth conditions against the specific growth rate, concluded that the species *Chlorella miniata* offered less productivity compared to the other *Chlorella* species, in the studied range of growth conditions and parameters. That being said, the other parameters such as different methods of aeration, culture modes, types of bioreactors, colors of light, growth mediums, and carbon sources were held constant for all experiments. Although the productivity of the microalgae was lower than other various *Chlorella* species in aforesaid studies, it is important to point out that even in the laboratory scale, which has a lower working volume and higher maintenance cost compared to a factory, the algae sold as a dry biomass is still profitable, theoretically.

The mathematical modelling that was carried out, gave an optimum growth temperature and light intensity value of 21,38 ^{o}C and 291,5 µmol photon m^{-2} s^{-1} respectively, and also proposed a light inhibition limit of 390,1 µmol photon m^{-2} s^{-1}, which was out of the scope of this study, but it can easily be said, due to the mathematical models utilized during the modelling of the optimum cultivation points of the *Chlorella miniata*, in terms of temperature and light intensity, had been calculated. It was also seen that with higher light intensities the effect of the temperature in between the values of the minimum and maximum limits of the temperature where growth can be observed, had decreased.

The next step of study was the extraction of the algae, where soxhlet extraction was chosen for this purpose using ethanol as the solvent, due to the success of other studies on extraction of phenolic compounds with the aforesaid method. An average extraction yield of 14,41% had been calculated, which was supposed to include the polar compounds, such as the phenolic compounds that the study was aiming for.

Analyzes of the antioxidant capacity and anti-inflammatory potential was accomplished on the next step of the study, it is certain to say that *Chlorella miniata* possessed both the antioxidant capacity and an anti-inflammatory potential. The results of the inhibition of hyaluronidase were outstanding, which increased with the temperature and light intensity in the studied ranges and when compared to other studies on vegetables, fruits and various plants, the potential was superior. However the same statement cannot be made for the total antioxidant capacity assay, which was easily, surpassed by other species of microalgae, and the results of the Trolox Equivalent Antioxidant Capacity and The Ferric Reducing Antioxidant of Potential assays were promising especially at the point of light saturation.

The LC MS/MS assay identified almost 30 different phenolic compounds and gave out satisfactory results in terms of coherency with the previous assays in this study. Quarcetin (3,27 μg/g dry algae at the highest) and Ellagic Acid (1,82 μg/g dry algae at the highest) showed coherency with the anti-oxidant activity assays of the TEAC and FRAP, Cyanidin (18,16 μg/g dry algae at highest) with hyaluronidase inhibition and Salicyclic Acid (944,09 μg/g dry algae at the highest) with Xanthine oxidase inhibition assays. Other than the previously mentioned phenolic compounds, Caffeic Acid (1115,20 μg/g dry algae at the highest), Trans-Cinnamic Acid (67,51 μg/g dry algae at the highest), Vanilic Acid (27,62 μg/g dry algae at the highest) also resulted in significant amounts.

In general the species *Chlorella miniata* possesses the low productivity though low cost of production with a good potential of anti-oxidant and anti-inflammatory effects, which includes high amounts of the phenolic compounds like salycyclic acid which is one key component and aspirin and caffeic acid which is proved to have high anti-oxidative capacity. Although compared to some species of plants the result of the assays might seem inferior but it should be kept in mind that microalgae grow faster, are not attached to soil nor do they need fertile ground, which is important especially now while with the world is fighting against starvation it is a crucial point that microalgae does not compete with agriculture for fertile soil.

The lower productivity rate of the algae compared to other *Chlorella* species, is the only real argument against the production of this species over them, although the anti-inflammatory effect of the other of Chlorella species are not comparable due to the lack of data. Studies on *Chlorella miniata* and other species of *Chlorella,* using different methods of cultivation which are previously mentioned in this chapter and assays on anti-oxidant and anti-inflammatory effects, respectively would give a better perspective of the feasibility of producing *Chlorella* with the aim of the previous stated pharmaceutical approach. Another research to be conducted for this purpose is of course to investigate the applicability of the algal extract as a medicine, food supplement or even nourishment, be it downstream processes to separate and purify the desired phenolic compounds or to carry the extract as a whole.

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