REPUBLIC OF TURKEY

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GRADUATE SCHOOL OF SCIENCE AND ENGINEERING

DEVELOPMENT OF MEDICAL SHAMPOO FORMULATION CONTAINING POLAR MICROALGAE

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MASTER OF SCIENCE THESIS

Department of Bioengineering

Bioengineering Program

Supervisor

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A thesis submitted by Başak AKIN in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE is approved by the committee on 09.02.2023 in Department of Bioengineering, Bioengineering Program.

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Dedicated to my family

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LIST OF SYMBOLS

α	Alpha
β	Beta
°C	Degree Celcius
ɣ	Gama
Rpm	Revolutions per minute
M	Specific growth rate

LIST OF ABBREVIATIONS

S. D.	Seboreik Dermatit
PV	Pitiriazis versicolor
MAA	Mycosporine-like amino acid
OD	Optical Density
pH	Potantial of Hydrogen
FID	Flame Ionization Detector

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ABSTRACT

Development of Medical Shampoo Formulation Containing Polar Microalgae

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Department of Bioengineering

Master of Thesis

Supervisor: Prof. Dr. Didem ÖZÇİMEN

Seborrheic dermatitis is known as one of the most common skin diseases in the world and it causes dandruff, scaly and inflamed patches most commonly on the scalp, oily areas of the face, nose, eyebrows, and chest areas. Furthermore, *Malassezia furfur* strain has been associated with this disease and it is necessary to use medicated shampoos or natural cosmetic products with antifungal effects for the treatment of this disease.

In the cosmetics industry, algae are already used for a variety of purposes, including skin conditioning, moisturizing, anti-aging, rejuvenating, and colorant. Polar microalgae have antioxidant, antimicrobial, antifungal and anticancer properties under favor of their valuable bioactive content.

In this thesis, a shampoo formulation was developed based on the antibacterial and antifungal properties of polar microalgae. By adding microalgae oil and the bioactive ingredient tea tree oil, which has an anti-dandruff effect in the literature and is currently available in anti-seborrheic shampoos, to the developed formulations, it was intended to compare the effects of these two bioactive

components with antifungal analyses. Additionally, the cosmeceutical potential of the developed formulation was assessed and the antifungal effects of the developed shampoo formulation were compared with the antifungal effects of the commercial selenium sulfide shampoo.

In this context, a shampoo formulation containing microalgae oil belonging to the *Chlorella variabilis YTU.ANTARCTIC.001*, which isolated and identified from Antarctica, and tea tree oil was developed. The stability of the shampoos was observed and their antifungal activities were compared.

In the thesis study, it was determined by biochemical analyzes that *Chlorella variabilis YTU.ANTARCTIC.001* contains oleic, linolenic, dihomo-γ-linolenic and eicosapentanoic fatty acids and has β-carotene amount of 2.52 μg/ml. As a result of the antifungal analysis, it was observed that the antifungal shampoo prepared with microalgae oil formed a 33.66 mm inhibition zone and showed a higher antifungal effect against *M. furfur* compared to the shampoo (23.66 mm) without microalgae oil and commercial shampoo. Also, it was observed that the antifungal shampoo prepared with tea tree essential oil had a similar inhibition rate against *M. furfur* to the shampoo prepared with microalgae extract. According to the obtained results, it is considered that polar microalgae may be an alternative as a new antifungal agent that can be used in anti-seborrheic dermatitis shampoos.

Keywords: Seborrheic dermatitis, Polar microalgae, *Chlorella variabilis YTU.ANTARCTIC.001*, shampoo formulation, antifungal effect, *Malassezia furfur*, cosmetics.

ÖZET

Kutup Mikroalgi İçeren Medikal Şampuan Formülasyonunun Geliştirilmesi

Başak AKIN

Biyomühendislik Anabilim Dalı

Yüksek Lisans Tezi

Danışman: Prof. Dr. Didem ÖZÇİMEN

Seboreik dermatit dünyadaki en yaygın cilt hastalıklarından biridir ve yaygın olarak kafa derisi, burun, kaş yüzün yağlı bölgeleri ve göğüs bölgelerinde kepek, pul pul ve iltihaplı lekelere neden olur. *Malassezia furfur* suşu bu hastalıkla ilişkilendirilmiş olup bu hastalığın tedavisi için ilaçlı şampuanlar veya antifungal etkiye sahip doğal ürünler kullanmak gerekmektedir.

Algler kozmetik endüstrisinde cilt iyileştirici, nemlendirici, yaşlandırma karşıtı, canlandırıcı, renklendirici gibi birçok özellikleri ile halihazırda kullanılmaktadır. Kutup mikroalgleri değerli biyoaktif madde içerikleri sayesinde antioksidan, antimikrobiyal, antifungal ve antikanser özellik göstermektedir. Kutup mikroalglerinin sahip olduğu antibakteriyel ve antifungal aktivitelerinden yola çıkılarak gerçekleştirilen bu tez çalışmasında, yeni bir şampuan formülasyonu geliştirilmiştir. Geliştirilen bu formülasyonlara mikroalg yağı ve literatürde anti-dandruff etkisi olan ve halihazırda anti-seborrheic şampuanlarında mevcut biyoaktif bileşen çay ağacı yağı eklenerek, bu iki biyoaktif bileşenin etkisinin antifungal analizler ile karşılaştırılması amaçlanmıştır. Ayrıca geliştirilen şampuan formülasyonunun antifungal etkileri, ticari selenyum sülfit içerikli şampuanın

antifungal etkileri ile de karşılaştırılarak, geliştirilen formülasyonun kozmetik potansiyeli değerlendirilmiştir.

Gerçekleştirilen tez çalışmasında, Antarktika'dan izole edilen ve tanımlanan *Chlorella variabilis YTU.ANTANTARCTIC.001* türüne ait mikroalg yağı ve çay ağacı yağı içerikli, baz bir şampuan formülasyonu geliştirilerek, şampuanların stabilitesi gözlemlenerek ve antifungal aktiviteleri karşılaştırılmıştır.

Yapılan tez çalışmasında, *Chlorella variabilis YTU.ANTANTARCTIC.001* türünün oleik, linolenik, dihomo-γ-linolenik ve eikosapentanoik yağ asitlerini içerdiği, 2,52 μg/ml β-karoten e sahip olduğu biyokimyasal analizler ile belirlenmiştir. Antifungal analizler sonucunda ise mikroalg yağı ile hazırlanan antifungal şampuanın 33.66 mm inhibisyon zonu oluşturduğu, mikroalg yağı içermeyen şampuana (23.66 mm) ve ticari şampuana (26.83 mm) kıyasla *M. furfur*'a karşı daha yüksek bir antifungal etki gösterdiği gözlemlenmiştir. Çay ağacı esansiyel yağı ile hazırlanan antifungal şampuanın ise *M. furfur*'a karşı mikroalg ekstraktı ile hazırlanan şampuana benzer inhibisyon oranına sahip olduğu görülmüştür. Elde edilen sonuçlara göre kutup mikroalglerinin değerli biyoaktif maddeler içerdiği ve bu nedenle anti-seboreik dermatit şampuanlarında kullanılabilecek yeni bir antifungal ajan olarak alternatif olabileceği düşünülmektedir. Bu çalışmada mikroalglerin dermokozmetik, ilaç ve kozmetik sektörlerinde yaygınlaşmasının artırılması ve kutup alglerinin sürdürülebilirliği ile ilgili çalışmaların artmasına katkı sağlayacağı gösterilmiştir.

Anahtar Kelimeler: Seboreik dermatit, Kutup mikroalgi, *Chlorella variabilis YTU.ANTANTARCTIC.001*, şampuan formülasyonu, antifungal etki, *Malassezia furfur*, kozmetik.

1
INTRODUCTION

1.1 Literature Review

Seborrheic dermatitis is a multifactorial disease caused by many factors such as stress, depression, fatigue, various vascular disorders, clothing, diet, *Malassezia furfur*, and the use of various drugs. The treatment method is aimed at controlling and inhibiting the symptoms, which vary according to the severity of the disease (Naldi & Rebora 2009).

Polar microalgae are extremophilic microorganisms that have unique biochemical contents compared to microalgae in temperate regions due to their adaptation to harsh environmental conditions. It is thought that microalgae living in the extremophilic region perform the biosynthesis of valuable bioactives and antioxidants as a metabolic adaptation mechanism in order to tolerate oxidative environmental conditions (Hoopes et. al., 2017). Microalgae has been widely used in many different fields such as food, medicine, pharmacy, cosmetics from past to present with its important bioactive compounds such as protein, carbohydrates, fatty acids, vitamins, minerals, pigments.

Biologically active compounds such as oleic acid, linoleic acid, palmitoleic acid, vitamin E, B_{12}, β-carotene, phycocyanin, lutein extracted from algae; play an important role in accelerating healing, reducing and preventing infections. The use of bioactive skin care products containing bioactive components is increasing day by day in the cosmetic product market, as the harmful effects of chemicals are more researched and public awareness is raised (Lyon and Mock 2014).

Choi et al., evaluated antifungal activity of 57 species of common seaweed from the coast of Korea against *Malassezia* species. It was reported that, 17 species exhibited 29.8% inhibitory activity. In agar disc diffusion method, the ether extracts of *Corallina pilulifera*, *Enteromorpha linza*, *Laminaria japonica*,

Symphyocladia latiuscula and *Ulva* sp. showed strong antifungal activity (Choi et.al., 2009).

It has been observed that polar microalgae isolated and identified from the Antarctic region have high antimicrobial activity against bacterial pathogens. It has also been proven by studies that various algae species grown in temperate conditions have antifungal effects on *Malassezia furfur*. Based on these results, in this study, it is aimed to determine the antifungal effect of the shampoo formulation containing *Chlorella variabilis YTU.ANTARCTIC.001* oil extract on *Malassezia furfur*, and to compare the antifungal effects of this shampoo with the commercial anti-seborrheic shampoo.

1.2 Objective of the Thesis

Microalgae are among the fastest growing organisms in the world and are considered among the potential biomass sources evaluated in several areas of industry. Among the bioactive materials detected from marine and freshwater microalgae, natural pigments, antioxidants and oils have received particular attention, and significant progress has been made in the evaluation of these substances in the last few years.

The following are the objectives of this thesis study:

Production and biochemical analysis of microalgae species isolated from the poles,

Investigation of the antifungal effect of *Chlorella variabilis YTU.ANTARCTIC.001*, a microalgae species adapted to harsh environmental conditions in polar regions, on *Malassezia furfur* strain,

By producing anti-seborrheic dermatitis shampoo with bioactive components, to pave the way for the application of polar algae as an industrial product for various skin disorders.

By adding microalgae oil and tea tree oil, which has an anti-dandruff effect in the literature and currently available in anti-seborrheic shampoos, to the formulations prepared by developing the shampoo formulation, comparing the effects of these two active ingredients with the experiments.

Comparison of the antifungal effect of shampoo formulation with algae oil prepared with shampoo containing selenium sulfide.

1.3 Original Contribution

While the common features of traditional shampoos are to soothe the scalp and slow the death of cells in the scalp, there is no specific commercial shampoo for the *Malassezia furfur* strain associated with this disease. This study will bring an innovative approach to the literature in this field.

Based on the algae species in the literature, there is no study in the literature on the antifungal effects of *Chlorella variabilis YTU.ANTARCTIC.001* on *Malassezia furfur*, this thesis will contribute to the literature as an article and will pave the way for similar studies. Also, there are a limited number of studies on microalgae species found in the poles, and studies on polar microalgae have originality and have the potential to contribute to the literature.

2
GENERAL INFORMATION

2.1 Algae

One of the key components of the ecosystem is algae. These adaptable microorganisms contribute to the ecology by photosynthesis and stand out by generating metabolites that are rich in bioactive compounds. According to their respective lifestyles, they are typically split into the micro and macro classes. Macroalgae settle new areas and develop into filaments to survive on. Single-celled algae called microalgae have a diameter of 2 to 10 μm (Ievina and Romagnoli, 2020, Yuan et al., 2020). They may live in waters that are hot, cold, salty, or fresh. As long as their primary food sources are nitrogen, water, and sunlight, algae can thrive.

In addition to these, other parameters influencing the life cycle of algae include temperature, pH, carbon dioxide, lighting duration, color, and intensity, as well as the amount of other nutrients. Algal morphology (such as cell size), bioactive production (diversity or quantity), and growth rate can show minimal or maximum changes under different conditions.

Algae have been a significant source of both food and medicine throughout history. Today, algae are utilized in a variety of industries, including those that produce food, medicine, and cosmetics.

When examining the reasons why algae are regarded as a source in these fields, note the usefulness of numerous bioactives they have, including those with anticancer, antioxidant, antibiotic, and antiviral properties. Additionally, its rapid productivity, environmental adaption mechanisms, contaminant resistance, and biodiversity offer additional advantages (Ievina and Romagnoli, 2020).

2.2 Polar Microalgae

The continent of Antarctica has a surface area of 14 million km^2, and there are numerous perennial frozen lakes in the midst of the world's largest desert (Lizotte 2001).

The polar regions make up a sizable section of the surface of the Earth and have a significant impact on the biogeochemical cycles on a global scale. Deep water formation in the Southern Ocean collects significant amounts of carbon (i.e., 30% of global uptake although only accounting for 10% of surface area) due to enhanced CO_2 solubility at low water temperatures (Sabine et al., 2004).

Terrestrial photoautotrophs are severely constrained by perennial permafrost, whereas algae and cyanobacteria have successfully adapted to polar freshwater and/or marine habitats. Despite being a physiologically challenging environment, they form the basis of these productive food chain by converting light energy and nutrients into chemical energy (Lyon and Mock 2014).

High latitudes, temperatures below freezing, full darkness with no light in the winter and continuous light with high UV conditions are just a few of the harsh characteristics of the poles. Additionally, during extended wintertime at the poles, oceans and lands freeze, there are significant salinity changes, little gas exchange, and extremely harsh conditions (Lyon and Mock 2014). It might be difficult to reaching nutrients for the microorganisms and bacteria that live in the poles. Different modifications have been developed by microorganisms to cope with food scarcity and to exist in darkness.

The polar food chain is assumed to be based on Antarctic microalgae, which have effectively adapted to the harsh and fluctuating polar ecosystem. The Antarctic ecology has numerous climatic difficulties such as freezing temperatures, including solar, osmotic, oxidative, and nutritional stress (Thomas & Dieckmann, 2002); (Mock & Thomas, 2008).

2.3 Applications of Algae in Cosmetics

The following is a summary of studies on utilization of microalgae in the cosmetics industry:

The brown macroalgae *Fucus vesiculosus*, which has great importance in the cosmetic sector, is a source of fucoidan. Using this biomass presents advantages like anti-aging and anti-wrinkle capabilities for cream and lotion cosmetic goods (Fujimura et al. 2000). By increasing the expression of integrin molecules, Fujimura et al. (2002) demonstrated that the administration of 1% *Fucus vesiculosus* aqueous extract facilitates contraction of collagen gels containing fibroblasts. Skin elasticity improved significantly, and skin thickness increased. As a result, it has been demonstrated that *F. vesiculosus* contains anti-aging properties.

Bioactive substances serve as antioxidants and photoprotectants. For instance, phenolic substances (like fluorotannins in brown algae), pigments (such astaxanthin and other carotenoids) and etc. (Bedoux et al. 2014; Maadane et al. 2015). The majority of polyunsaturated fatty acids obtained from algae also exhibit strong antioxidant activity (Maadane et al. 2015). Algae's phenolic chemicals play a role in a number of defense processes, including their ability to counteract the oxidative effects of UV exposure (Bedoux et al., 2014). Since their capacity to scavenge peroxyl radicals, carotenoids can interact with them and so prevent oxidative damage to cellular membranes and lipoproteins (Stahl and Sies, 2003, 2005). Carotenoids and tocopherols, which are well-known to be powerful antioxidants, can shield the skin against the harmful effects of UV light (Stahl et al. 2000)., Stahl et al. (2000) sought to determine the effectiveness of oral carotenoids supplementation and the effect carotenoids and vitamin E in preventing erythema in adults. *Microalga Dunaliella* salina was used in this investigation as a carotenoids source. It has been demonstrated that carotenoids alone do not effectively decrease erythema as effectively as carotenoids combined with vitamin E. Mycosporin-like amino acids (MAAs) are among nature's most effective UVA absorbers (Schmid et al. 2000). It has been demonstrated that a *Porphyra umbilicalis* extract that contains MAA can lessen the skin damage brought on by UVA (Schmid et al. 2006).

The two fundamental components of the anti-aging benefits of cosmetic products are skin hydration and skin softening (Ma'or et al. 2000). Algal extracts that are biologically active can be utilized as moisturize agent. These substances include fatty acids, polysaccharides, oligosaccharides, and natural moisturizing factors (NMF) (Agatonovic Kustrin and Morton, 2013).

Brown macroalgae's main polysaccharides are alginates, laminarins, sargans, and fucans. In order to investigate their chemical content and compare them to hyaluronic acid, Wang et al. (2013) isolated polysaccharides from several brown macroalgae species. Of all the polysaccharides investigated, moisture absorption calculated and discovered that the key active site for their capacities to absorb and hold moisture was the sulfated group.

Microorganisms, who are typically subjected to intense environmental circumstances, have the ability to create substances that raise their normal degree of stress tolerance. In the study of Stutz et al. (2012) CCryo 101-99 snow algae slows down transepidermal water loss and prevents aging (Stutz et. al., 2012).

Although lutein is one of the main carotenoids found in the macula and retina of the human eye, the functions of this carotenoid are known as light filtering and protecting eye from the damage of sunlight (www.webmd.com). In the clinical study which was conducted by Juturu et al. (2016), lutein and zeaxanthin were given orally to volunteers for 12 weeks. As a result of the supplementation, it was observed that the brightness of the skin of the volunteers increased and the skin conditions of the volunteers improved. It has been reported that the skin whitening function is due to its photoprotective properties and antioxidant properties (Juturu et. al., 2016).

In the study by Loganathan et al. (2020), it was reported that *Chlorella variabilis* can produce high concentrations of lutein and has potential as a commercial source (Loganathan et. al., 2020). Amrutkar et al. (2020) developed a formulation for the purpose of an anti-photo-aging facial gel with *Chlorella vulgaris* extract. They developed an anti-aging facial gel formulation against skin problems due to algae being a source of antioxidants, carotenoids and pigments (Amrutkar et. al., 2020).

Table 2.1 Function, detailed information of function and source (species, oil and extract) of *Chlorella* species (Cosmetic Database, https://ec.europa.eu/growth/tools-databases/cosing).

Function	*Chlorella* Species / Extract
Skin Conditioning	*Chlorella variabilis e*xtract, *Chlorella ellipsoidea* Extract, *Chlorella emersonii* Extract, *Chlorella minutissima e*xtract, *Chlorella minutissima o*il, *Chlorella Protothecoides, Chlorella Protothecoides o*il, *Chlorella pyrenoidosa e*xtract, *Chlorella pyrenoidosa p*owder, *Chlorella pyrenoidosa/sorokiniana e*xtract, *Chlorella sorokiniana e*xtract, *Chlorella sp. Ksf0006 e*xtract, *Chlorella sp. ksf0031 e*xtract, *Chlorella vulgaris e*xtract
Antimicrobial	*Chlorella ellipsoidea* extract
Hair Conditioning	*Chlorella protothecoides*
Skin Conditioning - Miscellaneous	*Chlorella protothecoides* extract, *Chlorella sp. hs2* extract, *Chlorella vulgaris* lysate filtrate, *Chlorella vulgaris* powder
Skin Conditioning - Emollient	*Chlorella protothecoides* oil, *Chlorella vulgaris* oil
Skin Protecting	*Chlorella protothecoides* oil, *Chlorella pyrenoidosa/sorokiniana* extract, *Chlorella sp. ksf0006* extract, *Chlorella sp. ksf0031* extract, *Chlorella vulgaris* callus culture extract
Antioxidant	*Chlorella pyrenoidosa/sorokiniana* extract, *Chlorella vulgaris* callus culture extract
Bleaching	*Chlorella pyrenoidosa/sorokiniana* extract
Skin Conditioning – Humectant	*Chlorella vulgaris* oil

2.4 Seborrheic Dermatitis

Seborrheic dermatitis is a widespread, chronic, inflammatory skin condition marked by scaling of the skin, which often returns despite effective therapy and significantly lowers quality of life (Clark et al., 2015). Its presence seems to be connected to the expansion of commensal *Malassezia* species (Garry G., 2013). It frequently affects more than one portion of the body; the scalp and face are the most frequently impacted regions. In order to effectively treat seborrheic dermatitis, a variety of antifungal, anti-

inflammatory, keratolytic, and immunomodulatory agents have been demonstrated; however, patients should be made aware that relapse is common and that treatment should continue (Kastarinen et al., 2014; Ooi and Tidman, 2014).

Symptoms are controlled using systemic, topical and combined methods. Antifungals, retinoids and phototherapy are systemic methods. As a topical method, various keratolytic agents such as salicylic acid, coal tar, juniper tar, selenium sulfide, zinc pyrithione are used. Combined treatments including topical steroids, lithium compounds, tea tree oil, aloevera extract, propylene glycol are also used for seborrheic dermatitis currently used in the treatment (Garry G., 2013).

2.5 Malassezia furfur

Malassezia furfur is a member of the lipid-dependent fungal species and is found in human and animal skin. It is known that *Malassezia* (M) species are responsible for various dermatological diseases, including seborrheic dermatitis (SD), pityriasis versicolor (PV) and *Malassezia folliculitis* (Vest and Krauland 2020). However, studies show that a reduction in the number of *Malassezia* with antifungal agents that heal symptoms of some skin conditions. The chosen treatment is more directed towards controlling exacerbations according to the severity of the disease, morphological changes and response to previous treatments (Garry G., 2013). In immunocompromised patients, *Malassezia* may act opportunistically and cause serious cutaneous and systemic infections (Vest and Krauland 2020).

2.6 Anti-Dandruff Effect of Tea Tree Oil

Umar et al. (2021) formulated an anti-dandruff shampoo with tea tree oil and performed its *in vitro* characterization. As a result of their study, they deduced that, it can compete with commercial anti-dandruff shampoos and has the potential to treat the dandruff problem (Umar et. al., 2021).

In a study by Nashwa et al. (2020), a comparative experiment was performed between irradiated human amniotic membrane and tea tree oil and topical tioconazole applied topically in the treatment of Pityrasis versicolor. As a result, they concluded that the therapeutic treatment developed by Nashwa et al. treated PV lesions and showed

higher activity than tioconazole which is an antifungal drug of the imidazole class used to treat infections caused by a fungus or yeast (Nashwa et. al., 2020).

In the study by Jain et al (2022), they summarized the recent findings regarding the antifungal properties of herbal essential oils against pathogens that play a role in dandruff prognosis. While essential oils of vegetable origin such as tea tree oil are gaining global importance in dermatology, these oils are rich in secondary aromatic metabolites, especially terpenes and phenolic compounds with important antimicrobial properties. As a result of the study, they deduced that essential oils, especially tea tree oil, have a very high potential to be developed as anti-dandruff products (Jain et. al., 2022).

2.7 Shampoo

Shampoo; although its main function is to clean the hair, to make the hair smell good and to improve the appearance of the hair, is a viscous substance that various components can be added (conditioner agents etc.) according to the function of the product.

In addition to the basic substances that should be in a shampoo, there are some substances that are added according to the function of the shampoo. These are detergents, foaming agents, conditioners, thickeners, opacifiers, fragrances, preservatives, bioactive components. Detergent removes dirt and excess sebum from the hair and hair scalp. Although foaming agents are used in formulations to increase the foam, the excess foam creates the perception of the consumer that the shampoo cleans better. While the hair conditioner makes the hair soft and smooth, it also provides easy styling to the hair. The thickening agents are used in shampoo formulation because consumers believe that a thick shampoo cleans hair better than a thin shampoo. Opacifying agents improve the appearance of the product by making the shampoo opaque in its translucent appearance. The fragrance substance is added to the shampoo to give a fragrance that will satisfy the consumer. Preservatives are added to extend the shelf life of the shampoo and prevent microbial contamination (Draelos 2013).

3
MATERIAL METHODS

3.1 Cultivation and Extraction of *Chlorella variabilis YTU.ANTANTARCTIC.001*

In this study, *Chlorella variabilis YTU.ANTARCTIC.001* (Genbank Access No: MN372092) which were isolated and described from samples obtained from Antarctica, Horseshoe Island, Skua Lake by the 3rd National Antarctic Science Expedition held on February 22, 2019 under the auspices of the Presidency and the Ministry of Industry and Technology, were grown and characterized in the Algal Biotechnology and Bioprocessing laboratory of Yildiz Technical University.

BG-11 medium was used for the cultivation of *Chlorella variabilis YTU.ANTARCTIC.001*. This medium is a general environment for freshwater blue green algae. In accordance with the procedure, the chemicals specified in Table 3.1 were added to 829 ml of pure water and the pH value was optimized to 7.1 with 1M NaOH and HCl. 15 g/L agar was added for the solid medium (Inan, 2019). The prepared media were sterilized by autoclaving at 121°C for 15 minutes, then the solid media were poured into petri dishes. In the first stage, stock cultures of *Chlorella variabilis YTU.ANTARCTIC.001* were prepared and solid medium was used for this purpose. Microalgae planted in solid media for stock preparation were left to grow at room temperature (~25 °C) and under 24-hour white light for 15-20 days (Figure 3.1). The grown microalgae were washed from petri dishes with distilled water and stored at a temperature of +4 C to be used as a stock.

Table 3.1 Chemicals used in BG-11 medium and concentrations

Chemical	Concentration (g/L)
NaNO3	1,5
K_2HPO_4	0,04
$MgSO_47H_2O$	0,075
$CaCl_2.2H_2O$	0,036
Citric Acid	0,006
Amonnium ferric citrate	0,006
Na_2EDTA	0,001
Na_2CO_3	0,02
H_3BO_3	0,00286
$MnCl_2.4H2O$	0,00181
$ZnSO_4.7H2O$	0,00022
$Na_2MoO_4.2H2O$	0,00039
$CuSO_4.5H2O$	0,000079
$Co(NO_3)_2.6H_2O$	0,00000494

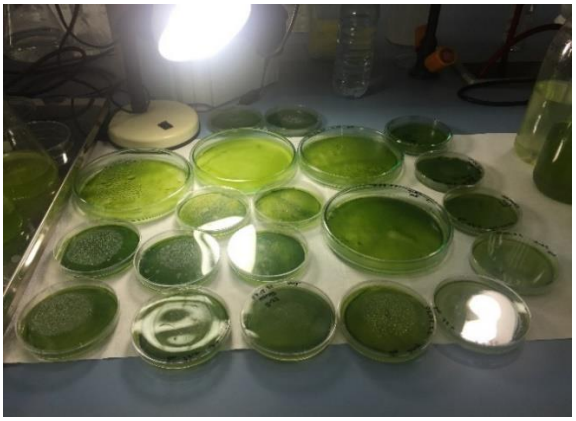
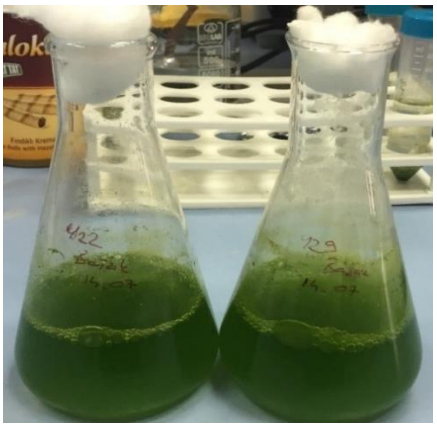


Figure 3.1 *Chlorella variabilis YTU.ANTARCTIC.001* grown in petri dishes

After the stock cultures were prepared, production was started in 250 mL flasks. At this stage, 10% inoculation was performed on 250 mL (working volume 100 mL) flasks (Figure 3.2). The solutions obtained after the pH values were adjusted to 7.01 and placed in a shaking water bath at 27°C. Microalgae were mixed for 10 days at a shaking speed of 150 rpm.

The growth of *Chlorella variabilis YTU.ANTARCTIC.001* was monitored by taking measurements at a wavelength of 680 nm with a PG Instruments T60 brand and model UV-Visible Spectrophotometer device (Koçer et al., 2021). Cultures reaching the appropriate concentration was grown in 5 L photobioreactors (Figure 3.2) (Sert et al. 2018). *Chlorella variabilis YTU.ANTARCTIC.001* under the microscope is given in Figure 3.3.



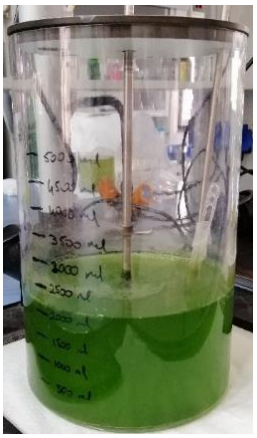


Figure 3.2 *Chlorella variabilis* grown in erlenmeyer and bioreactor

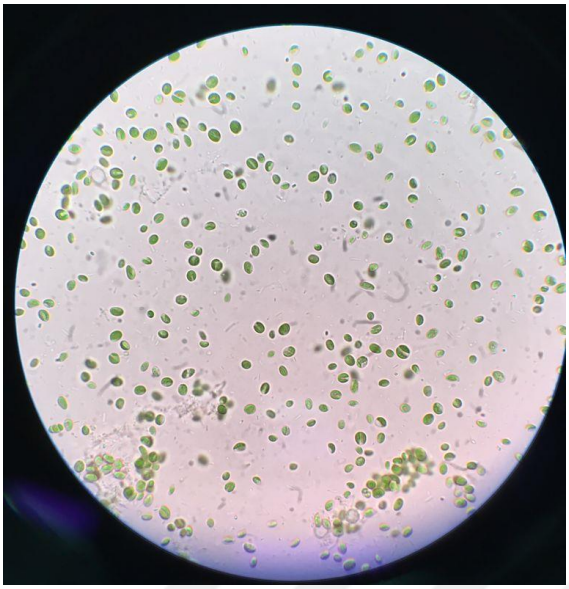


Figure 3.2 Microscope image of *Chlorella variabilis*

For measurement, 3 ml were taken from microalgae samples grown in Erlenmeyer flasks in liquid medium using a Pasteur pipette. Pure water was used as the 'blank' for this measurement. The growth rate (μ) and doubling time (td) of microalgae grown in liquid medium were calculated using the following equation 3.1 and 3.2 (Wang et al., 2010).

$$\mu = (lnxt - lnx0) / t1 - t0 \quad (3.1)$$

$$td = ln2 / \mu \quad (3.2)$$

$$t0 = 0$$

xt = optical density value measured at time t

x0 = optical density value measured at start time

μ = specific growth rate

3.2 Characterization of *Chlorella variabilis YTU.ANTARCTIC.001*

3.3 Carbohydrate Content

The phenol-sulfuric acid method was used to determine the total carbohydrate content of microalgal cells. For this, 80 gr phenol and 20 gr distilled water (80% w/w) were used. Glucose was used as the standard for this evaluation. 0.05 mL of phenol was added to glucose solutions of varying concentrations, then the sample tubes were kept at room temperature for 15 minutes by adding 5 mL of sulfuric acid (H_2SO_4). The absorbances of the samples were measured using a UV spectrophotometer at 490 nm and the results were determined by constructing a standard curve with the data obtained (Dubois et al., 1956). Analyzes were performed in triplicate.

3.4 Protein Content

The Lowry method was used to determine the protein content of the isolated *Chlorella variabilis YTU.ANTARCTIC.001* (Lowry et al., 1951). Briefly, standards in the range of 70-140 mg protein/L were obtained when the standard amount of BSA solution specified in the experiment was placed in glass test tubes and added to distilled water and 1 N NaOH solution.

After adding 1 N NaOH to the grown microalgal cells, the samples were kept in a 100 °C water bath for 5 minutes and then cooled in a cold water bath. Reagents C (reagent C is formed from a 100:1 mixture of reagents A and B. Reagent A is Na_2CO_3, NaOH, Na-tartrate; reagent B is $CuSO_4.5H_2O$) and reagent D (300 µl Folin-Phenol reagent) are added to the samples and mixed vortex was used. Absorbance values were measured with a spectrophotometer (750 nm) after a 30-minute rest period at room temperature (Lowry et al., 1951). Standards and reagents are given in Table 3.2.

Table 3.2 Standards and reagents used in the modified Lowry method

Unit: *ml*	Blank	St1	St2	St3	St4	St5	St6	Sample

Table 3.2 Standards and reagents used in the modified Lowry method (continued)

Standard BSA Solution	0	0.05	0.1	0.15	0.2	0.25	0.3	-
Distilled water	0.5	0.45	0.4	0.35	0.3	0.25	0.2	-
1 N NaOH	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Sample	-	-	-	-	-	-	-	0.5
Reagent C	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Reagent D	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

3.5 Lipid Content

For the extraction; the *Chlorella variabilis YTU.ANTARCTIC.001* were centrifuged at 8000 rpm after production, dried at 60°C and stored for the next step, extraction. For microalgae oil extraction, 10 g of dry biomass was weighed and reduced by pounding in a mortar. Microalgae samples were crushed into powder and extracted using ethanol as a solvent (Figure 3.4). For this, the samples were extracted with 100% ethanol solvent (MERCK brand) for 3 hours, the extraction process was carried out 3 times in total. The obtained extract was removed by evaporation at 78°C under vacuum in the evaporator to evaporate the ethanol. The amount of oil obtained was determined by weighing. The extract was stored in a closed and dark environment against any possibility of oxidation.

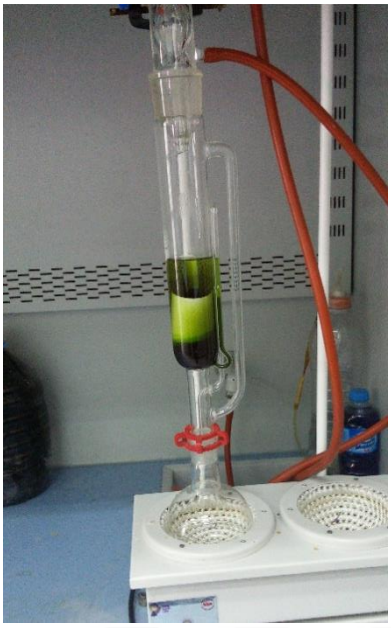


Figure 3.3 Extraction process of microalgae by Soxhlet method

Gas chromatography device was used for the characterization of the extracted oil. The gas chromatograph device used (YL Instruments 6100 GC) is a flame ionization detector (FID) and 30 m x 0.32 mm x 0.25 μm. Hydrogen was chosen as the carrier gas and internal standard methyl heptadeconate (C17:0) was used for its control. The sample was prepared using methyl heptadeconate and n-heptane (Bligh and Dyer, 1959). In the program, the starting temperature is determined as 50 °C and the other settings are as follows:

Initial high temperature: 175°C

Second high temperature: 230°C

Ramp speed: 15°C/min

Second ramp speed: 5°C/min

The temperature of the injector was set to 230°C and the flow rate was 1.8 mL/min. The detector temperature was kept constant at 280°C.

3.6 Spectrophotometric Pigment Analysis

β-carotene and chlorophyll-α are important bioactive compounds found in microalgae. Evaluation was made using the method of Zou and Richmond, and Macías-Sánchez et al. (Macías-Sánchez et al., 2005; Zou and Richmond, 2000). To determine the β-carotene and chlorophyll-α content, a 5 mL sample of microalgae was centrifuged and the supernatant discarded. Afterwards, the remaining pellet was homogenized with 5 mL of acetone in an ultrasonic bath for 5 minutes, then placed in a 50-60°C water bath and vortexed for 15 seconds after waiting for 5 minutes. The sample was then centrifuged again. The supernatant was analyzed using a spectrophotometer at 666 nm and 475 nm. Chlorophyll-α and β-carotene content were calculated by equations 3.3 and 3.4.

$$\text{Chlorophyll } \alpha \text{ (µg/ml)} = 13{,}9 \text{x} A_{666} \quad (3.3)$$

A_{666} =666 nm absorbance value (Macías-Sánchez vd., 2005)

$$\beta\text{-Carotene (µg/ml)} = 4{,}5 \text{x} A_{475} \quad (3.4)$$

A_{475} =475 nm absorbance value (Zou ve Richmond, 2000)

3.7 Shampoo Production

As for the formulation of shampoo, firstly, the determined amount of water was put into the beaker, then lauryl glycoside was added and the raw material was dissolved, and then xanthan gum was added slowly (Brooks G. and Franklin S., 2017). Then, in order, other raw materials; betaine PEG-40, Cocamide DEA, coconut oil, tea tree oil, *Chlorella variabilis YTU.ANTARCTIC.001* oil, sodium chloride, Polyquaternium-7, vitamin E, glycerin and EDTA were added. The pH-measured formulations were adjusted to the pH range of 5.5-6.5. The purpose was to optimize the amounts of lauryl glycoside and betaine by testing various shampoo formulations and observing the viscosity of the formulations.

Table 3.3 Raw material and concentration limits used in shampoo formulation

Madde	Leave-on	Rinse-off	Reference
Lauryl glucoside	There is no limit in literature.	There is no limit in literature.	https://www.cosmeticsinfo.org/ingredients/lauryl-glucoside/ http://ec.europa.eu/enterprise/cosmetics/inci/inci_2006.pdf
Xanthan gum	There is no limit in literature.	There is no limit in literature.	Food Ingredients and Packaging Substances Generally Recognized As Safe (GRAS) EU Cosmetics Inventory
Betaine	Max. 3%	-	https://www.cosmeticsinfo.org/ingredients/cocamidopropyl-betaine/ EU Cosmetic Ingredients Inventory
Cocamide DEA	Max. 10%	Max. 10%	Food and Drug Administration Food Ingredients and Packaging Substances Generally Recognized As Safe (GRAS) EU Cosmetic Ingredients Inventory https://www.cosmeticsinfo.org/ingredients/cocamide-dea/
PEG-40	There is no limit in literature.	There is no limit in literature.	https://www.cosmeticsinfo.org/ingredients/peg-40/ Food Contact Substances Search the Code of Federal Regulations EU Cosmetics Inventory
Polyquaternium-7	5%	5%	Search the Code of Federal Regulations EU Cosmetics Inventory https://www.cosmeticsinfo.org/ingredients/polyquaternium-7/
EDTA	There is no limit in literature	There is no limit in literature	https://www.cosmeticsinfo.org/ingredients/tetrasodium-edta/ Food Additives by the Food and Drug Administration Food Ingredients and Packaging Food Contact Substances FDA Overview of Food Ingredients, Additives & Colors EU Cosmetics Inventory http://ec.europa.eu/consumers/cosmetics/cosing/”>Search the Code of Federal Regulations

Table 3.3 Raw material and concentration limits used in shampoo formulation (continued)

Vitamin E (Tocopherol)	There is no limit in literature.	There is no limit in literature.	https://www.cosmeticsinfo.org/ingredients/tocopherol/ Food Contact Substances: Substances Generally Recognized As Safe (GRAS) Office of Dietary Supplements Search the Code of Federal Regulations EU Cosmetics Inventory
Tea tree oil (*Melaleuca alternifolia* oil)	-	2% (Hair products)	SCCS Opinion onTea Tree Oil
Citric acid	There is no limit in literature.	There is no limit in literature.	https://www.cosmeticsinfo.org/ingredients/citric-acid/ Food Ingredients and Packaging Food Contact Substances Code of Federal Regulations EU Cosmetics Inventory
Glycerin	79%	99%	https://www.cosmeticsinfo.org/ingredients/glycerin/ Listing of FDA-approved OTC drug active ingredients Search Title 21 of the U.S. Code of Federal Regulations (CFR) EU Cosmetics Regulations
Coconut oil	There is no limit in literature	There is no limit in literature	https://www.cosmeticsinfo.org/ingredients/hydrogenated-coconut-oil/ Food Ingredients and Packaging Food Contact Substances Substances Generally Recognized As Safe (GRAS) Search the Code of Federal Regulations EU Cosmetics Inventory

3.8 Stability of Shampoo Samples

The stability assessment was carried out in accordance with the ICH guidelines. Cosmetic products containing *Chlorella variabilis YTU.ANTARCTIC.001* extract and tea tree oil are placed in 50 ml centrifuge tubes and kept at 4±1°C in the refrigerator, at 25±1°C room temperature and in the incubator at 45±1°C for 8, 14 and 28 days, 3 months.

Organoleptic characteristics as appearance, color, smell, phase separation, viscosity and chemical (pH) and microbial changes will be evaluated (Adejokun and Dodou 2020, Moulai Mustefa et al. 2006);(Mahmood et al., 2018).

Determination of pH for each formulated cosmetic product model was performed using a benchtop pH meter with a single electrode, measuring temperature and pH, continuously stored in a 0.1 M HCl solution. After 8 days of product preparation, the measurement was obtained by rinsing the probe with deionized water after removing it from the 0.1 M HCl storage solution and placing it in the diluted test sample (0.05 mL of product is dissolved in 5 mL of deionized water using a 1 mL graduated syringe). The probe was held in place until a stable pH is reached. All measurements will be made in triplicate, taken at 25°C and repeated after 14 and 28 days, 3 months.

3.9 Antifungal Effect of Shampoo Samples

3.10 *Malassezia furfur* Growth Conditions and Inoculum Preparation

Malassezia furfur (KCCM 12679) was supplied from the KIMEKS. This strain was grown on Sabouraud Dextrose Agar (SDA) (Merck), following incubation at 30°C during 2-7 days. *Malassezia* strains were maintained on the same medium for antimicrobial activity assay. The same medium was used in all the experiments. Inoculum suspensions prepared by 1 ml of 48 h culture was centrifuged (3000 g at 4°C for 1 min), followed by washing the pellets with phosphate buffered saline (PBS). *Malassezia* furfur were formed upon preparation of inoculum suspensions and more accurate turbidity measurements (Lee and Lee 2010).

3.11 Agar Well Diffusion Assay

In this method, an agar plate (SDA) is inoculated by the pathogenic fungal strain using the spreading plate technique. A well (6 mm in diameter) is then created aseptically with a sterile cork drill. Next, the well should be filled with antifungal and control shampoo, and algae extract. Then petri dishes are incubated at 30°C for 24 hours. When incubated, the antimicrobial extract solution slowly diffuses from the agar medium and inhibits the growth of the tested fungal species. Finally, the zone of inhibition can be observed and the diameter of the zone taken as a measurement (Filip et al., 2010).

3.12 Statistical analysis

A one-way analysis of variance (ANOVA) (JMP version 6.0.0) was performed and Student's t-test was used to reveal statistical differences ($p<0.05$) between samples.

4
RESULTS AND DISCUSSION

4.1 Cultivation of *Chlorella variabilis YTU.ANTARCTIC.001*

Absorbance values of the growth of *Chlorella variabilis YTU.ANTARCTIC.001* for 10 days are given in Figure 4.1. According to these values, the growth rate (μ) of *Chlorella variabilis YTU.ANTARCTIC.001* was 0.11 day^{-1}; doubling time (td) was calculated as 6.18 day.

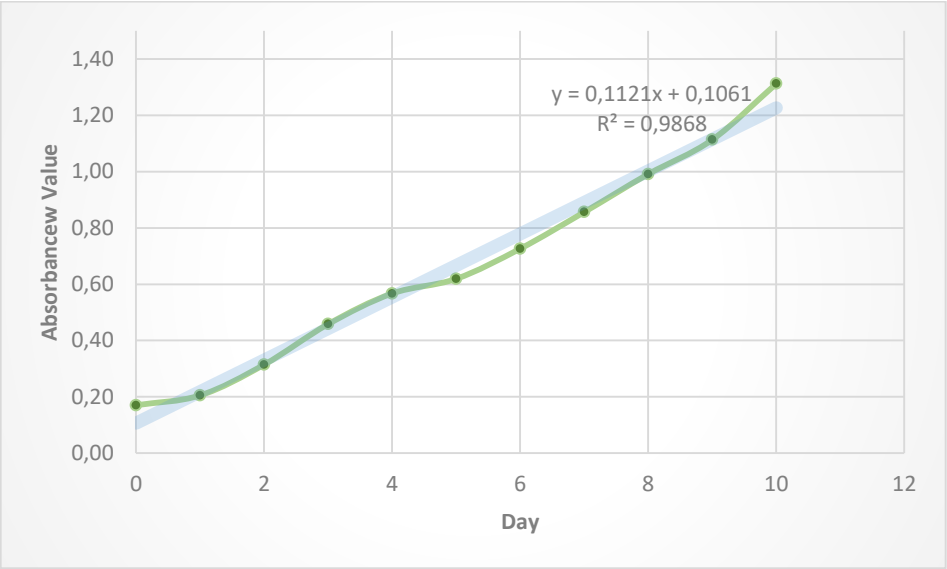


Figure 4.1 *Chlorella variabilis YTU.ANTARCTIC.001* growth graph (absorbance value versus day)

4.2 Characterization of *Chlorella variabilis YTU.ANTARCTIC.001*

The carbohydrate, lipid and protein contents of the prepared stock cultures were determined using the Phenol-Sulfuric Acid method (Dubois et al., 1956), The Bligh-Dyer method (Bligh and Dyer, 1959) and Lowry Method (Lowry et al., 1951), respectively. According to these analyzes, carbohydrate, lipid and protein contents of stock *C. variabilis* were determined as 28%, 49% and 21%, respectively (Table 4.1).

Table 4.1 Biochemical content of *Chlorella variabilis YTU.ANTARCTIC.001*

	Carbohydrate(%)	Protein (%)	Lipid (%)	Clorophyll-α (μg/ml)	β-Carotene (μg/ml)
C. variabilis	28±1.3	21±1.2	49±1.1	3,14±0.1	1,51±0.01

When the chromatogram obtained as a result of gas chromatography analysis was examined, the presence of palmitic (16:0), oleic (18:1), linoleic (18:2) acids in the content of *Chlorella variabilis YTU.ANTARCTIC.001* oil is observed. Other fatty acids were not evaluated in the results as they were present in trace amounts. According to the obtained chromatogram, the highest fatty acid content was seen as linoleic acid (Figure 4.2).

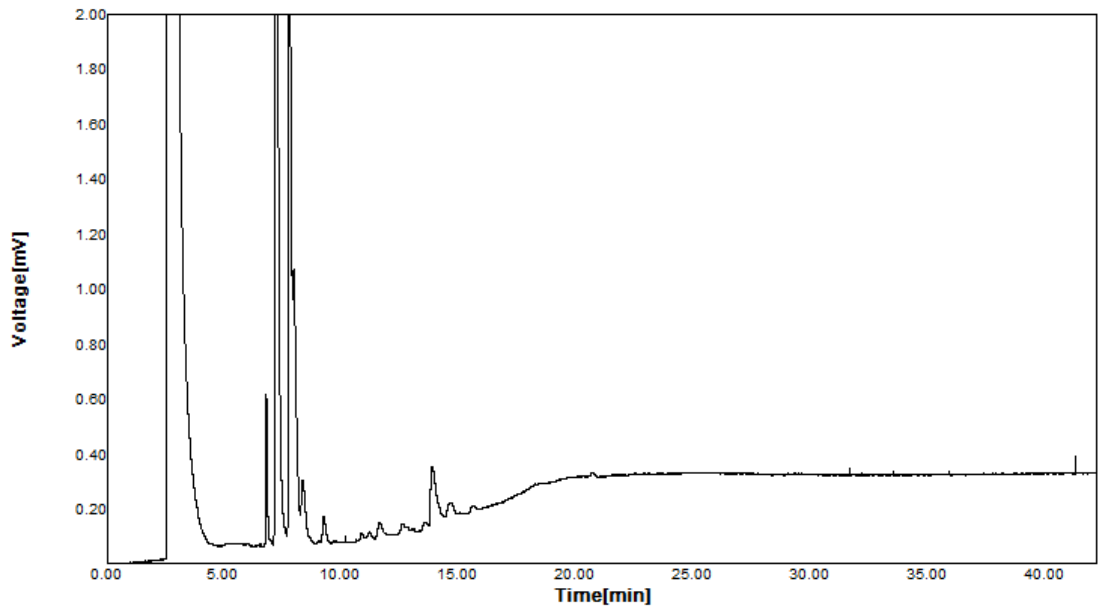


Figure 4.2 Chromatogram of methyl ester of *Chlorella variabilis* oil

4.3 Shampoo Production

Based on the formulations and patents in the literature, it is aimed to obtain a good viscosity by optimizing the amounts of lauryl glycoside and betaine in shampoo formulations, taking into account the conformed concentrations of the raw materials (Brooks G. and Franklin S., 2017).

The first shampoo trials, raw materials and used concentrations are shown in Table 4.2.

Table 4.2 Shampoo trials (1)

No	1	2	3	4	5	6	7	8
Aqua	75	74.35	71.35	73.35	72.35	73.35	76.1	74.1
Lauryl glucoside (8-12%)	10	12	15	13	14	14	12	12
Betaine (2-5%)	4	5	5	5	5	4	5	5
PEG-40 (2-3%)	3	1	1	1	1	1	3	3
Citric Acid (qs)	qs	qs	qs	qs	qs	qs	qs	qs
Cocamide DEA (0.1-1%)	1	1	1	1	1	1	1	1
Coconut Oil (0.1-1%)	1	1	1	1	1	1	-	-
Tea Tree Oil (0.05-0.2%)	0.1	0.1	0.1	-	-	-	0.1	0.1
Chlorella variabilis YTU.ANTANTARCTIC.001 (0.1-1.5)	1.5	-	-	-	-	-	-	-
Sodium Chloride (0.1-0.3%)	0.9	0.3	0.3	0.3	0.3	0.3	0.3	0.54
Polyquaternium-7 (0.1-1)	1	1	1	1	1	1	1	1
Vitamin E (0.1-0.5)	0.1	0.1	0.1	0.1	0.1	0.1	-	-
Xanthan gum	1	1	1	2	2	2	2	2.3
Glycerin (1-3%)	1	3	3	3	3	3	1	3
EDTA (0.05-0.15)	0.15	0.15	0.15	0.15	0.15	0.15	0.5	0.5

It was observed by organoleptic methods that the 2nd formulation was more similar to commercial shampoos in terms of appearance, color, homogeneity and viscosity compared to other formulations. In other trials, optimization was made based on the 2nd trial. Citric acid was added to adjust the pH of the shampoo between 4.5 and 5.5.

As a result of the first trials, it was seen that Xanthan Gum did not dissolve. The viscosity of the shampoo was not as desired. When by slowly adding xanthan gum after the lauryl glycoside addition, problem was solved. It has been reported in the literature that Xanthan Gum increases viscosity when added with salt (Zatz and Knapp 1984).

By adding *Chlorella variabilis YTU.ANTARCTIC.001* to the 1st formulation, its appearance was observed clearly. Therefore, it was decided to add *Chlorella variabilis YTU.ANTARCTIC.001* oil by optimizing the base formulation. (Figure 4.3).

Figure 4.3 Shampoo trials appearance (1,2,3)

A1-A2-A3 samples were prepared according to the ratio of the 2nd sample, and different amounts of cocamide DEA were used to test the effect of the amount of A1-A2-A3 cocamide DEA on the consistency. The expected viscosity of A3 was better than compared with A1 and A2 (more viscous, non-flowing).

As a result, the viscosity of A1 was closer to commercial shampoos than other samples. From here, the following inferences can be made with the amount of cocamide DEA:

Cocamide DEA (diethanolamide) is a common ingredient in personal care products such as shampoos, soaps, and lotions. It is used as a foam booster, emulsifier, and thickening agent.

One of the properties of cocamide DEA is its ability to increase the viscosity, or thickness, of a product. This makes it useful for creating products with a desired texture, such as thick and creamy shampoos or lotions.

Cocamide DEA works by interacting with other ingredients in the product to create a network of molecules that traps and holds water, making the product thicker and more viscous. The amount of cocamide DEA used will affect the viscosity of the final product, so it is important to use the appropriate amount for the desired texture (Cocamide, D. E. A., 1986).

Densities of raw materials may vary depending on the company from which they are purchased. The purity of Cokamide DEA purchased for this thesis study was not sufficient to increase the viscosity.

It can be said that the viscosity decreases as the amount of cocamide DEA increases.

According to the results of the trials made here, it was found that the amount of cocamide DEA can be used at most 2%.

In addition, when the pH values are examined, we can say that the pH increases as the amount of cocamide DEA increases.

A6, A7, A8 and A9 samples were prepared. Other prepared samples were stored at three different temperatures at +4 °C, 25 °C and 45 °C for 28 days. Organoleptic properties such as pH, viscosity were investigated for 7, 14 and 28 days, 3 months.

The appearance of the samples was quite good, their semi-transparent viscosities are quite good, but if we list them;

A8 > A9 > A7 > A6 (the most fluid A6)

It can be seen that the amount of betaine in sample A8 is the element that improves the viscosity more than the others (Vander et. al., 2013).

Chlorella variabilis YTU.ANTARCTIC.001 oil was added to A6, A7, A8 and A9 formulations and AA6, AA7, AA8 and AA9 shampoo trials were carried out, respectively. AA6, AA7, AA8 and AA9 shampoo samples were shown in Figure 4.4.

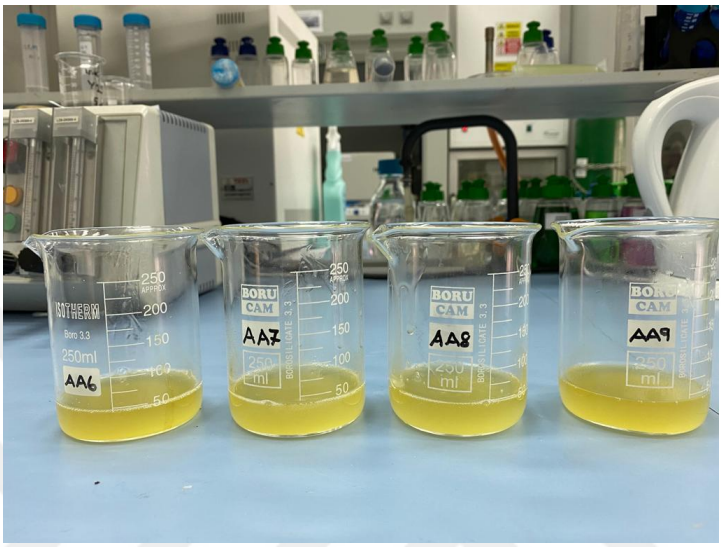


Figure 4.4 Shampoo trials with *Chlorella variabilis YTU.ANTARCTIC.001* oil

Tea tree oil was added to A6, A7, A8 and A9 formulations and TA6, TA7, TA8 and TA9 shampoo trials were carried out, respectively.

Shampoo formulations A8, AA8 and TA8 with the best appearance, viscosity and pH were placed in eppendorf tubes for stability tests.

4.4 Stability of Shampoo

The stability of the prepared base shampoo, algae oil shampoo and tea tree oil shampoo was investigated. The stability results of the samples are given in the Table 4.3, Table 4.4 and Table 4.5.

Table 4.3 Base shampoo stability results

	Parameters	**Product Specification**	**Initial**	**1st week**	**4th week**	**3 months**
+4°C (Refrigerator)	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic

Table 4.4 Base shampoo stability results (continued)

	pH	**3.5-5.5**	**5.5**	**5.5**	**5.5**	**5.5**
	Colour	White	White	White	White	White
25°C Room temperature	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5.5	5.5	5.5	5.5
	Colour	White	White	White	White	White
45°C (Incubator)	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5.5	5.5	5.5	5.5
	Colour	White	White	White	White	White

Table 4.5 Tea Tree Oil Shampoo Stability Results

	Parameters	**Product Specification**	**Initial**	**1st week**	**4th week**	**3 months**
+4°C (Refrigerator)	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5	5	5	5
	Colour	White	White	White	White	White
25°C Room temperature	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5	5	5	5
	Colour	White	White	White	White	White
45°C (Incubator)	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5	5	5	5
	Colour	White	White	White	White	White

Table 4.6 *Chlorella variabilis YTU.ANTANTARCTIC.001* oil shampoo stability results

	Parameters	**Product Specification**	**Initial**	**1st week**	**4th week**	**3 months**
+4°C (Refrigerator)	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None

Table 4.7 *Chlorella variabilis YTU.ANTANTARCTIC.001* oil shampoo stability results (continued)

	Odour	**Characteristic**	**Characteristic**	**Characteristic**	**Characteristic**	**Characteristic**
	pH	3.5-5.5	5.5	5.5	5.5	5.5
	Colour	Pale green	Pale green	Pale green	Pale green	Pale green
25°C Room temperature	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5.5	5.5	5.5	5.5
	Colour	Pale green	Pale green	Pale green	Pale green	Pale green
45°C (Incubator)	Appearance	Semitransparent	Semitransparent	Semitransparent	Semitransparent	Semitransparent
	Settle	None	None	None	None	None
	Odour	Characteristic	Characteristic	Characteristic	Characteristic	Characteristic
	pH	3.5-5.5	5.5	5.5	5.5	5.5
	Colour	Pale green	Pale green	Pale green	Pale green	Pale green

According to the findings of the stability test, the manufactured shampoo samples are stable for 3 months. As a result of the accelerated stability test, the shelf life of the products corresponds to 3 years.

4.5 Antibacterial and Antifungal Effect of Shampoo

According to the results antibacterial activity increased with the increase in the concentration of *Chlorella variabilis YTU.ANTARCTIC.001* oil (Table 4.6). When the results were evaluated, *Chlorella variabilis YTU.ANTARCTIC.001* showed more antibacterial effect on gram positive bacteria compared to gram negative bacteria. In direct proportion to the increase in the amount of addition, an increase in the inhibition zone was also observed. The inhibition zone diameters for *E.coli* of the extract obtained from 30, 20 and 10 μL microalgae oil were 1.9 cm, 1.7 cm and 1.0 cm, respectively; it was recorded as 2.5 cm, 1.7 cm and 1.2 cm for *S.aureus*, respectively.

Table 4.8 Inhibition zones of *Chlorella variabilis YTU.ANTARCTIC.001* against *E. coli* and *S. aureus*

Extract(µL)/ Bacteria species	***E. Coli (cm)***	***S. aureus (cm)***
10 µL	1.0	1.2

Table 4.9 Inhibition zones of *Chlorella variabilis YTU.ANTARCTIC.001* against *E. coli* and *S. aureus* (continued)

20 µL	1.7	1.7
30 µL	1.9	2.5

Few studies have been carried out in the literature the biological activity of essential oils on *M. furfur* (Lee and Lee 2010) (Pooja, A., Arun, N., & Maninder 2013). Lee et al. evaluated the inhibitory effect of essential oils on the growth of *M. furfur* using the disk diffusion method and reported that 17 plant essential oils had inhibitory activity against *M. furfur* at 2 mg/ml. (Lee and Lee 2010).

Figure 4.5 shows the inhibition zones formed by the base shampoo, algae oil and algae oil shampoo formulation.

It is seen that the inhibition zone of the shampoo with *Chlorella variabilis YTU.ANTARCTIC.001* extract shows greater inhibition than the inhibition zone of the base shampoo and algae oil.

While it is expected that the algae oil alone will show a higher inhibition zone, it is thought that the higher inhibition of the algae oil shampoo formulation may provide more accurate data for the interpretation of the experiments by repeating.

Figure 4.6 shows the inhibition zones of the shampoo formulation prepared with commercial shampoo, tea tree oil and tea tree oil.

Tea tree oil appears to show a greater zone of inhibition against Malassezia furfur than the tea tree oil shampoo formulation.

Figure 4.7 shows the inhibition zones of the shampoo formulation prepared with base shampoo, tea tree oil and tea tree oil.

It is seen that the base formulation also creates a zone of inhibition on its own.

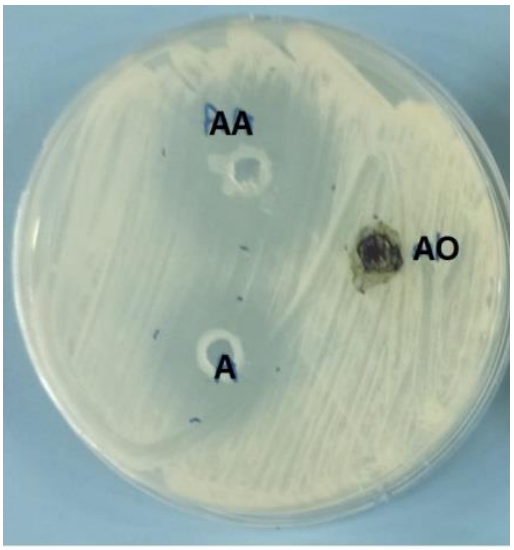


Figure 4.5 Antifungal activity of samples against *M. furfur* by agar well diffusion assay (base shampoo, *Chlorella variabilis YTU.ANTARCTIC.001* extract, shampoo with *Chlorella variabilis YTU.ANTARCTIC.001* extract)

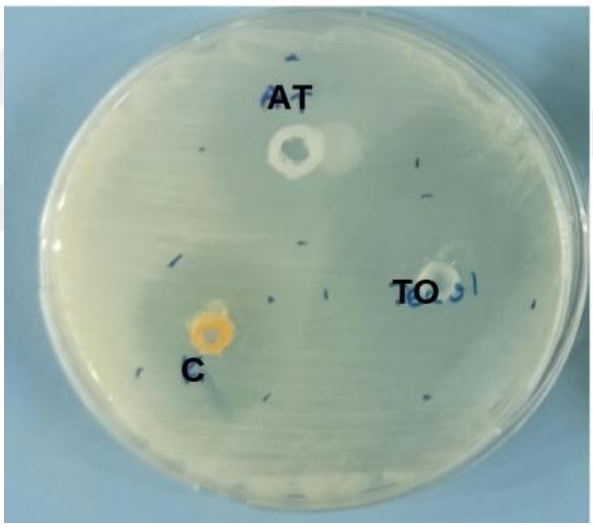


Figure 4.6 Antifungal activity of samples against *M. furfur* by agar well diffusion assay (commercial shampoo, tea tree oil, shampoo with tea tree oil)

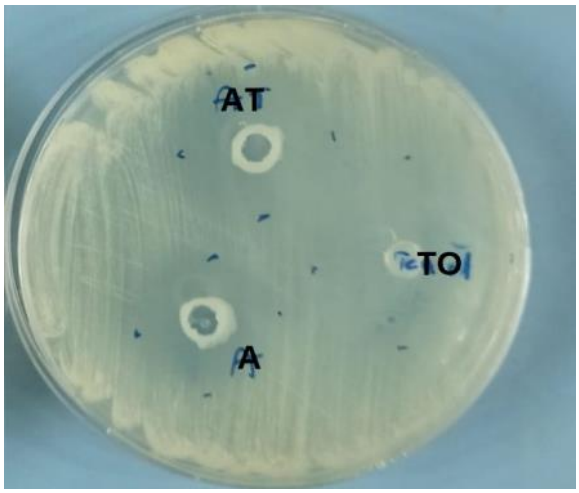


Figure 4.7 Antifungal activity of samples against *M. furfur* by agar well diffusion assay (base shampoo, tea tree oil, shampoo with tea tree oil)

A: Base shampoo Formulation

AO: *Chlorella variabilis YTU.ANTARCTIC.001* oil extract

AA: Shampoo with *Chlorella variabilis YTU.ANTARCTIC.001* oil extract

C: Commercial anti-dandruff shampoo

TO: Tea tree oil

AT: Shampoo with tea tree oil

Inhibition zone of each sample against Malassezia furfur is given in Table 4.7.

Table 4.10 Inhibition zone (mm) of each samples against *M. furfur* (n=3)

Samples	Inhibition zone (mm)
AA	$33,66 \pm 0,57^{A}$
AO	$10,33 \pm 0,57^{C}$
A	$23,66 \pm 1,52^{B}$
AT	$32,33 \pm 2,51^{A}$
TO	$32,00 \pm 0,00^{A}$
C	$26,83 \pm 0,76^{B}$

*A–C: Within each column, different superscript uppercase letters show differences between the samples ($p < 0.05$).

According Table 4.7, in the thesis, antifungal shampoo prepared with algae extract (AA=33.66 mm) showed a higher inhibition zone against *M. furfur* compared to shampoo without *Chlorella variabilis YTU.ANTARCTIC.001* oil (A=23.66). As a result, it has been observed that algae extract has an enhancing effect on the antifungal effect of the shampoo. In addition, higher inhibition was observed in the antifungal shampoo prepared with algae extract compared to the commercial shampoo (C).

Tea tree oil is a natural anti-dandruff ingredient commonly used in many formulations such as anti-dandruff shampoos, creams, and lotions (Pooja, A., Arun, N., & Maninder 2013). Nenoff et al., in their study in 1996, showed that tea tree oil inhibited the growth of *M. furfur*.

In the Rekha & Prabhu's study, it was found that shampoos containing piroctone olamine and zinc pyrithione were more effective than other herbal shampoo samples. However, according to the Cosmetic regulation 1223/2009, the use of zinc pyrithione in cosmetic products is prohibited (Regulation (EC) No 1223/2009). It was found that shampoos with herbal active ingredients in the study showed anti-pityrosporum activity. It was concluded that herbal shampoos are safer and more effective than synthetic dandruff inhibitors (Rekha, & Prabhu 2022).

In this study, another antifungal shampoo (AT) was prepared using tea tree essential oil (TO). It has been observed that this prepared shampoo has a similar inhibition zone against *M. furfur* with the shampoo prepared with *Chlorella variabilis YTU.ANTARCTIC.001* extract.

In another study, it is reported that anti-dandruff shampoo containing 5% tea tree essential oil is effective in the treatment of dandruff (Andrew et al., 2002). According to SCCS opinion 5% tea tree oil concentration is not safe (SCCS).

Anova results of samples is given in Table 4.8.

Table 4.11 Analysis of variance ANOVA values was performed using the JMP (release 6.0.0, SAS) and the significance between the averages of samples

Source	Sum of Squares	df	Mean Square	F Ratio
Model	878,41	5	175,68	56,97
Error	18,50	6	3,08	Prob > F
C. Total	896,91	11		0.0001*

*$p<0.05$ was considered significant

As a result of the ANOVA analysis, a significant difference was found between the samples.

Although synthetic anti-dandruff shampoos have a better anti-dandruff effect than herbal shampoos, their usage area is limited because they cause skin burning, skin irritation and hair loss. For this reason, while showing an anti-dandruff effect in the consumer, on the other hand, the tendency to shampoos with non-synthetic herbal ingredients that do not cause irritation to the skin has increased (Rekha, & Prabhu 2022).

While shampoo with *Chlorella variabilis YTU.ANTARCTIC.001* extract has both anti-dandruff effect, it is predicted that it will attract the attention of consumers with its moisturizing and antioxidant properties.

Consequently, algae-derived anti-fungal agents are of increasingly interest for the development of new, more effective and specific anti *Malassezia furfur* agents.

Why tea tree oil show a greater zone of inhibition against *Malassezia furfur* than the tea tree oil shampoo formulation is that the effectiveness of tea tree oil in inhibiting the growth of *Malassezia furfur* may be influenced by various factors, such as concentration, purity, and formulation. One reason why tea tree oil may show a greater zone of inhibition against *Malassezia furfur* than a tea tree oil shampoo formulation is due to the concentration of the active ingredient.

Tea tree oil is typically used in a highly concentrated form, whereas shampoo formulations often contain lower concentrations of the active ingredient to reduce the risk of skin irritation. The lower concentration of tea tree oil in the shampoo may therefore result in a weaker inhibitory effect on *Malassezia furfur*.

Additionally, the formulation of the shampoo may also affect its effectiveness. The other ingredients in the shampoo formulation may interfere with the ability of tea tree oil to penetrate the cell wall of the fungus and inhibit its growth. Moreover, the duration of contact of the shampoo on the skin may be shorter than the duration of contact of the tea tree oil used directly, which could also contribute to the difference.

For example, coconut oil has been shown to have antimicrobial activity against fungi and other microorganisms, and can help moisturize the scalp. Citric acid can help regulate the pH of the shampoo formulation, which can help inhibit fungal growth. Xanthan gum can help thicken the shampoo and provide a better consistency, while betaine and glycerin can help moisturize and soothe the scalp.

However, to have a significant antifungal effect, an additional active ingredient would need to be added to the shampoo formulation. Common antifungal agents used in shampoos include ketoconazole, zinc pyrithione, and selenium sulfide. These agents work by inhibiting the growth and replication of fungi, and can help control conditions such as dandruff and seborrheic dermatitis.

Therefore, while the shampoo formulation you listed contains ingredients that can help support healthy hair and scalp, it would need to be combined with an antifungal agent to provide an antifungal effect. This may explain the higher inhibition zone of the algae oil shampoo formulation compared to the base formulation.

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PUBLICATIONS FROM THE THESIS

Conference Papers

Akın, B., İnan, B., Koçer, A. T., Eyier, Ş. M., Ünlü, İ. D., Vehapi, M., Özçimen, D., " Developing Anti-Seborrheic Effective Shampoo Formulation with Polar Algae Extract And Review Antifungal Activity " 6th International Cosmetic Congress, 1 December 2022-3 December 2022 Antalya, Istanbul.

Eyier, Ş. M., Akın, B., İnan, B., Koçer, A. T., Özçimen, D., " Kutup Mikroalgleri: Medikal Kremler için Yeni Bir Biyo-etken Madde Kaynağı" 5. Ulusal Kutup Bilimleri Çalıştayı 30 Kasım 2021 Gebze, Istanbul

İnan, B., Akın, B., Ünlü, İ. D., Koçer, A. T., Çelik, A., Vehapi, M., Özçimen, D., "Growth and biochemical response of Chlorella variabilis YTU.ANTANTARCTİC.001 to temperature and Nitrogen Stress" Algal Biomass, Biofuels & Bioproducts, 14 June 2021-16 June 2021 Online.

Paper 1 İnan, B., Çelik, A., Akın, B., Ünlü, İ. D., Koçer, A. T., Vehapi, M., Özçimen, D., "Determination of Antioxidant Activity and Phenolic Content of Antantarctic Microalgae" Algae Biomass Summit 14 October 2021 Online.

Papers

İnan, B., Akın, B., Ünlü, İ. D., Koçer, A. T., Çelik, A., Vehapi, M., ... & Özçimen, D. (2023). Interactive effects of cold and temperate conditions on growth and biochemical content of Antantarctic microalga *Chlorella variabilis YTU.ANTANTARCTIC.001.* Journal of Applied Phycology, 1-13.

Projects

1. 120Z292, Antarktika Bölgesi Mikroalglerinden Elde Edilen Biyoaktif Bileşenlerce Zengin Mikroalgal Yağ Yüklü Nanopartiküllerin Üretimi ve Nöroprotektif Etkilerinin Araştırılması, 1001 - Araştırma, Burslu, Yürürlükte, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 6/1/21 - 8/15/22, Project Start/End Dates: 2/15/21 - 8/15/22.

2. Project No: FYL-2021-4805, Kutup Alglerinin Kozmetik Uygulamaları, Thesis Project Yildiz Technical University The Scientific Research Projects Coordination Unit