ISTANBUL MEDENIYET UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENTS OF MOLECULAR BIOLOGY AND GENETICS

Characterization of Exopolysaccharides Produced by Psychrotolerant *Chlamydomonas* sp. ASYA25 for Biomaterial Production

Master's Thesis

Kader Bozuklu

January, 2024

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THESIS JURY APPROVAL

This master's thesis titled "Characterization of Exopolysaccharides Produced by Psychrotolerant *Chlamydomonas* sp. ASYA25 for Biomaterial Production." written by Kader Bozuklu at the Department of Molecular Biology and Genetics was accepted by our jury.

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STATEMENTS

Style and Reference Manual Statement

Having reviewed this thesis written under my supervision, I confirm that it has been written in accordance with APA Manual of Style and used its in text reference format consistently throughout the entire text.

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Declaration of Originality

I hereby declare that all information in this dissertation has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conducts, I have fully cited and referenced all material and results that are not original to this work.

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Kader BOZUKLU

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LIST OF SYMBOLS

°C: Degree Celsius

CFU/ml: Colony Forming Unit per Milliliter

g: Gram

g: g force or rcf

km^2: Square Kilometer

kDa: Kilodalton

L: Liter

µg: Microgram

µl: Microliter

mg: Milligram

ml: Milliliter

min: Minute

M: Molarity

mM: Millimolar

Nm: Nanometer

%: Percentage

w/w: Weight by weight

LIST OF ABBREVIATIONS

ATP: Adenosine Triphosphate

BBM: Bold Basal Medium

BG-11: Blue-Green Medium

BLAST: Basic Local Alignment Search Tool

BSA: Bovine Serum Albumin

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 2,2-Difenil-1-Pikrilhidrazil

DSC: Differential Scanning Calorimetry

EPS: Exopolysaccharide

FTIR: Fourier Transform Infrared Spectrophotometry

HPLC: High Pressure Liquid Chromatography

LB: Lightly Bound

m-HDP: meta-Hydroxyphenyl

NMR: Nuclear Magnetic Resonance

PCR: Polymerase Chain Reaction

PEG: Polyethylene Glycol

PMP: 1-Phenyl-3-methyl-5-pyrazolone

rDNA: Ribosomal deoxyribonucleic acid

SDS: Sodium Dodecyl Sulfate

TAE: Tris-Acetate-EDTA

TAP: Tris Acetate Medium

TB: Tightly Bound

TCA: Trichloroacetic Acid

TGA: Thermogravimetric Analysis

TFA: Trifluoroacetic Acid

UV: Ultraviolet

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ABSTRACT

Characterization of Exopolysaccharides Produced by Psychrotolerant *Chlamydomonas* sp. ASYA25 for Biomaterial Production

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Supervisor: Prof. Dr. Turgay ÇAKMAK

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Microalgae are photosynthetic microorganisms that can survive in various habitats like oceans, freshwater, deserts, and snow surfaces with their adaptation capabilities. Microalgae have been exploited in biotechnology industry especially food, medical, pharmaceutical, and energy sectors due to their rich metabolite profiles. Exopolysaccharide (EPS) is amongst the value-added metabolites that microalgae produce to protect themselves against unfavorable conditions. EPS production is not pronounced in green microalgae; yet, some extremophilic microalgae were reported to produce substantial amount of EPSs. This thesis covers the characterization of EPSs synthesized by psychrotolerant *Chlamydomonas* sp. ASYA25 isolated from Antarctica and evaluation of their potential for biomaterial formation.

The microalgae were isolated from Horseshoe Island during the IV National Antarctic Science Expedition and identified as *Chlamydomonas* sp. ASYA25 by Sanger Sequencing method was maintained in the IMU-ASYA culture collection. Firstly, the growth behavior was monitored at different temperatures in the media determined to be suitable for growth by growing in different nutrient media and thus growth conditions were optimized. *Chlamydomonas* sp. ASYA25 was considered to have EPS production potential due to its gel-like appearance, and this was confirmed by a screening study with species in the culture collection. Then, the cultivation conditions were optimized by Plackett-Burman experimental design method to maximize EPS production. As a result, salinity and high light intensities were identified as factors that had a significant effect on EPS production. Within the scope of characterization studies, the composition of EPS was evaluated by measuring total saccharide, protein, sulfate, and uronic acid contents. The monosaccharide profile was evaluated by HPLC

analysis, while structural and thermal properties were evaluated by FTIR, TGA, and DSC analyses. After the evaluation of antioxidant and antibacterial activities, the potential of EPS to form biomaterials was evaluated. Lastly, the potential of EPS to form film material by solvent casting method and to form a hydrogel by the addition of physical or chemical crosslinkers were evaluated.

As a result of this study, it was observed that EPS production could be increased by 48% with salt and high light stress applications. The main composition of EPS was determined as 35% total saccharide, 4.5% protein, 1.6% sulfate, and 6.2% uronic acid. HPLC analysis showed that the monosaccharide profile of EPS was composed mainly of mannose, galactose, ribose, arabinose or xylose, and glucose. Moreover, EPS showed 70% DPPH scavenging activity at 2 µg/ml concentration and antibacterial activity against *S. aureus* at 10 mg/ml concentration with a 13 mm inhibition zone. In addition, the potential of using EPSs obtained from microalgae, which are considered as renewable resources, for the creation of biomaterials has been examined and confirmed. For film formation, 3% EPS concentration was determined as optimal level. In order to induce hydrogel formation, 1.5% and 1% EPS concentration was convenient as shown by physical crosslinking and chemical crosslinking methods, respectively. Overall, the bioactive exopolysaccharides produced by psychrotolerant green microalga *Chlamydomonas* sp. ASYA25 exhibit potential to be exploited for biomaterial production.

Keywords: Antarctica, Biomaterial, Exopolysaccharides, Microalgae, Polymer

ÖZET

Biyomateryal üretimi için psikrotolerant *Chlamydomonas* sp. ASYA25 tarafından üretilen ekzopolisakkaritlerin karakterizasyonu

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Mikroalgler, adaptasyon yetenekleri ile okyanuslar, tatlı sular, çöller ve kar yüzeyleri gibi çeşitli habitatlarda yaşayabilen fotosentetik mikroorganizmalardır. Mikroalgler, zengin metabolit profilleri nedeniyle biyoteknoloji endüstrisinde, özellikle gıda, tıp, ilaç ve enerji sektörlerinde kullanılmaktadır. Ekzopolisakkarit (EPS), mikroalglerin kendilerini olumsuz koşullara karşı korumak için ürettikleri katma değerli metabolitler arasındadır. EPS üretimi yeşil mikroalglerde yaygın değildir; ancak bazı ekstremofilik mikroalglerin önemli miktarda EPS ürettiği bildirilmiştir. Bu tez, Antarktika'dan izole edilen psikrotolerant *Chlamydomonas* sp. ASYA25 tarafından sentezlenen EPS'lerin karakterizasyonunu ve biyomateryal oluşturma potansiyellerinin değerlendirmesini kapsamaktadır.

IV Ulusal Antarktika Bilim Seferi sırasında Horseshoe Adası'ndan izole edilen ve Sanger Dizileme yöntemi ile *Chlamydomonas* sp. ASYA25 olarak tanımlanan mikroalg IMU-ASYA kültür koleksiyonunda muhafaza edilmektedir. Öncelikle, farklı besi ortamlarında büyütülerek belirlenen uygun besi ortamında farklı sıcaklıklarda büyüme davranışı izlenmiş ve böylece büyüme koşulları optimize edilmiştir. *Chlamydomonas* sp. ASYA25'in jelli görüntüsünden ötürü EPS üretim potansiyeline sahip olduğu düşünüldü ve bu durum kültür koleksiyonundaki mikroalgler üzerine yapılan tarama çalışması ile doğrulandı. Ardından, kültür koşulları EPS üretimini maksimize etmek için Plackett-Burman deney tasarım yöntemi kullanılarak optimize edildi. Sonuç olarak tuzluluk ve yüksek ışık şiddetinin EPS üretimi üzerine belirgin bir etkiye sahip olduğu görüldü. Karakterizasyon çalışmaları kapsamında, EPS'nin kompozisyonu total sakkarit, protein, sülfat ve üronik asit içeriklerinin ölçülmesiyle belirlendi. Monosakkarit profili HPLC analizi ile

belirlenirken, yapısal ve termal özellikleri FTIR, TGA ve DSC analizleri ile belirlendi. Antioksidant ve antibakteriyel aktivitelerinin değerlendirilmesinin ardından, biyomateryal oluşturma potansiyeli değerlendirildi. Son olarak, EPS'nin çözücü döküm yöntemiyle film oluşturma ve fiziksel veya kimyasal çapraz bağlayıcıların eklenmesiyle hidrojel oluşturma potansiyeli değerlendirilmiştir.

Bu çalışma sonucunda, EPS üretiminin tuzluluk ve yüksek ışık yoğunluğu stresleri ile 48% oranında artırılabileceği görülmüştür. EPS'nin ana bileşimi 5% toplam sakkarit, 4.5% protein, 1.6% sülfat ve 6.2% üronik asit olarak belirlenmiştir. HPLC analizi, EPS'nin monosakkarit profilinin esas olarak mannoz, galaktoz, riboz, arabinoz ya da ksiloz ve glikozdan oluştuğunu göstermiştir. Ayrıca, EPS 2 µg/ml konsantrasyonda 70% DPPH süpürme aktivitesi ve 10 mg/ml konsantrasyonda *S. aureus'*a karşı 13 mm çapında inhibisyon zonu ile antibakteriyel aktivite göstermiştir. Ayrıca, yenilenebilir kaynaklar olarak değerlendirilen mikroalglerden elde edilen EPS'lerin biyomateryal oluşturulması için kullanımı değerlendirildi ve doğrulandı. Film oluşumu için 3% EPS konsantrasyonu optimum seviye olarak belirlenmiştir. Hidrojel oluşumunu indüklemek için, fiziksel çapraz bağlama ve kimyasal çapraz bağlama yöntemleriyle 1.5% ve 1% EPS konsantrasyonu uygun bulunmuştur. Genel olarak, psikrotolerant yeşil mikroalga *Chlamydomonas* sp. ASYA25 tarafından üretilen biyoaktif ekzopolisakkaritler, biyomateryal üretimi için kullanılma potansiyeli sergilemektedir.

Anahtar kelimeler: Antarktika, Biyomateryal, Ekzopolisakkarit, Mikroalg, Polimer

1. INTRODUCTION

Microalgae are photosynthetic microorganisms that can live in environments such as lakes, rivers, seas, oceans, and terrestrial surfaces with advanced adaptation capabilities (Metting, 1996). Microalgae, which form the basis of the food chain, are also rich in biotechnological resources with the metabolites they contain. Their pigments, polysaccharides, protein, and saturated or unsaturated fatty acid contents are seen as valuable resources in a wide range from the food industry to the energy industry (Borowitzka, 2015; Spolaore et al., 2006).

Microalgae can enrich their metabolite profiles by changing them in response to environmental stimuli. Among these metabolites, exopolysaccharides (EPSs), which have attracted much attention in recent years, are a metabolite produced by microalgae to protect themselves from adverse environmental conditions (Li et al., 2001). This extracellular polymeric structure, which is synthesized inside the cell and then secreted outside the cell, surrounds the cell like a protective barrier. EPS are known for their high content of saccharides and proteins and can be synthesized by many organisms such as bacteria, fungi, microalgae, and macroalgae (Delattre et al., 2016).

Bacteria in particular stand out as EPS producers and the underlying reason for this is that they have lower cultivation costs and produce higher amounts of EPSs compared to other producers. Nevertheless, microalgae-based EPS studies have increased rapidly in the last decade, as shown in a review of the literature (Pierre et al., 2019). The main reason behind this is the development of production systems that allow the production of microalgae in high volumes with developing technology and the development of alternative nutrient media such as the use of wastewater. EPSs can be produced in extreme or normal conditions to protect themselves under adverse conditions. However, an increase in EPS production was observed when microalgae were exposed to environmental stress. Therefore, microalgae, which can respond rapidly to environmental variables and show these responses by enriching their metabolite profile, show suitable potential for EPS production.

The main component of EPS is polysaccharides while the protein is the second component (Pereira et al., 2009). The ratio between polysaccharide and protein can be varied according to producer species and culture conditions. The monosaccharide

content is also showing variation between species and glucose, galactose, arabinose, rhamnose, mannose, fucose are the most common monosaccharide types. In some situations, EPS contains only one of these monosaccharides and they are attributed as homopolysaccharides. The heteropolysaccharides contain more than one monosaccharide. Moreover, some non-sugar groups like sulfate and uronic acid can be found in EPS structures. The physical properties of EPSs also show differences, and the biological functions of EPSs are affected by these variations.

The EPSs are known for their antibacterial, antioxidant, anticancer, anti-inflammatory, and immune-stimulatory activities (Kumar et al., 2018). With these biological properties, EPSs show high potential for use in food, cosmetics, and biomedical fields. In addition, the gelling, thickening, and stabilizing activities of EPSs are important for their use in the food industry. There are commercially available EPSs in the food industry such as xanthan gum and agar. In cosmetic applications, hyaluronic acid and alginate are the most known examples.

In recent years, the usage potential of EPS in biomaterial formation has been an innovative approach that increases its value (Lin et al., 2022; Popyrina et al., 2023). The film, fiber, and hydrogel materials that are produced by EPS gain attention due to their advantages nature. Synthetic polymers are used for the formation of these materials, but their toxicity and non-biodegradability limit their use. The EPSs with their natural, non-toxic, and biodegradable nature gained the attention of the biomaterial industry. In addition to these advantages properties, the fact that they are derived from renewable resources such as microalgae makes EPSs more attractive. The film material produced from EPSs can be used in food packaging applications and the edible food industry. The hydrogel or fiber produced from EPSs can be used in wound healing, tissue engineering, drug delivery, and cosmetic applications.

In this thesis, the main aim is to obtain EPS from Antarctic microalgae *Chlamydomonas* sp. ASYA25 and evaluate its biomaterial formation potential after the characterization studies. For this purpose, it is aimed to evaluate the EPS production potential of *Chlamydomonas* sp. ASYA25 and to optimize the culture conditions that will maximize EPS production. Characterization of the obtained EPSs and evaluation of their antioxidant and antibacterial activities are also among the objectives. This master's thesis will contribute to the limited number of studies on

EPSs of Antarctic microalgae in the literature and will be an exemplary study evaluating the use of microalgal EPSs as an alternative polymer source for biomaterial formation.

1.1.Microalgae

Microalgae are photosynthetic microorganisms with advanced adaptation capabilities that can thrive in various habitats such as ponds, lakes, rivers, and soil. Microalgae are considered suitable sources for obtaining high-value metabolites such as fatty acids, pigments, carbohydrates, proteins, and vitamins. Microalgae convert the energy they receive from the sun using the photosynthesis mechanism into chemical energy and store this energy in their cells as proteins, carbohydrates, lipids, and nucleic acids (Magierek & Krzemińska, 2018; Pulz & Gross, 2004). These components vary depending on the species of microalgae and the cultivation conditions. Microalgae development, culture growth, and photosynthetic metabolite production rates can be influenced by many environmental factors such as nutrient type and concentration, CO_2 availability, temperature, and light.

Microalgae, due to their high content of fat, protein, and carbohydrates, can be utilized in the fields of food, animal feed, and energy. In addition to these contents, these microalgae synthesize numerous valuable metabolites such as fucoxanthin, astaxanthin, beta-carotene, chlorophylls, polyunsaturated fatty acids, sterols, and peptides, both inside and outside the cell, to adapt to the conditions of their environment (Pulz & Gross, 2004).

1.2.Biotechnological Importance of Microalgae

Microalgae attracts the attention of the biotechnology field such as biofuel, biomass, wastewater treatment, and value-added products such as antioxidants, polyunsaturated fatty acids, pigments, and vitamins. There are 22,000 to 26,000 species of microalgae, but only a few of these are commercially available. *Spirulina*, *Chlorella*, and *Dunaliella* are the most widely known of these microalgae (Raja et al., 2008).

In the food industry, microalgae can be considered as alternative food with valuable components. Microalgae can be rich in nutritional components such as healthy fats like omega-3 fatty acids, proteins, vitamins (e.g. A, C, D, E), and minerals. These

properties enable them to be used in the production of food supplements and nutritional support products. Nowadays, *Spirulina* and *Chlorella* are commercially available with a rich profile in the food industry as a powder or pill form (Loy & Chu, 2012). *Nannochloropsis oculata* is evaluated in food and feed industries with high polyunsaturated fatty acids (Mohamad et al., 2020).

Microalgae are also considered a rich source of high-value products. Moreover, these valuable bioactive components show some biological activities like antioxidants, antibacterial, and antiviral. Such components can be used in the pharmaceutical industry to develop new drugs. The polyunsaturated fatty acids, carotenoids, phycobiliproteins, and polysaccharides are the most pronounced products of microalgae. The carotenoid obtained from *Dunaliella salina* can be evaluated in the food industry with food colorant activity while the antioxidant and anticancer activities can be evaluated in the pharmaceutical industries (Jin & Melis, 2003).

Microalgae have attracted attention as biomass sources for biofuel production with high oil content and so are considered potential sources for biodiesel production and can also produce organic matter that can be used in biogas production (Mata et al., 2010). The lipids of *Chlorella protothecoides* and *Botryococcus braunii* are evaluated in the biofuel industry (Loy & Chu, 2012).

Microalgae can be used to remove organic pollutants in water treatment (Matamoros et al., 2015). Some microalgae can absorb pollutants or remove them through biosorption (Priya et al., 2014; Subashchandrabose & Ramakrishnan, 2013). They can also play a potential role in reducing greenhouse gases through their carbon absorption. Some microalgae species can absorb and remove heavy metals from soil and water. Thanks to these properties, they can be used to clean up contaminated areas. Microalgae can grow by absorbing carbon dioxide from the atmosphere. This can help reduce carbon emissions and contribute to a sustainable future. Microalgae can be used as potential sources to produce bioplastics and biopolymers. This could help develop sustainable alternatives in the plastics industry.

1.3.Antarctica

All these metabolites and activities mentioned in the previous parts show variation from one species to another. The isolation area of microalgae is important because the

metabolite profile changes according to its environment. The microalgae which is the subject of this thesis study was isolated from Antarctica and so the metabolite profile is adapted to extreme conditions of this region.

Antarctica, which covers 98% of its surface with ice and spans an area of 13,209,000 km^2, is the most arid, windiest, and coldest continent on Earth. It is known that various microorganisms persist in the continental climate prevalent on the continent and can easily adapt to extreme conditions. Antarctic microalgae contribute to biodiversity in this region by successfully adapting to extreme environmental conditions. Antarctica is considered a stressful environment for the survival of microalgae due to cold temperatures, UV radiation, osmotic pressure, and limited nutritional conditions. Therefore, microalgal species living in Antarctica have undergone some physiological and morphological changes to adapt to adverse living conditions (Burkholder & Mandelli, 1965; Torstensson et al., 2018; Van Leeuwe et al., 2018).

The changes in membrane fluidity and enzyme kinetics, as well as the production of metabolites such as compatible solutes, cryoprotectants, and extracellular components, are some of the adaptive properties that are improved (Lyon & Mock, 2014). Since the cell membrane plays a role in nutrient exchange and removal of cellular wastes, it is important to maintain membrane fluidity at freezing temperatures. For this reason, the high content of polyunsaturated fatty acids in Antarctic microalgae has been confirmed by studies and since these fatty acids are considered as omega-3 and omega-6 sources, they increase the value of microalgae as a food source. Furthermore, in freezing conditions, cells need to maintain the aqueous environment outside the cells, and for this purpose cells increase the production of metabolites such as ice-binding proteins that prevent ice formation and crystallization around the cell.

Another important metabolite produced to protect against freezing conditions is extracellular substances containing polysaccharides and proteins (Ewert & Deming, 2013). In a study conducted with snow algae *Chlamydomonas*, the effect of cold stress was examined through transcriptomic analyses (Peng et al., 2021). In another study, the cold stress tolerance of *Coccomyxa subellipsoidea* was associated with EPSs and antifreeze glycoproteins (Blanc et al., 2012). Metagenomic analysis of microorganisms from the Antarctic and Arctic regions revealed that genes related to EPS and membrane adaptations are common (De Maayer et al., 2014). EPSs act as a

cryoprotective mechanism and EPS production increases in psychrophiles under cold temperatures (Mancuso Nichols et al., 2005; Qin et al., 2007). The snow algae *Chlamydomonas nivalis* produced higher EPS when it was grown at 4 °C instead of 22 °C (Peng et al., 2021). Therefore, the Antarctic microalgae have the potential to be EPS producers.

1.4.Exopolysaccharides

EPSs are high molecular weight polymeric structures, and these polymers are secreted outside following the synthesis inside the cells (Xiao & Zheng, 2016). Microorganisms like microalgae, cyanobacteria, bacteria, fungi, and some macroalgae species produce these polymeric structures to protect themselves against unfavorable environmental conditions such as high or low temperatures, salinity, or nutrient limitations (Delattre et al., 2016). Nevertheless, bacteria are known as primary EPS producers on the industrial scale due to their fast growth cycle and easily manipulated characteristics. Moreover, the yield of EPS from bacteria is higher than the other species (Pierre et al., 2019). However, in recent years, microalgae and cyanobacteria have gained attention as potential EPS producers due to their green biosynthesis advantages. Microalgae are suitable sources for EPS production as they are sustainable natural resources and can respond rapidly to environmental stimuli.

EPSs are classified as homopolysaccharides and heteropolysaccharides based on the type of monosaccharide they contain (Rossi & De Philippis, 2016). Homopolysaccharides contain one monosaccharide type while heteropolysaccharides contain more than one type of monosaccharide. In addition to this, EPSs can be differentiated according to their location and physical properties (Xiao & Zheng, 2016). EPSs are categorized into two groups: released polysaccharides (soluble EPS) and cell-bound polysaccharides (capsular EPS) based on their adhesive properties (Figure 1). Despite released polysaccharides being released into the growth medium, cell-bound polysaccharides are attached to the cell surface at different strengths and categorized as slime, sheath, and capsule forms according to the attachment strengths. Slime forms loosely attach to the cell surface and do not represent the cell shape. However, sheath and capsule forms strongly attach to the cell surface and represent the cell shape, but the capsule form is thicker than the sheath form (Kumar et al., 2018).

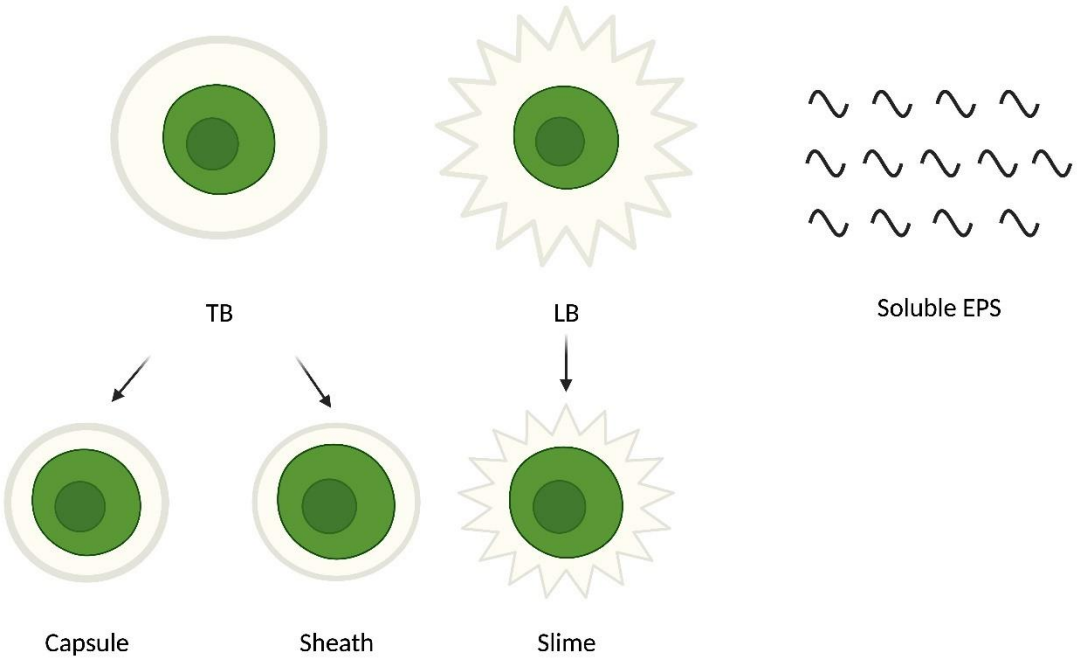


Figure 1. Categorization of EPSs according to the location and attachment strength (TB: tightly bound, LB: lightly bound)

EPSs mainly form carbohydrates but can contain protein as a secondary metabolite at different ratios according to species (Delattre et al., 2016; Xiao & Zheng, 2016). EPSs also contain a small amount of lipid and nucleic acid, but the ratio of these metabolites is less than %1 so these metabolites are not measured during the characterization studies of EPS. In addition, EPSs contain some non-sugar compounds like sulfate, pyruvate, o-methyl groups, and uronic acids. As seen in literature studies, sulfate content gives some functional activities and anionic nature to EPSs.

To date, EPS production potentials of various microalgae have been evaluated and confirmed through research studies. These research studies have revealed that the yield, content, and activity of EPSs vary from species to species. The diversity in the monosaccharide content of EPS obtained from green microalgae is a fitting example of this phenomenon (as seen in Table 1).

Table 1. The monosaccharide content of EPSs obtained from green microalgae

Species	*Monosaccharide Content*	*References*
Botryococcus braunii	*Arabinose, rhamnose, galactose, and fucose*	(Casadevall et al., 1985)
Botryococcus braunii	*Arabinose, galactose, glucose, fucose*	(W. N. Wang, Li, Li, et al., 2021)
Chlamydomonas reinhardtii	*Arabinose, rhamnose, galactose, glucose, xylose, and ribose*	(Bafana, 2013)
Chlamydomonas mexicana	*Arabinose, rhamnose, galactose, glucose, xylose, mannose, fucose, and ribose*	(Barclay & Lewin, 1985)
Chlamydomonas sajao	*Arabinose, rhamnose, galactose, glucose, xylose, mannose, fucose, and ribose*	(Barclay & Lewin, 1985)
***Chloroccum* sp.**	*Galactose, xylose, mannose, fucose, and ribose*	(Harun et al., 2011)
***Chloroccum* sp.**	*Arabinose, rhamnose, galactose, glucose, and mannose*	(Zhang et al., 2019b)
***Scenedesmus* sp.**	*Rhamnose, galactose, glucose, and mannose*	(Zhang et al., 2019b)
Chlorella zofingiensis	*Galactose, glucose, and mannose*	(Naveed et al., 2019)
Chlorella vulgaris	*Arabinose, rhamnose, and galactose*	(Barboríková et al., 2019)
***Chlorella* sp.**	*Arabinose, glucose, and fucose*	(Yalcin et al., 1994)
Chlorella pyrenoidosa	*Arabinose and galactose*	(Naveed et al., 2019)
Chlorella pyrenoidosa	*Arabinose, rhamnose, galactose, glucose, mannose, fucose, and ribose*	(Zhang et al., 2019b)
Chlorella sorokiniana	*Galactose, glucose, and mannose*	(Koçer et al., 2021)
Chlorella minutissima	*Galactose, glucose, and mannose*	(Koçer et al., 2021)
***Cosmarium* sp.**	*Arabinose, rhamnose, galactose5, xylose, mannose, and fucose*	(Kiemle & Domozych,

		2019)
Desmococcus olivaceus	*Arabinose, rhamnose, galactose, glucose, xylose, and mannose*	(Hokputsa et al., 2003)
Dunaliella tertiolecta	*Galactose, glucose, xylose, and ribose*	(Templeton et al., 2012)
Dunaliella salina	*Galactose, glucose, and xylose*	(Mishra & Jha, 2009)
***Dunaliella* sp.**	*Arabinose, rhamnose, galactose, glucose, xylose, and mannose*	(Concórdio-Reis, David, et al., 2023)
***Graesiella* sp.**	*Arabinose, galactose, glucose, xylose, mannose, and fucose*	(Trabelsi et al., 2016)
***Nannochloropsis* sp.**	*Glucose, xylose, mannose, and fucose*	(Templeton et al., 2012)
Netrium oblangum	*Arabinose, rhamnose, glucose, xylose, mannose, fucose, and ribose*	(Kiemle & Domozych, 2019)
Netrium interruptum	*Arabinose, rhamnose, galactose, glucose, mannose, fucose, and ribose*	(Kiemle & Domozych, 2019)
Palmella mucosa	*Arabinose, glucose, fucose, and ribose*	(Tischer & Moore, 1964)
Penium cylindrus	*Arabinose, rhamnose, galactose, glucose, xylose, mannose, and ribose*	(Kiemle & Domozych, 2019)
Penium margaritaceum	*Arabinose, galactose, glucose, mannose, and fucose*	(Domozych et al., 2005)
Penium spirostriolatum	*Rhamnose, galactose, glucose, xylose, mannose, fucose and ribose*	(Kiemle & Domozych, 2019)
Pleurotaenium trabecula	*Arabinose, rhamnose, galactose, glucose, mannose, and ribose*	(Kiemle & Domozych, 2019)
Tetraselmis suecica	*Glucose and mannose*	(Parra-Riofrío et al., 2020)

Although the monosaccharide content of EPSs obtained from green microalgae varies, arabinose, rhamnose, glucose, and galactose are the most common monosaccharides. Environmental or cultural conditions and strain of the culture can influence monosaccharide contents so they can vary even for the same species, as in the case of *Botryococcus braunii* and *Chloroccum* sp. species. These environmental factors may lead to differences in the quantitative distribution of monosaccharides, even if they do not vary in monosaccharide type.

1.4.1. Extraction and Characterization of Exopolysaccharides

The extraction procedure is one of the important factors affecting the EPS yield and varies according to the type of EPS to be obtained. As mentioned in the previous part, considering their location, EPSs can be categorized as released and cell-bound polysaccharides. While released polysaccharides can be obtained simply by centrifugation or filtration, some physical or chemical pretreatments are required for cell-bound polysaccharides. Figure 2 shows the different extraction methods that have been applied for EPS extraction until now.

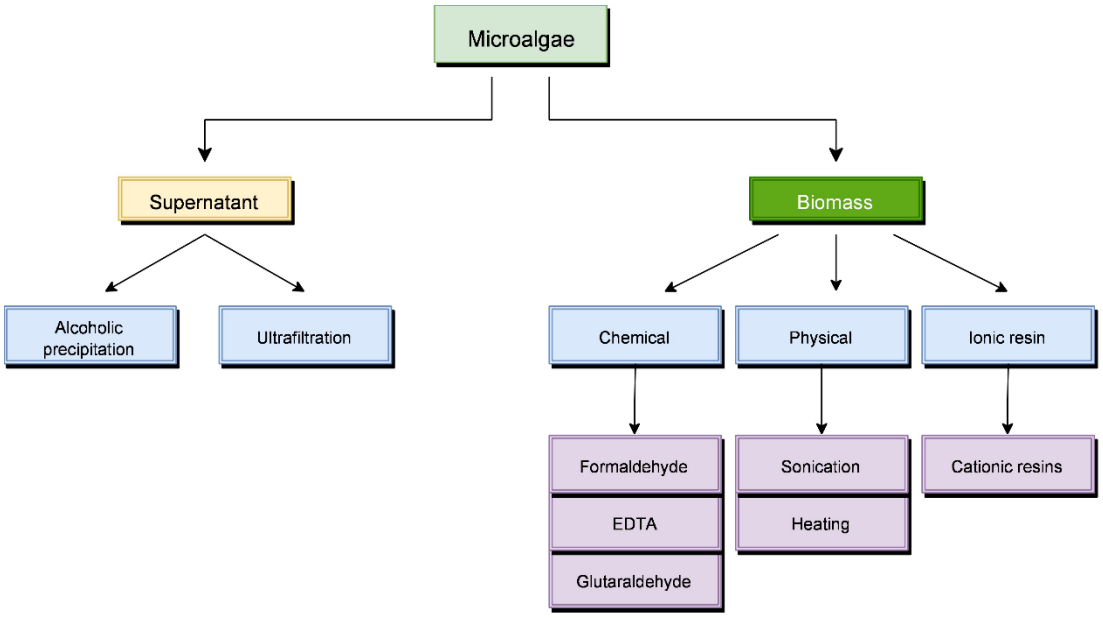


Figure 2. Strategies for EPS extraction from microalgae

1.4.1.1.Extraction of Released Exopolysaccharides

The released polysaccharides are in the surrounding environment without attaching to the cell surface and this form of the EPSs can be separated from cell biomass via centrifugation or filtering process. After the separation process, crude EPSs can be extracted via alcoholic precipitation or ultra-filtration process (Delattre et al., 2016; Kumar et al., 2018). Commonly alcoholic precipitation follows the precipitation of EPSs via the addition of alcohol onto the supernatant to provide approximately 65-70% alcohol concentration. This is done by adding generally, two or three volumes of pure or 96% ethanol for one volume of supernatant. Afterward, the alcoholic solution is incubated at 4 Cº overnight. Then the solution is centrifuged to obtain the EPS fractions. After evaporation of the alcohol, the final stage is dialysis of crude EPS and lyophilization. One of the advantages of alcoholic precipitation is the recovery of alcohol via distillation. The EPSs can be extracted by following the ultra-filtration technique, but the extraction yield is less than the alcoholic precipitation in this procedure (Cruz et al., 2021; Kumar et al., 2018). The efficiency of the membrane filtration technique differentiates depending on the viscosity and concentration of EPS, pore size, and membrane morphology (Delattre et al., 2016). Even if the ultrafiltration procedure seems to be an alternative to alcoholic precipitation, it needs to be improved for scale-up production.

1.4.1.2.Extraction of Capsular Exopolysaccharides

The structure, composition, and physico-chemical properties of cell-bound polysaccharides may show strain-specific differentiation (Delattre et al., 2016; Rossi & De Philippis, 2016). Therefore, there is no straightforward extraction procedure for these polymeric structures. The cell-bound polysaccharides need a chemical or physical treatment to separate the EPS structure from the cell. For the extraction of cell-bound polysaccharides, formaldehyde, EDTA, and NaOH are used as chemical treatments while sonication, physical agitation, and heating are used as physical treatments (Delattre et al., 2016). The efficiency of these methods on microalgal EPSs was analyzed in previous studies and the efficiency difference between chemical and physical methods was compared. Chemical treatments (EDTA, formaldehyde, and glutaraldehyde) were reported as superior to physical (sonication, heating, and cation exchange resin) treatments for the extraction of cell-bound EPSs (Comte et al., 2006).

In one study, twenty different Chlamydomonas strains were used for EPS extraction and characterization purposes, and cell-bound EPSs were extracted via washing technique (Katona et al., 2018). In washing technique, cell biomass was mixed with ultra-pure water and incubated at 30 °C for 1 hour. However, this method also has some drawbacks due to the intracellular component contamination as seen in the EPS extraction from *Navicula jeffreyi* (Takahashi et al., 2009). *Neocystis mucosa SX* was tested for an efficient cell-bound polysaccharide extraction. Researchers applied cation exchange resin, EDTA, heating, acidic treatment, and alkaline treatment. The content and chemical composition of extracted EPSs were analyzed. The results showed that the cation exchange resin and heating treatments provide a higher and chemically rich EPS yield than the other treatments (Lv et al., 2019).

1.4.1.3.Characterization of Exopolysaccharides

Since the usage areas and biological activities of EPSs vary according to their chemical and structural properties, the characterization of EPSs is considered as an important step. The workflow of EPS obtaining is represented in Figure 3.

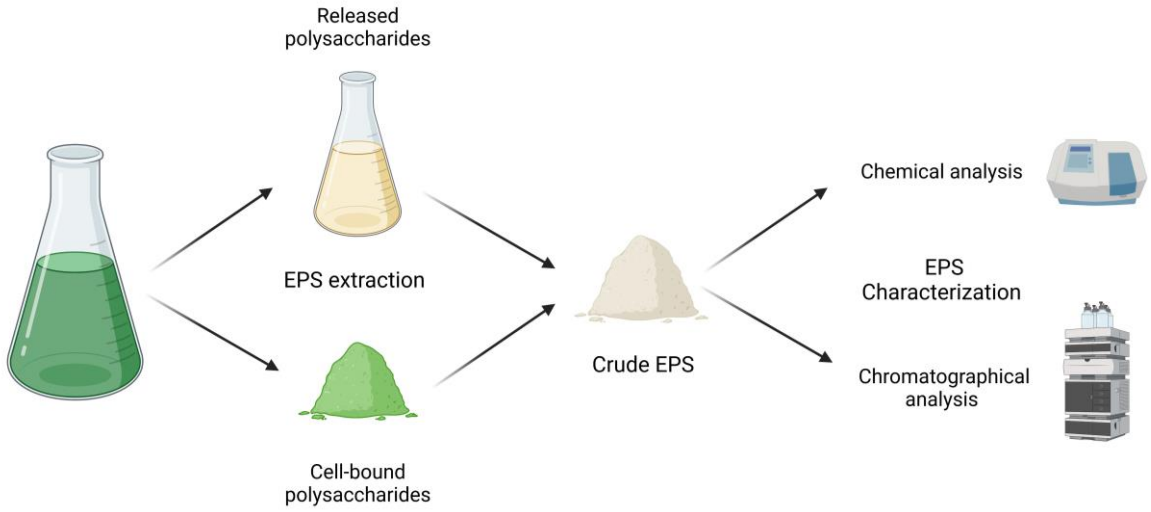


Figure 3. The workflow of EPS obtaining

As it is known from the studies carried out so far, polysaccharides constitute the majority of EPSs, but proteins, lipids, and nucleic acids can also be found in the structure. However, since their lipid and nucleic acid content can be found below 1%,

they are generally not considered in characterization studies (Rossi & De Philippis, 2016). When measuring the saccharide content of EPS phenol-sulfuric acid is used, while methods such as Lowry and Bradford can be employed for measuring the protein content (Delattre et al., 2016; Dubois et al., 1956; Lowry et al., 1951). The monosaccharide contents of EPS vary depending on the strain they are derived from and environmental conditions. For the characterization of monosaccharide content, chromatographical analysis is commonly accepted gas and liquid chromatographies are the most reliable methods, and coupling this analysis with mass spectrometry increases the efficacy of the results (Decamp et al., 2021; Vieira et al., 2021).

Additionally, non-sugar groups such as sulfate and uronic acid can be found in the structure of EPS, and the presence of these groups not only confers an anionic nature to EPS but also can affect their biological activities. For the characterization of EPS, the meta-hydroxy diphenyl method is used to measure the uronic acid content, while turbidimetric or chromatographic methods can be employed for measuring the sulfate content (Blumenkrantz & Asboe-Hansen, 1973; Craigie et al., 1984; Filisetti-Cozzi et al., 1991). Fourier transform infrared spectroscopy (FTIR) analysis is a method that provides information about the components and structures of EPS, helping to understand its physical and chemical properties (Castro et al., 2014; Şentürk, 2020). Moreover, nuclear magnetic resonance (NMR) can be used to determine the chemical structures, molecular arrangements, and components of EPS (Vieira et al., 2021). Another distinguishing feature of EPS is its molecular weight, which can be characterized by gel permeation chromatography (Patel et al., 2013).

1.5. Biological Activities of Exopolysaccharides

In addition to their protective barrier activities, EPSs also stand out with their various biological activities such as antibacterial, antioxidant, anticancer, and anti-inflammatory activities. Although clinical use of these activities is not yet available, many studies in this field are found in the literature.

1.5.1. Antioxidant Activity

One of the most extensively studied properties of EPSs is their antioxidant activity. Due to the molecules in the backbone, EPSs can bind to reactive oxygen species and

show antioxidant activity. The antioxidative activity was analyzed and identified on EPSs of *Botryococcus braunii* (W. N. Wang, Li, Li, et al., 2021). In the literature, there are many studies about the antioxidant activity of microalgal EPSs. In a study, researchers analyzed the antioxidant activity of *Chlorella pyrenoidosa*, *Scenedesmus* sp., and *Chlorococcum* sp. EPSs (Zhang et al., 2019b). The results confirmed the antioxidative properties of EPSs. Another study demonstrating the antioxidant property of EPSs was conducted using EPS obtained from *Picochlorum* sp. microalgae and results showed 85% antioxidant activity (Mousavian et al., 2022). In the same study, *Chlorella sorokiniana* and *Chlorella* sp. species showed scavenging activity against ABTS radicals. The antioxidant properties of EPSs are considered effective attributes for their use in the food, cosmetic, and medical fields. In a study, EPS extracted from *Arthrospira platensis* was used in body cream formulation due to its antioxidative activity (Pierucci et al., 2017).

1.5.2. Anticancer Activity

In a study, EPSs extracted from *Chlorella pyrenoidosa*, *Scenedesmus* sp., and *Chlorococcum* sp. species show anticancer activity against colon cancer cell lines (Zhang et al., 2019b). EPSs have dose-dependent reduced the viability of colon cancer cells, and it was observed that extracellular polysaccharide obtained from all three species reduced cell viability by approximately 20% at a concentration of 0.6 mg/ml. Anticancer activity of EPSs on colon cancer was studied for EPSs of *Chlorella vulgaris* and *Chlorella zofingiensis* species. At the 0.6 mg/ml concentration of *Chlorella vulgaris* and *Chlorella zofingiensis* EPSs showed 29% and 18% decrease in colon cancer cell viability (Zhang et al., 2019a). In another study, EPSs extracted from *Tribonema* sp. decreased the cell viability of HepG2 (human liver cancer cell line) 66% ratios at the concentration of 250 μg/ml. Therefore, EPS of *Tribonema* sp. was evaluated as an alternative to commercial anticancer drugs (X. Chen et al., 2019). The anticancer activity of EPSs extracted from a diatom *Nitzschia palea* was studied and a 82% decrease in cell viability of human lung adenocarcinoma was reported at the concentration of 250 μg/ml (Sanniyasi et al., 2022). Another study investigated the anticancer activity of EPS extracted from *Porphyridium cruentum* on Hela and Ht29 (human colorectal adenocarcinoma cell line). The results of the study showed that EPS at the concentration of 100 μg/ml decreased cell viability of the Ht29 cell line by nearly

40% ratios while did not show activity on the Hela cell line (Ivanova et al., 2022).

1.5.3. Antibacterial Activity

Microalgae secrete EPSs to protect against abiotic and biotic stress factors. Therefore, microalgal EPSs are pronounced with their antibacterial activities that provide the potential to be used in food, medicine, and cosmetic industries. In a research study, the antibacterial activity of EPSs extracted from *Botryococccus braunii* and *Chlorella pyrenoidosa* was pronounced against *E. coli* and *S. aureus* (Gallón et al., 2019). In another study, the antibacterial activity of EPSs extracted from *Porphyridium cruentum* was tested against *S. aureus*, E. coli , and S. enteritidis (Raposo et al., 2014). The results showed that EPSs show antibacterial activity against *S. enteritidis* while no significant antibacterial activity was observed against *S. aureus* and *E. coli*.

1.5.4. Bio-flocculant Activity

Extraction cost can be considered among the limiting factors for the biotechnological use of microalgae. In this context, the potential use of EPSs in the harvesting process is being evaluated due to their bio-flocculant activities. The flocculant activity of EPS extracted from *Scenedesmus acuminatus* was tested in the culture of the same species and bio-flocculant activity was confirmed (Yang et al., 2020).

1.5.5. Cryoprotective Activity

The microorganisms can secret EPS at the lowest temperature to protect their metabolism against the effect of cold temperatures. Therefore, the cryoprotective activity of EPS can be evaluated. In sea ice, diatoms produce EPS to protect themselves (Aslam et al., 2012). In recent years, the use of EPSs as cryoprotectants has gained attention for the storage of microalgae. The cryoprotective role of EPSs was known for bacteria but the microalgae and cyanobacteria can be preserved by using EPS as a cryoprotective agent. In a valuable research, EPS extracted from *Pseudomonas* sp. BGI-2 was applied in the cryopreservation of some cyanobacteria and microalgae cultures (Ali et al., 2021). In the result of the study, EPS shows a potential to be replaced with other chemical cryoprotectants like methanol, glycerol, and dimethyl sulfoxide.

1.5.6. Anti-inflammatory and Immunostimulatory Activities

Microalgal or cyanobacterial EPSs show anti-inflammatory activities and are demonstrated in animal models. The capsular EPS of *Matigocladus laminosus* showed anti-inflammatory activity (Gloaguen et al., 2007). In a valuable research study, the anti-inflammatory activity of EPS obtained from *Chlorella vulgaris* was studied and showed a potential therapeutic activity for asthma (Barboríková et al., 2019). In another study, the anti-inflammatory activity of EPSs extracted from *Phormidium* sp. was demonstrated on Zebrafish embryo and larva models (Zampieri et al., 2020). The immunomodulatory activity of EPSs obtained from *Cyanobacterium aponinum* showed a positive therapeutic effect on psoriasis disease (Gudmundsdottir et al., 2015).

1.5.7. Anti-viral Activity

Another intriguing biological activity of EPSs is their antiviral activity and especially the high sulfate-containing EPSs show these activities (Kumar et al., 2018). The role of sulfated polysaccharides in the inhibition of some human pathogens development was demonstrated in research studies (Chakraborty et al., 2015). Therefore, these EPSs, referred to as sulfated polysaccharides, have been evaluated as a potential therapy method to block pathogen adhesion. The EPSs extracted from *Arthrospira platensis* and *Porphyridium purpureum* were demonstrated with anti-viral activities against HIV-1 and HIV-2 (Delattre et al., 2016). In another study, the anti-viral effect of EPS extracted from *Gyrodinium impudicum* was designated against encephalomyocarditis virus (Yim et al., 2004).

1.5.8. Wastewater Treatment and Soil Conditioning Activities

EPSs can have an anionic nature due to their non-sugar groups like uronic acid and sulfate groups. Therefore, EPS can help to remove positively charged heavy metals and be used in the treatment of wastewater (De Philippis et al., 2011). The cyanobacterial EPS forms a cluster on the surface of the soil and can decrease the loss caused by erosion(Mager & Thomas, 2011).

1.5.9. Bio-stimulant Activity

In recent years, new biofertilizers and bio-stimulants have been investigated to replace chemical and harmful substances so the environment and human health can be

protected. At this point, the EPSs obtained from renewable and natural sources of microalgae gain attention as bio-stimulators (Chanda et al., 2019). The EPSs protect plants against abiotic and biotic stress factors. In a valuable study, the bio-stimulatory activity of EPSs obtained from *Dunaliella salina* was analyzed for tomato plants under salinity stress, and a positive effect was demonstrated (El Arroussi et al., 2018). In another study, EPSs of *Spirulina* were applied to plants at different concentration ranges and the growth of plants was followed (Chaudhuri & Balasubramanian, 2023). The results showed that *Spirulina* EPS shows bio-stimulatory activity at 1.5 mg/ml concentration.

1.6. Factors Affecting Exopolysaccharide Production

EPSs are produced from various microorganisms to protect themselves against biotic and abiotic stress factors so act as a defense mechanism. EPS yield shows variation according to source species but also the environmental and cultural factors influence EPS production. Nutrient limitation, pH level, light intensity, and temperature are common manipulative parameters to maximize EPS production.

1.6.1. Carbon Source

In a study conducted with *Nostoc flagelliforme* species in the literature, the production profile of EPS is evaluated by applying a mixed carbon source and nitrogen starvation. When nitrogen starvation stress was combined with the addition of 4 g/L glucose and 2 g/L acetate as carbon sources, the maximum EPS yield was obtained as 879 mg/L. Comparing EPS production under conditions without a carbon source to those with added carbon sources, it was observed that the addition of carbon sources led to an approximately 8-fold increase in EPS yield. However, under these optimally determined conditions, cell growth is reduced by 50% compared to the group with the added nitrogen source. Moreover, EPS production is affected by the growth conditions according to mixotrophic, autotrophic, and heterotrophic conditions (Babiak, Energies, et al., 2021). This situation was tested on *Chlorella vulgaris* and the highest EPS yield was obtained in mixotrophic growth conditions when compared to autotrophic growth conditions (Zhang et al., 2019a).

1.6.2. Nitrogen

Nitrogen is used in the synthesis of vital components like proteins, amino acids, and enzymes, so it is evaluated as an essential nutrient source (Turpin, 1991). Therefore, nitrogen deficiency directly affects cellular metabolism by increasing the accumulation of reserve compounds like carbohydrates and lipids (Bellou & Aggelis, 2013). In addition to the limitation or starvation of nitrogen, the source of nitrogen affects the metabolism of EPS production. In a study, the effect of different nitrogen sources on EPS production was investigated for *Botryococcus braunii* (Lupi et al., 1994). Results showed that the maximum EPS yield was obtained in nitrate-containing medium instead of ammonium and urea. The EPS yield became 2.4 g/L for nitrate, 2.1 g/L for ammonium, and 1.8 g/L for urea. The limitation of nitrogen also causes an increase in the production of EPS. In a study, nitrogen starvation increased the EPS productivity in *Chlamydomonas mexicana* (Kroen & Rayburn, 1984).

1.6.3. Phosphate

Another important nutrient is phosphorus, which is used in the synthesis of important compounds like DNA and ATP (Lakatos et al., 2019). The starvation or limitation of phosphate increases the production of EPS-like metabolites. In a research study conducted with *Spirulina subsalsa,* EPS yield increased under phosphate starvation (Chakraborty et al., 2015). In another study, *Cylindrotheca fusiformis* EPS productivity was evaluated under phosphate-limited culture conditions, and a positive effect was observed (Magaletti et al., 2007).

1.6.4. Aeration

The aeration is another factor that shows efficacy on EPS production and in a research study, the different aeration rates were applied to *Porphyridium cruentum* culture, and the EPS productivity was followed (Iqbal & Zafar, 1993). The results showed that the EPS productivity can reach 1.8 g/L when the aeration rate was arranged to 500 ml/min.L. The positive effect of aeration on EPS productivity was observed in *Anabaena* sp. species and EPS synthesis increased with increasing airflow rate (Moreno et al., 1998). In another study, the application of aeration increased the EPS production of *Cyanothece* sp. with the combination of other optimized culture conditions (Su et al., 2007).

1.6.5. pH

The pH is also another important parameter for EPS synthesis because the maximum EPS production occurs in the stationary growth phase and the pH reaches generally 8-9 values (Babiak, W., et al., 2021). Therefore, the different pH values were evaluated during the growth process. In a valuable study, the maximum EPS yield was obtained at pH 7 value for *Chlamydomonas reinhardtii* (Bafana, 2013). When the effect of pH on EPS yield was investigated in different cyanobacteria, it was observed that the maximum EPS yield was achieved at a pH of 8.5, with pH values ranging from 5 to 10 being evaluated (Khangembam et al., 2016).

1.6.6. Light

A study conducted with the species *Arthrospira platensis* examined the combined effect of temperature and light factors on EPS production. The light intensities of 50, 115, and 180 μmol photons $m^{-2}s^{-1}$ were applied (Trabelsi et al., 2009). As a result, the lowest growth rate was obtained under the conditions of 180 μmol photons $m^{-2}s^{-1}$ while the optimum condition for maximum EPS production was determined to be 180 μmol photons $m^{-2}s^{-1}$. In another study, the positive effect of high light irradiance on the EPS productivity of *Anabaena* sp. was confirmed (Moreno et al., 1998). The EPS productivity of *Nostoc* sp. shows an increase with increasing light intensity (Ge et al., 2014). The effect of light intensity on the productivity and component of EPS was analyzed for *Microcoleus vaginatus* and results showed that the EPS productivity increased, but any significant change was not observed in the composition of EPS (Ge et al., 2019). In addition to light irradiance, EPS yield is changeable according to the source of light. For instance, red lights provide higher EPS yield for *Nostoc flagelliform* when compared with white light (Han et al., 2014).

1.6.7. Temperature

The temperature effect on EPS production shows variation according to selected microorganisms. In a study investigating the effects of temperature on EPS production of *Oscillatoria formasa*, the productivity of EPS at different temperatures is compared (Jindal et al., 2011). The result of the study showed that when comparing the EPS yield at 35°C and 21°C, an increase in temperature resulted in a two-fold increase in yield.

In another study, the EPS productivity of *Anabaena* sp. could be increased 4-5 times by increasing the culture temperature to 40-45 °C (Moreno et al., 1998). When temperatures of 30, 35, and 40°C were applied to *Arthrospira platensis,* the maximum EPS yield was obtained at 40 °C (Trabelsi et al., 2009). The highest temperature effect on EPS productivity was analyzed on *Graesiella* and the maximum yield was obtained at the highest temperature 40 °C (Mezhoud et al., 2014). In another study, the highest EPS yield was obtained from *Botryococcus braunii* at 25-30 °C temperature range (Lupi et al., 1991).

1.6.8. Salinity

In another literature study, the effect of salt stress on EPS production is analyzed in different strains of the Synechocystis species (Ozturk & Aslim, 2010). An increase in EPS production was observed with increasing salt stress; however, beyond 0.4 M NaCl stress, there was significant growth inhibition. Therefore, applying 0.2 M NaCl stress resulted in an approximately 10% increase in EPS production. The salt stress can induce EPS productivity according to previous studies. In one study, the EPS productivity of *Dunaliella salina* was induced by increasing salt concentration from 56 to 944 mg/L (Concórdio-Reis, David, et al., 2023). The EPS production of *Chlamydomonas reinhardtii* increased under the 100 and 150 mM NaCI stress (Khona, Shirolikar, Gawde, Hom, et al., 2016).

1.6.9. Growth Phase

In addition to cultural factors that show different efficacy based on species, the growth phase is also important for EPS yield. For instance, some *Nostoc* species show the maximum EPS productivity in the exponential growth phase while *Cyanothece* and *Rhodella* species produce EPS only in the stationary phase (Delattre et al., 2016). However, in general, the maximum EPS yield is obtained in the stationary phase because the nutrient is finished, and cells enhance new strategies like EPS secretion to survive. In another study, for *Chlamydomonas mexicana* the EPS production increased in stationary phase (Kroen & Rayburn, 1984).

The culture parameters such as high temperature, nutrient starvation, and salt stress, which can increase EPS production, can have a negative impact when they suppress the growth of microalgae. In such cases, a two-stage cultivation system emerges as a

favorable method. In the two-stage cultivation process, cells are cultivated under normal conditions until they reach the exponential phase, after which the specified stress parameters are applied (Delattre et al., 2016).

1.7. Application Areas of Exopolysaccharides

EPSs have been used in various application areas such as food, cosmetics, pharmaceutical, and biomedical industries (Figure 4). Despite the potential of microalgal EPSs in these areas, it is seen that the use of bacterial EPSs is preferred when examined on an industrial scale.

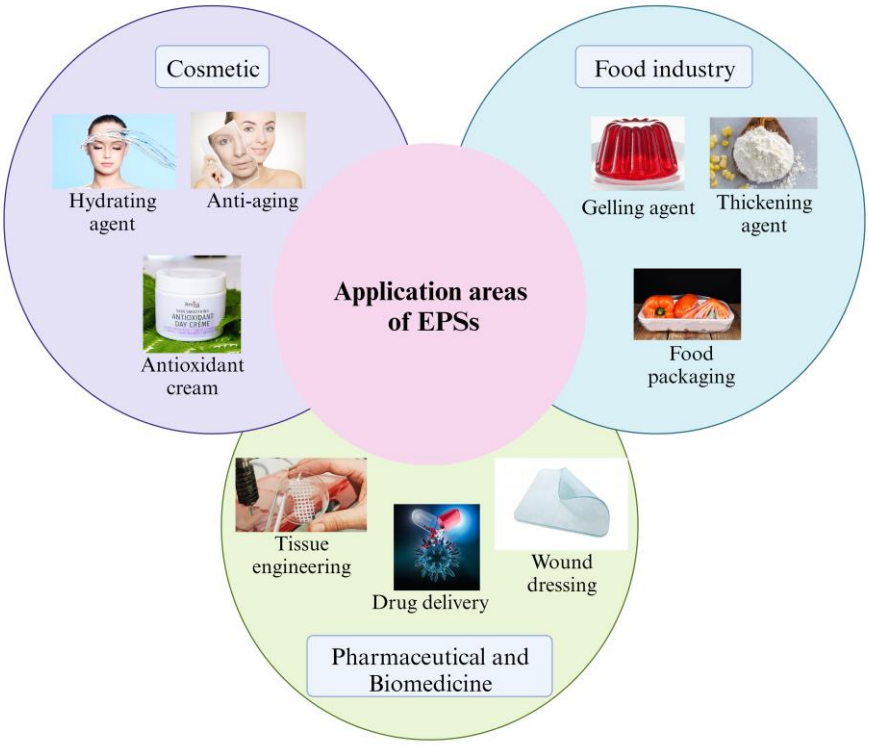


Figure 4. The application areas of EPSs

1.7.1. Food Industry

EPSs can be used in the food industry due to their thickening and gelling agent properties. In the food industry, bacterial EPSs are used as emulsifiers, gelling agents, stabilizers, and viscosifying agents. Researchers are evaluating EPSs as natural

components that could replace synthetic food stabilizers (Suryawanshi et al., 2022). Curdlan is an example of a food additive that is used for stabilization and viscosity (Osmałek et al., 2014). Dextran, a microbial-derived EPS known for its use as a gelling, texture, and emulsifier in the food industry, is classified as GRAS. In another study, the good emulsifier activity of *Lactobacillus sakei* L3 EPS was confirmed (B. Wang et al., 2019). In addition to food additive potential, EPSs also have the potential to be used for food packaging. Pullulan, gellan, alginate, and xanthan are the most known examples of this situation.

1.7.2. Cosmetic

EPSs can be used in cosmetic applications due to their antioxidant and anti-aging properties. In a valuable research study, the EPS produced by *Spirulina* was evaluated for cream formulation due to its antioxidant activity (Pierucci et al., 2017). Alguronic acid, which has been patented by Algenist, exhibits anti-aging activity that can be cited as a commercial use example. Astaderm and Acqualift, EPSs obtained and purified from two different species of Porphyridium, are used as ingredients in the cosmetics industry (Morais et al., 2022). The EPS of *Porphyridium* sp. is evaluated as a hydrating agent in cosmetic applications and the product is known as Hydrintense^{TM} (Tounsi et al., 2024).

1.7.3. Pharmaceutical and Biomedicine

EPSs have gained attention in pharmaceutical industries due to their antibacterial, antiviral, anticancer, anti-inflammatory, and immunostimulatory activities. For instance, a sulfated polysaccharide obtained from *Spirulina platensis* and known as *Calcium spirulan* is known with antitumor activity.

In medical applications, EPSs can be evaluated for use in tissue engineering and drug delivery systems. The easy preparation procedure, and non-toxic, biocompatible nature of EPSs make them advantageous alternatives to commercial equivalents. EPSs can form hydrogel materials that are unsolved in water and these structures are evaluated as suitable drug carrier systems. Drug delivery systems are important for the efficiency of treatment because they protect against degradation and application of needed dosages without decreasing during reach to the targeted area (Asgher et al., 2020).

1.8.Commercial Applications and Market Potential of Exopolysaccharides

Despite their significant characteristics, the commercial use of EPSs obtained from microalgae is limited. Bacterial EPSs have a higher potential for commercial use compared to microalgal EPSs. Despite the advantages and properties of microalgal EPSs, industrial application generally depends on bacterial EPSs. The main reason why they are not widely used on an industrial scale is that the amount of EPS obtained from bacteria is higher (Pierre et al., 2019). To obtain high amounts of EPS from microalgae, it needs to be cultivated on a large scale and this is not preferred due to its high cost. However, studies in this field have been increasing rapidly with the increasing interest in microalgae-derived EPSs in recent years. Strategies are being developed to increase the production efficiency of EPS from microalgae and reduce production costs. One of these strategies involves reducing production costs by using chemicals at a technical level instead of analytical grade chemicals in open or closed pond systems. In addition, wastewater can be evaluated as growth media for microalgae and so the cultivation cost can be decreased. The selected strain, cultivation conditions, and extraction methods are important parameters that affect EPS yield. Therefore, after selecting the appropriate strain, applying factors such as nutrient deprivation, salt stress, and light stress to the culture conditions, and utilizing extraction methods that minimize the reduction in yield, can enhance EPS production for maximum yield.

Dextran, Xanthan, Pullulan, Alginate, and Hyaluronic acid are the most known bacterial EPSs and have applications in food, pharmaceutical, cosmetic, and medicine areas (Rana & Upadhyay, 2020). The market value of xanthan gum has been increasing and is estimated to reach ~US$ 1.5 billion in 2027 (Nadzir et al., 2021).

1.9.Biomaterial Formation Potential of Exopolysaccharides

EPSs gain attention with their antibacterial, antioxidant, and anticancer-like activities in different application areas. However, in recent years, EPS has gained the attention of the biomaterial industry based on biopolymers. There has been a rapid increase in interest in polysaccharide-based products in the fields of biomedicine and food packaging. Many polymers used in this field being petroleum-based and non-biodegradable, posing environmental hazards, have led to the scientific community's

interest in natural, renewable, and biodegradable sources to replace them. Polysaccharides are considered suitable sources for this purpose, and research in this context has shown rapid growth in recent years. Numerous studies have shown that polysaccharides are incorporated as raw materials or by-products in the structure of many products that can be used in tissue engineering, food packaging, and biomedical fields. In this concept, EPSs can be evaluated for the formation of film, hydrogel, and nanofiber materials that have usage potential in food packaging, wound healing, drug delivery systems, and tissue engineering.

1.9.1. Film

Nowadays, packaging created using various resources is widely used to protect foods from environmental conditions and mechanical stresses. In food packaging applications, generally, petroleum-based plastics are used and preferred because of their cost-effective and easy-to-process properties. Moreover, their excellent mechanical strength, thermal stability, and barrier properties make them suitable candidates for food packaging applications. The volume of plastic used in food packaging constitutes 20% of global plastic production. However, due to some disadvantageous properties of synthetic-based plastics and packaging, the search for alternative materials has been increasing rapidly in recent years. Synthetic plastics, especially those used in the field of food packaging, constitute a major portion of environmental pollution. Moreover, there is a risk that the chemicals in the structure of food packaging made from synthetic plastics may mix with the food and pose a threat to human health.

Currently, biopolymers are gaining attention as the most potential candidates for food packaging applications due to being natural, non-toxic, and biodegradable polymers. Biopolymers, also regarded as biodegradable plastics are comprised of polysaccharides, proteins, and aliphatic polyesters. Moreover, the antibacterial and antioxidant nature of biopolymers increases the value of film materials that are used for food packaging. In addition to these advantageous properties of biopolymers, they are also non-harmful to the environment, unlike synthetic polymers.

In recent years polysaccharides have been evaluated as an alternative polymer source for the food packaging industry due to their biodegradable natures. Polysaccharides not only serve as suitable polymers for food packaging due to their barrier function for

gas permeation but also enhance the quality of the film structure by providing additional protection through their antioxidant and antibacterial activities. Another advantage of polysaccharide-based films is that they can be obtained from renewable sources such as cyanobacteria and microalgae. Synthetic polymer-based films are considered harmful to the environment not only because they do not biodegrade in nature but also because they are produced from finite resources like petroleum (Anis et al., 2021). In the polysaccharide category, EPSs obtained from renewable sources like microalgae and cyanobacteria have an increasing interest (Perera et al., 2023). The main cause of this interest is easy to obtain these polymers because EPSs generally secreted to growth media or remain attached to the cell surface. Moreover, the production of EPS can be increased easily by manipulating culture conditions.

Polysaccharide films are used in the food industry, but another potential of polysaccharides is their suitability for use as edible film and coating materials. Edible film and coating materials can not only protect food but also be consumed along with food and serve as a functional food. Packaging materials should ensure the preservation of food without deterioration during transportation, storage, and the sales process. Water vapor and oxygen are factors that affect the shelf life and quality of food; therefore, it is expected that the food packaging material to be used exhibits a barrier property against these factors (Popyrina et al., 2023).

Despite the advantages nature of biopolymers, the use of polysaccharides in food packaging is limited due to certain characteristics. The polysaccharide-based materials fall short in terms of their physicochemical properties for use in food packaging. The mechanical properties such as tensile strength and percentage of elongation of film materials created from polysaccharides are also important parameters for their use in food packaging. While some polysaccharide films exhibit mechanical properties like those created from synthetic polymers, the low mechanical strength of certain polysaccharides can be a limiting factor for their use in food packaging. Moreover, the high susceptibility to moisture limits the use of these polymers.

In recent research, studies have been made to improve the structure by adding some synthetic polymers in small amounts, plasticizers, and natural agents like essential oils. Modifications can be made to the polysaccharide structure to enable the formation of film structures that can be used for food packaging purposes. At this point, various

synthetic polymer additives or plasticizers are being used to enhance the structural strength. Glycerol and sorbitol are the most used plasticizers (Cazón et al., 2017). Glycerol is commonly used for plasticization, which is one of the most common modifications. Thus elongation, distribution, flexibility, elasticity, and rigidity of film can be enhanced (Janik et al., 2022).

Cellulose, chitosan, starch, and alginate are the most pronounced polysaccharides in food packaging and coating studies. The commercial products of cellulose and starch are available in food packaging. Despite being considered for food packaging due to its gel-forming capacity and high chemical stability, the use of cellulose, one of the most prevalent polysaccharides, is restricted because it does not completely dissolve in water. However, by performing derivatization on the structure, limitations like these can be overcome, and additionally, the thermal properties of the structure can be enhanced. Cellophane, made from regenerated cellulose and produced on an industrial scale, is used in food packaging due to its barrier properties against air, water, and bacteria.

In a valuable study, the EPS of *Graesiella* sp. was evaluated for biodegradable film production to use in meat packaging (Gongi et al., 2022). The results showed that the EPS can form a film when it is mixed with polyethylene glycol and the properties of the film are suitable for packaging. In another study, the EPS extracted from kefir grains was evaluated for biodegradable edible film (Ghasemlou et al., 2011). Due to the poor mechanical properties and hydrophilic nature of the polysaccharide film, the use of these films is limited. In this study, to overcome these limitations and enhance the structure of EPS film, glycerol was used as a plasticizer. The results showed that the addition of plasticizers enhances the film properties to be used in packaging applications. In another study, the EPS obtained from *Enterobacter* was mixed with citric acid to produce a biodegradable film, and the obtained film structure showed good mechanical properties (Ferreira et al., 2014). The EPS of *Alteromonas* strains was also evaluated for biodegradable film formation by the addition of glycerol, and suitable film structures that can be used in biomedicine or food packaging applications were obtained (Concórdio-Reis et al., 2022). Moreover, the EPSs can be used as an additive to other polysaccharide-based film formulations to enhance the properties of film. In a research study, the EPS extracted from the fungi *Bacillus velezensis* P1 was

added to the chitosan film solution to gain antifungal activity (Binmad et al., 2022). Then, this film formulation was evaluated for the coating of mangoes compared with the only chitosan-containing film formulation. The results showed that the shelf life prolongs in both conditions but in the EPS-containing formulation, extra antifungal activity was observed.

1.9.2. Hydrogel

Hydrogels are polymeric materials that can absorb water and other liquids in large quantities and have a high holding capacity against water (Chelu & Musuc, 2023). These materials are used in many different industrial, medical, and environmental applications due to their water absorption capacity and retention properties. For example, in the medical field, they are used in areas such as wound care, drug delivery, and tissue engineering. In the agricultural field, there are many applications such as polymeric irrigation gels used in the development of soil moisturizing and irrigation systems.

Hydrogels can be classified into different categories according to various parameters like source, type of crosslinking, response, and preparation. Hydrogels can be formed from natural or synthetic polymers as well as hybrids by mixing these two polymer types (Peppas & Hoffman, 2020). Hydrogels show various advantages and disadvantages depending on whether they are formed from natural or synthetic sources. Although hydrogels made of natural polymers are considered advantageous due to their non-toxicity, biodegradability, and biocompatibility, they are considered disadvantageous compared to hydrogels made of synthetic polymers in terms of mechanical strength and controllability of production. Polymers such as cellulose, starch, and alginate are commonly used as natural polymers for hydrogel formation, while synthetic polymers such as polyvinyl alcohol and polyethylene glycol can be used as synthetic polymers.

Another classification parameter is their formation mechanism because hydrogels can be formed via physical crosslinking or chemical crosslinking mechanisms and different crosslinkers are used for both procedures. Physically crosslinked hydrogels are referred to as physical hydrogels and produce hydrophobic associations, crystallization, polymer chain complexation, or hydrogen bonding. However, chemically crosslinked hydrogels are formed with covalent bonding and these hydrogels are permanent and irreversible while the physical hydrogels are reversible.

The toxicity of chemically crosslinked hydrogels due to toxic crosslinker is a major disadvantage of this type of hydrogel (Ullah et al., 2015). For the chemically crosslinked hydrogel formation, the addition of a chemical crosslinker, photo- or radiation-initiated free radical polymerization, or enzyme-assisted reaction can be performed (Lin et al., 2022).

Synthetic-based hydrogels have some disadvantages like non-biodegradable and potential toxicity. At this point, natural polymers gain attention in the biomaterial field due to their biocompatible and non-toxic natures. Polysaccharides are considered one of the most suitable candidates for natural polymer-based biomaterial formation. Among the common polysaccharides used in this field, cellulose, chitosan, alginate, and starch can be mentioned. Hydrogel structures formed from these polysaccharides stand out due to their high water-retaining capacities, renewability, biocompatibility, and non-toxicity. The polysaccharides have functional groups like carboxylic acid and hydroxyl groups that are important for crosslinking mechanisms (Laubach et al., 2021).

1.9.2.1.Hydrogels in Drug Delivery Applications

In recent years, the use of hydrogels as drug delivery systems that protect against drug degradation has become one of the intriguing topics (Far et al., 2022). Hydrogels can absorb and transport the drug placed inside. Its chemical structure and porous structure can create an ideal environment to hold drug molecules. Hydrogels can provide a controlled release of the drug. This can increase the effectiveness of the medicine by ensuring that it is released into the body at a specific rate and in a stable manner. Since polysaccharides are naturally occurring components, they are generally compatible with the body. This means they have high biocompatibility and can minimize foreign substance reactions (Sanjanwala et al., 2022).

1.9.2.2. Hydrogels in Wound Healing

Wound healing is a complicated process that contains coagulation, inflammation, cell migration and proliferation, and remodeling steps. Hydrogels are considered an effective method in wound healing because they can provide a biocompatible, moisture-retaining environment around the wound and prevent the formation of microbial infections. Hydrogels can accelerate wound healing by keeping the wound

moist because a moist environment can allow cells and tissues to recover, which can promote faster healing. Hydrogels can create a barrier to protect the wound from external agents. Therefore, they can reduce the risk of wound infection and help protect the wound surface against external factors (Sulastri et al., 2021).

1.9.2.3. Hydrogels in Tissue Engineering

Polysaccharide-based hydrogels are used in a wide range of applications in the field of tissue engineering. Tissue engineering is the process of developing artificial tissues to replace or repair damaged or lost tissues in the body. Hydrogels can provide a suitable environment for cells to grow, proliferate, and form a specific structure. In this way, they can be used to create artificial tissues in tissue engineering applications. In tissue engineering applications, scaffold structures that mimic the extracellular matrix are used for tissue development. These scaffold structures are expected to be non-toxic, biocompatible, and possess suitable mechanical properties. Within this scope, polysaccharide-based hydrogel structures gain attention due to the non-toxic, biodegradable, and biocompatible nature of polysaccharides. For this purpose, cellulose, chitosan, alginate, and starch are used for hydrogel formation which can be used in tissue engineering (T. Zhu et al., 2019).

Considering this information, the usage potential of EPSs in biomaterial formation will be evaluated. The potential use of bacterial EPSs in this field is more widely evaluated than microalgal EPSs. This is caused by the high EPS production capacity of bacteria, but the rich content of microalgae can provide additional properties to the biomaterial. Moreover, microalgae have been evaluated as renewable and green production systems in recent years. Therefore, the optimization of biomaterial production from microalgal EPS increases the competition in the polymer market. The biodegradable, non-toxic, and biocompatible nature of EPS-based materials can be safely applied to the human body.

2. MATERIALS and METHOD

2.1.Sampling and Identification

The microalgae were sampled from Horseshoe Island (67°49'36.6"S, 67°13'38.7"W) in Antarctica by Prof. Dr. Turgay ÇAKMAK. The samples were collected into 50 ml falcon tubes and later cultivated in five different growth media (BBM, BG-11, TAP, FW, and F2). The growth characteristic of microalgae was evaluated by comparing the situation in five different mediums and so suitable media was determined. The purity of culture was investigated by microscopic examination and to obtain axenic culture microalgae was seeded in agar plates. The streak plate method was used to purify the microalgae culture. In this method, the cells were seeded in an agar plate, and after microscopic examinations single colony was transferred to a new agar plate.

Figure 5. The area where the microalgae was isolated on Horseshoe Island

The identification was carried out with a modified CTAB method (Aljanabi & Martinez, 1997; Winnepenninckx & Backeljau, 1993). The molecular analysis and microalgae identification were carried out according to 18 rDNA regions. The DNA extraction was performed with 100 mg of fresh biomass that was stored at -80 °C after being frozen with liquid nitrogen. The CTAB extraction buffer was heated to 60 °C before use. 100 mg of microalgae biomass was dissolved in 200 µl CTAB solution and nearly 70 mg of glass beads were added to the solution for the homogenization process during 1 min. Afterward homogenization, 200 µl extraction buffer was put into the mixture and later, 40 µl SDS (10%) and 8 µl Proteinase K (20 mg/ml) were added. The final solution was mixed with vortex for 5 seconds and incubated at 60 °C for 1 h with a thermoshaker. Then, the samples were cooled to room temperature and gently mixed after 2 min. The 400 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added

into the solution and mixed with a rotator (20 rpm) for 2 min. Following the incubation, the solution was separated into three phases via centrifugation at 14000×g for 10 min. The bottom layer contains an organic phase with green pigment, the middle layer contains proteins or not lysed cell residues and the upper layer contains DNA fragments. The upper phase containing DNA fragments was transferred into a new tube to wash by using 400 µl of Phenol: chloroform: isoamyl alcohol (25: 24: 1) solution. The washing procedure was applied by mixing the samples for 2 min and washed DNA samples were obtained via centrifugation at 14000×g for 10 minutes. The upper phase containing the washed DNA fragments was collected into a new centrifuge tube and the same volume of cold isopropanol was added. Then, the samples were mixed via a rotator for 3 min and samples were stored at -20 °C to obtain visible DNA samples. The solutions were centrifuged at 14000×g for 10 minutes at 4 °C and visible DNA samples were obtained by removing the supernatant part. The DNA pellets were dissolved in 750 µl of iced-cold ethanol (70%) and mixed via rotator for 2 min. Following the centrifugation procedure at 14000×g for 1 min, after the removal of the supernatant part remaining ethanol was evaporated in a sterile cabinet.

The purity and concentration of DNA were controlled by a nanodrop spectrophotometer. The dissolved DNA pellets in 100 µl of nuclease-free distilled water were measured at 260/280 nm absorbance for this aim. After quantification, 50 µl of DNA sample was stored at 4 °C or -20 °C according to the usage time. After the purity and concentration were confirmed, PCR amplification of DNA fragments was performed.

For PCR amplification, 100 ng/ml of DNA was used, and different primers were evaluated as seen in Table 2. In the PCR solution, 25 µl master mix (EcoTaq2xPCR master mix), and 0.5 µl of primer were added for both reverse and forward primer. The final volume was completed to 50 µl by adding nuclease-free distilled water.

Table 2. The primers used for PCR amplification.

Primers	**Sequence 5'- 3'**	**Reference**
EAF3	TCGACAATCTGGTTGATCCTGCCAG	(Marin et al., 2003)
ITS055R	CTCCTTGGTCCGTGTTTCAAGACGGG	(Marin et al., 2003)

The PCR amplification was run in a thermocycler at the conditions represented in

Table 3.

Table 3. The conditions for PCR amplification

Stage	Temperature	Time	Cycles
Initial Denaturation	95 °C	2 min	1 cycle
Denaturation	95 °C	1 min	30 cycles
Annealing	55 °C	1 min	
Extension	68 °C	3 min	
Final extension	72 °C	5 min	1 cycle

The PCR-amplified samples were run on an agarose gel to assess the size, quantity, and purity of the amplified DNA fragments. For this aim, 1% agarose gel was prepared by using 1 X Tris-Acetate-EDTA (TAE) buffer containing 10 mg/ml ethidium bromide. The electrophoresis process was run at 80 volts for 100 minutes and gels were photographed using a gel imaging system (Azure Gel İmaging Systems, VWR). At the end of the procedure, PCR-amplified DNA samples were sent to an institution for Sanger sequencing analysis. The culture of the isolate, which was identified as Chlamydomonas sp. ASYA25 according to Sanger sequencing and blast results, is maintained in the IMU-ASYA culture collection at Istanbul Medeniyet University (https://www.imuasya.org/collection).

2.2. Morphological Identification and Growth Characteristic

Following the identification of microalgae as *Chlamydomonas* sp. ASYA25, the suitable growth medium was tested again by controlling the growth kinetics in TAP (Andersen, 2005), BG-11 (Stanier et al., 1971), and 3N-BBM medium (No, 1963). The 3N-BBM was selected most suitable media for the growth of *Chlamydomonas* sp. ASYA25 and cultures were continued in this medium (Table 4).

Table 4. The content of the BBM medium

BBM (Bold Basal Medium)		
Stock Solution	**Component**	**Final Concentration in Medium**
S1	$NaNO_3$	$2.94 . 10^{-3}$ M
S2	$MgSO_4.7H_2O$	$3.04 . 10^{-4}$ M
S3	NaCl	$4.28 . 10^{-4}$ M
S4	K_2HPO_4	$4.31 . 10^{-4}$ M
S5	KH_2PO_4	$1.29 . 10^{-3}$ M
S6	$CaCl_2$	$1.70 . 10^{-4}$ M
S7 Trace element solution	$ZnSO_4.7H_2O$	$3.07 . 10^{-5}$ M
	$MnCl_2.4H_2O$	$7.28 . 10^{-6}$ M
	MoO_3	$4.93 . 10^{-6}$ M
	$CuSO_4.5H_2O$	$6.29 . 10^{-6}$ M
	$Co(NO_3)_2.6H_2O$	$1.68 . 10^{-6}$ M
S8	H_3BO_3	$1.85 . 10^{-4}$ M
S9	$Na_2EDTA.2H_2O$	$1.71 . 10^{-4}$ M
	KOH	$5.53 . 10^{-4}$ M
S10	$FeSO_4.7H_2O$	$1.79 . 10^{-5}$ M
S11 (soil extract)	garden soil	50 ml
S12	Vit. B_1 (Thiamine HCl)	$2.97 . 10^{-6}$ M
	Vit. H (Biotin)	$1.02 . 10^{-9}$ M
	Vit. B_{12}	$1.11 . 10^{-10}$ M
For 3N-BBM preparation triple quantity of S1 was added The soil extract was prepared by boiling 150 g of soil in 1.5 L distilled water The pH of the medium should be 5.5-6.5 and sterilized by autoclaving at 121 °C for 20 min		

After determining a suitable medium, the growth of cells was followed at 4, 15, and 25 °C conditions. The *Chlamydomonas* sp. AYSA25 was cultivated in 20 ml 3N-BBM media. The cultivation was carried out in shakers at 120 rpm under 50 µmol/m^2.s light intensity during 30 days. The growth characteristic was evaluated by measuring the OD at 680 nm and the morphology of Chlamydomonas sp. ASYA25 was evaluated by light microscopy imaging during growth monitoring.

2.3.Investigation of Exopolysaccharide Productivity

EPS productivity of *Chlamydomonas* sp. ASYA25 was evaluated in comparison with 3 different *Chlamydomonas* species (*Chlamydomonas moewusii* ARAS123, *Chlamydomonadaceae* ARAS124, and *Chlamydomonas reinhardtii* ARAS125) by

using two different approaches. In the first approach, the petri images of the species were comparatively analyzed, and the cultures were evaluated in terms of EPS production potential according to whether they were in mucous form or not. In the second approach, the EPS productivity was compared with EPS extraction by growing the species in a liquid media until the stationary phase. According to the results, *Chlamydomonas* sp. ASYA25 was selected as an EPS producer with a higher EPS yield.

2.4. Optimization of Exopolysaccharide Production via Plackett-Burman Experiment Desing

The literature studies show EPS content and yield are changeable based on species, culture parameters, and environmental stresses. In this study, experimental groups were created using the Plackett-Burman experimental design method to increase EPS production. To maximize EPS production, nutrient limitations (nitrogen and phosphate starvation), high light intensity, basic pH level, different temperature ranges, and salt (NaCI) stress were applied to cells (Cruz et al., 2020; Jindal et al., 2011; Ozturk & Aslim, 2010; Trabelsi et al., 2009). For this aim, 12 experiment sets as seen in Table 5 were applied, and EPS extraction yield with growth characteristics of *Chlamydomonas* sp. ASYA25 was analyzed.

Table 5. The experiment sets formed via the Plackett-Burman experiment design in Minitab

RunOrder	Nitrogen (g/L)	Phosphate (g/L)	NaCI (M)	pH	Temperature (°C)	Light (µmol photon)
1	0,25	0,00	0,200	6	25	50
2	0,25	0,25	0,025	9	25	50
3	0,00	0,25	0,200	6	15	50
4	0,25	0,00	0,200	9	25	150
5	0,25	0,25	0,025	9	15	50
6	0,25	0,25	0,200	6	15	150
7	0,00	0,25	0,200	9	25	150
8	0,00	0,00	0,200	9	15	50
9	0,00	0,00	0,025	9	15	150
10	0,25	0,00	0,025	6	15	150
11	0,00	0,25	0,025	6	25	150
12	0,00	0,00	0,025	6	25	50

The *Chlamydomonas* sp. ASYA25 was grown for 10 days at an initial cell density of 0.5 absorbance in 1L BBM media prepared according to the conditions in Table 7. The beginning and final situations of the cultures are represented in Figure 6.

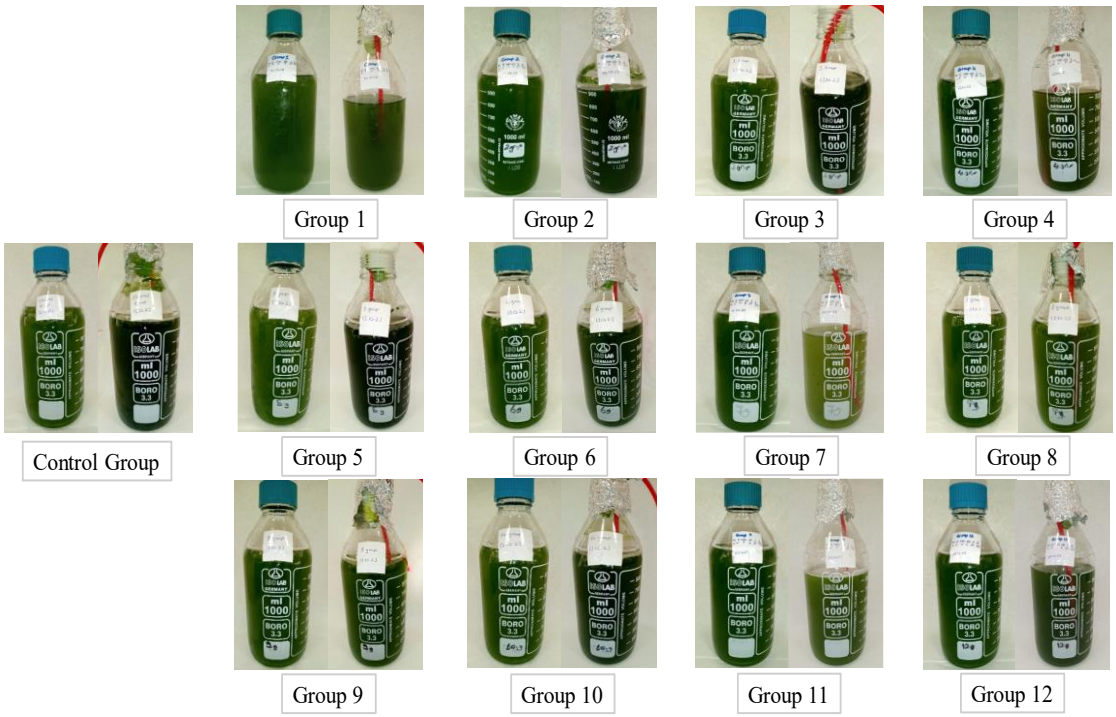


Figure 6. The groups of Plackett-Burman experiments before and after of cultivation process

In the second part of the optimization study, the Minitab18 software was used to determine the optimum condition for the maximization of EPSs. According to the results obtained from the software, the conditions of Group 6 were determined to be suitable for maximum EPS production. Then, the control group, optimized group, and some groups (Group 3, Group 7, and Group 10) that show higher EPS yield were set up to confirm the results.

2.5. Cultivation of Chlamydomonas sp. ASYA25 Under Optimized Conditions

After the determination of optimized conditions, *Chlamydomonas* sp. ASYA25 was cultured in salt stress and high light intensity applied BBM medium at 15 °C.

Figure 7. The cultivation of *Chlamydomonas* sp. ASYA25 under optimized conditions

2.6.Extraction of Exopolysaccharides

EPS extraction varies according to the forms that are released into the environment or found attached to the cell. The conventional alcohol precipitation method was used for the extraction of EPS samples released into the medium (Kumar et al., 2018). The EPS extraction was performed from cells grown to stationary phase under conditions determined by optimization studies. The extraction of released EPS was performed according to the alcoholic precipitation method (represented in Figure 8). For this aim, the cell-free supernatant was recovered via centrifugation at 5000×g, for 15 minutes at 4 °C. For the precipitation of polysaccharides, 2 volumes of 96% ethanol were applied to each 1-volume supernatant. The ethanol-applied supernatant was kept at 4 °C for 24-48 hours and the EPSs were precipitated by decreasing the solubility of polysaccharides. Then, the ethanol-supernatant solution was centrifuged at 5000 ×g, for 15 minutes at 4 °C.

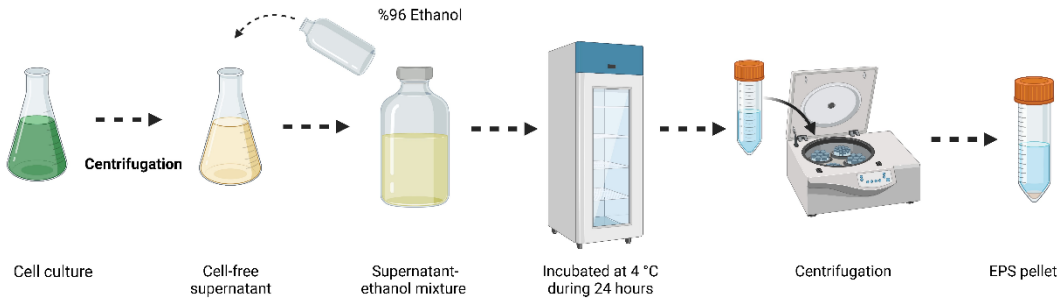


Figure 8. The extraction procedure of released EPS

The cell-bounded EPSs were extracted by using a different method because this type of EPS needs physical or chemical treatments to separate from cells. In this study, we applied a modified method as represented in Figure 9 (Bafana, 2013). To release the cell-bound EPS into the supernatant, fresh cell biomass (1 g) was dissolved in 1% NaCl solution (5 ml) and heated to 80 °C for 2-3 hours. After the heat application, the capsular EPS containing supernatant part was obtained via centrifugation at 5000 ×g, for 15 minutes at 4 °C, and 2 volumes of 96% ethanol was added to 1 volume of supernatant. The supernatant-ethanol solution was incubated at 4 °C for 24-48 hours to precipitate the EPSs and later the precipitated EPS fragments were obtained via centrifugation at 5000 ×g, for 15 minutes at 4 °C.

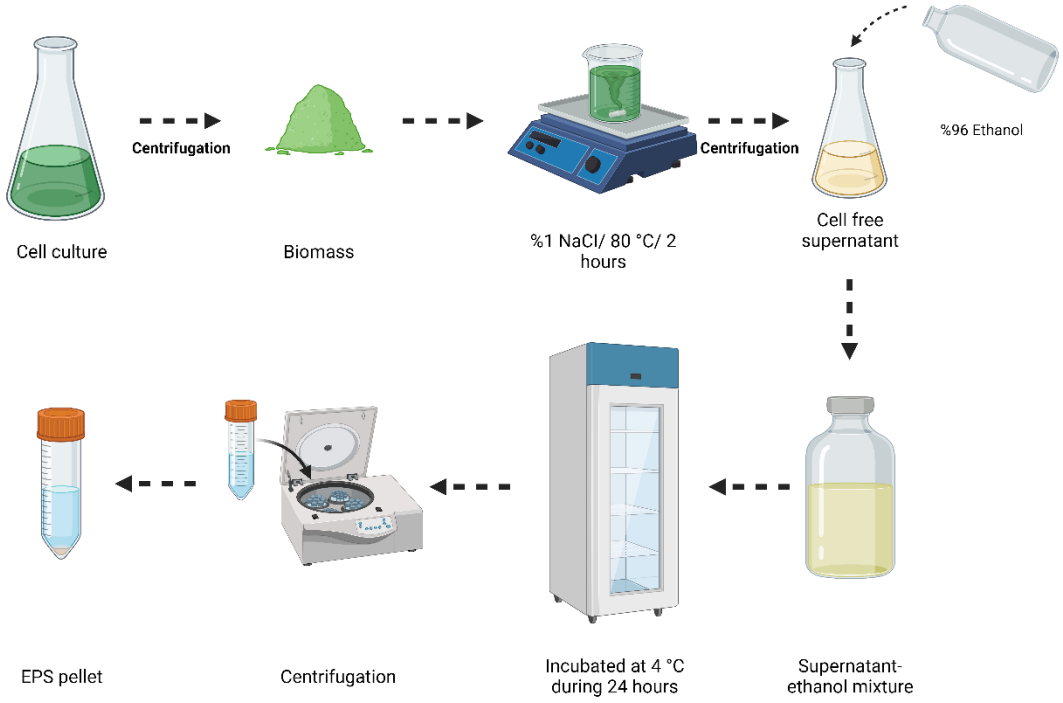


Figure 9. The extraction of capsular EPSs

2.7. Purification of Exopolysaccharides

For the purification, the EPS solution that was prepared by dissolving in distilled water was dialyzed against distilled water and a dialysis membrane with 3.5 kDa pore size was used to eliminate the remaining salt contaminants. The dialysis process was performed at 4 °C for 48 hours. The dialyzed EPS samples were lyophilized and stored

as dry powder for further analysis (W. N. Wang et al., 2022).

2.8. Characterization of Exopolysaccharides

2.8.1. Total Saccharide Content Measurement

The total saccharide content of EPSs was measured according to the phenol-sulphuric acid method (Zavřel et al., 2018). The 10 mg dry EPS biomass was dissolved in 1 ml of distilled water. Then, 500 µl of EPS solution was taken from the stock polysaccharide solution and mixed with 500 µl 5% phenol solution (prepared by dissolving 1 gram of phenol in 19 ml distilled water). The solution was incubated in a rotator for 15 minutes and later 60 µl sample was loaded onto 96 well plates from this solution. After the addition of 150 µl sulphuric acid to the samples in well plates solution was mixed by pipetting. Afterward, the reaction was completed, and the solution reached room temperature, total saccharide content was measured at 490 nm with a spectrophotometer. For the calculation of total saccharide content, a calibration curve prepared with glucose was used (Figure 10). The different glucose concentrations were prepared as seen in Table 6 and phenol-sulfuric acid protocol was applied to these samples.

Table 6. The glucose concentrations for the standard curve

From glucose stock solution (500 ug/ml)	Distilled water	Glucose (ug)
25	475	1.5
50	450	3
75	425	4.5
100	400	6
300	200	18
500	0	30

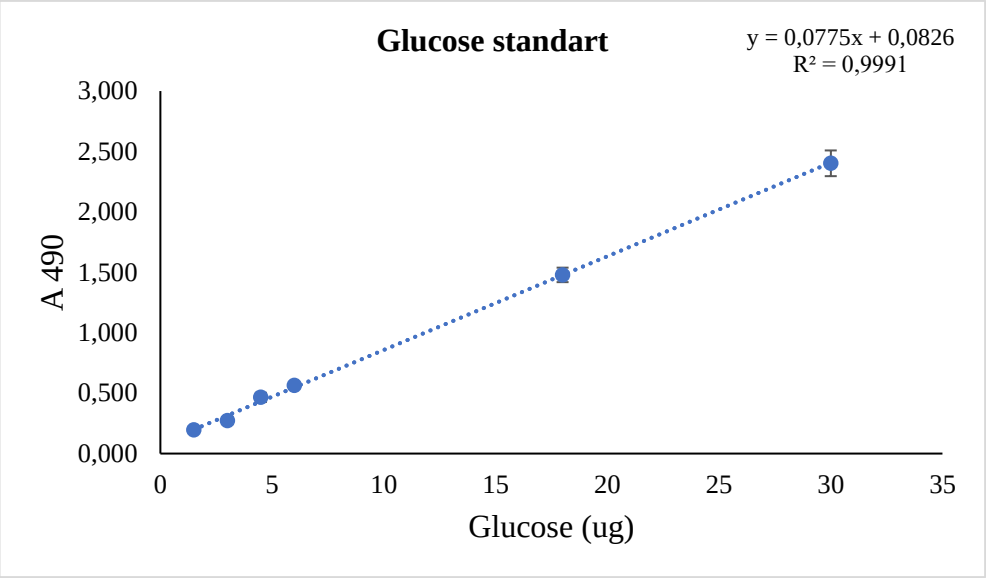


Figure 10. The standard curve of glucose

2.8.2. Total Protein Content Measurement

The protein content of EPS was measured according to a modified method (Weis et al., 2002). The 10 mg dry EPS pellet was dissolved in 1 ml of distilled water and the solution was mixed until the homogenous solution was obtained. Then 5 µl EPS sample was loaded to 96 well plates and the final volume was arranged as 10 µl by the addition of 5 µl distilled water. Then, the 200 µl of working reagent solution was added to the samples and kept at 60 °C for 30 min. The working reagent was prepared by mixing 1 part copper sulfate with 50 parts bicinchoninic acid solution and stored under dark conditions at room temperature. After the solutions reached room temperature, the absorbance was measured at 562 nm, and total protein content was calculated according to a standard curve prepared with bovine serum albumin at different ratios (as seen in Table 7 and Figure 11).

Table 7. The BSA concentration for the protein standard curve

BSA (0.5 mg/ml)	Water
µl	µl
0	10
1.5	8.5
3	7
5	5
7.5	2.5
10	0

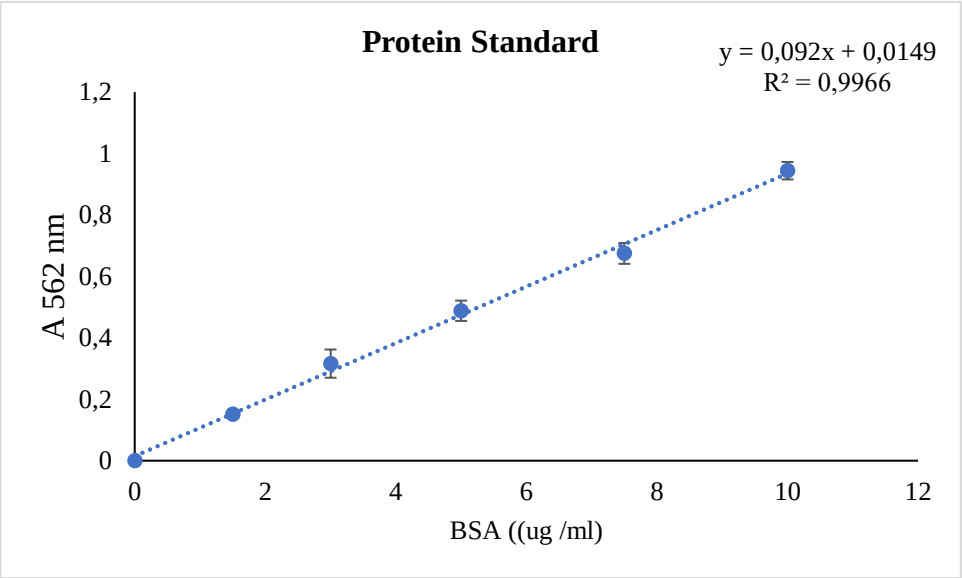


Figure 11. The standard curve of bovine serum albumin

2.8.3. Monosaccharide Content Determination

The method for the HPLC analysis of EPS was optimized by utilizing information from two different articles (Bai et al., 2015; Uhliariková et al., 2021). First, 5-10 mg of EPS sample was hydrolyzed to monosaccharides by incubating in 1 ml of 3M TFA at 110 °C for 5 hours. After the incubation, the remaining residues were discarded via centrifugation at 1000 rpm for 5 minutes at room temperature and the supernatant part was collected in a new tube. The excess TFA was removed under the airflow and the remaining residues that contained monosaccharides of EPS were dissolved in 0.5 ml of distilled water, followed by derivatization of the monosaccharides with a 0.5 ml 0.5 M PMP-methanol solution.

The 0.5 ml of 0.3 M NaOH was added into the EPS solution to create the appropriate alkaline environment for the reaction to occur. After the mixing via vortex, the solution was incubated at 70 °C for 30 min in a thermoshaker. Then, the solution was cooled until reached room temperature, and the solution was neutralized by adding 0.3 ml of 0.3 M HCl. Afterward, the solution was transferred to a new falcon tube, and excess PMP was removed by washing with the same volume of diethyl ether. The solution was centrifuged at 3000 rpm for 15 minutes after being mixed with a vortex and the organic layer (upper phase) was removed. After repeating this washing process at least 5 times, the solution was filtered by using a 0.22 µm membrane filter and transferred to vials.

The analyses were conducted using the Dionex Ultimate 3000 HPLC (Thermo scientific) system with an Acclaim C18 column (150mm×4.6 id, 3 µm). The acetonitrile and ammonium acetate (0.1 M, pH 5.5) were used as a mobile phase at a 1 ml/min flow rate and the injection volume of the sample was arranged as 2 µl. The ratio of ammonium acetate to acetonitrile was arranged as 80:20 and measurements were performed at 245 nm and 250 nm. A commercial monosaccharide kit was used as a standard and subjected to the derivatization process.

2.8.4. Sulfate Content Measurement

The sulfate content of EPSs was analyzed using a turbidimetric method containing barium chloride-gelatin reagent application (Concórdio-Reis, Ferreira, et al., 2023). Firstly, 2 mg EPS was dissolved in 500 µl TFA (2 M) and hydrolyzed by incubation at 120 °C for 1 hour. After incubation, 3.8 ml TCA (3%, trichloroacetic acid) was mixed with 200 µl of the hydrolyzed sample. Then, 1 ml barium chloride-gelatin reagent was added to the mixture and incubated at room temperature for 15-20 minutes. In the preparation of barium chloride-gelatin reagent, firstly, 75 mg gelatin was dissolved in distilled water by incubating at 80 °C for 10 minutes. Afterward, 75 mg of barium chloride was added to the gelatin solution, and the reagent kept at 4 °C was brought to room temperature before use. The samples to which only gelatin solution was added instead of barium chloride-gelatin reagent were considered as blank. The K_2SO_4 solutions (1mg/ml stock solution concentration) prepared at different concentrations were used as standards and the absorbance was measured at

360 nm. For the interpretation of the sulfate content of EPSs, a standard graph (as seen in Figure 12) prepared by using the K_2SO_4 ratios shown in Table 8 was used.

Table 8. The K_2SO_4 concentrations for sulfate content standard curve

K_2SO_4 Concentrations (µg/ml)	**K_2SO_4 Stock solution (1mg/ml) (µl)**	**Distilled water (µl)**
0	0	1000
50	50	950
100	100	900
150	150	850
200	200	800
250	250	750
300	300	700

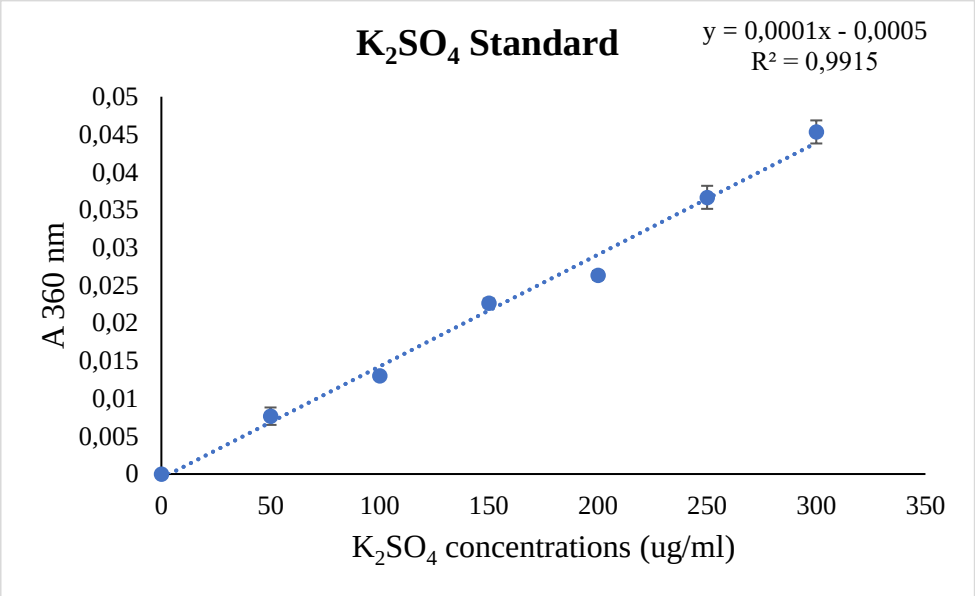


Figure 12. The standard curve of K_2SO_4

2.8.5. Uronic Acid Content Measurement

In the measurement of uronic acid content, the meta-hydroxy diphenyl method was

applied (Gargouch et al., 2021). EPS samples were prepared by dissolving in distilled water and 200 µL EPS solution was mixed with 1 ml 0.12 M borax ($Na_2B_4O_7$,10 H_2O) solution. The mixture was incubated at 90 °C for 1 hour and later 200 µl meta-hydroxyphenyl reagent (was prepared by dissolving 100 mg m-HDP in 1 ml DMSO) that diluted with 80% sulphuric acid was added. The results were evaluated according to the standard curve (as seen in Figure 13) prepared with glucuronic acid (Table 9).

Table 9. The glucuronic acid concentrations for uronic acid content standard curve

From glucuronic acid stock solution (5 mg/ml)	Distilled water	Glucuronic acid (ug)
0	200	0
10	190	7,14
20	180	14,3
30	170	21,4
40	160	28,6
50	150	35,7

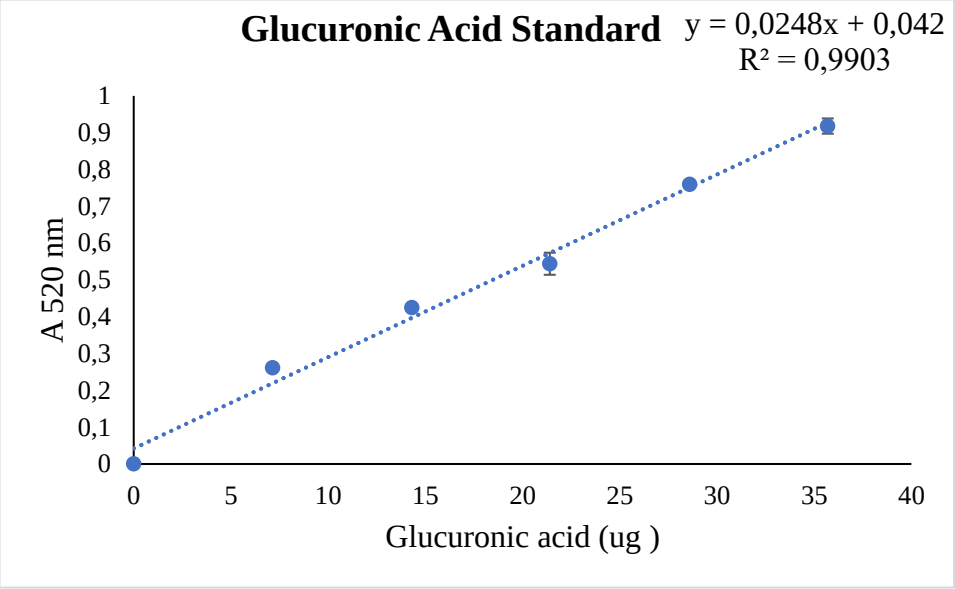


Figure 13. The standard curve of glucuronic acid

2.8.6. Fourier Transform Infrared Spectroscopy Analysis

FTIR analysis was used to characterize of general structure of EPS and the Perkin Elmer Spectrum Two machine was used for this analysis (Díaz Bayona & Garcés, 2014). The dry EPS pellet was put onto the sampler of the ATR module FTIR machine, and

the scanning was made between 400-4000 cm^{-1} at a resolution of 4 cm^{-1} and 128 scans.

2.8.7. Thermogravimetric Analysis

The thermogravimetric analysis (TGA) of EPSs was done on the Perkin Elmer TGA Instrument (Parikh & Madamwar, 2006). The thermal stability of EPSs was evaluated using nitrogen as a carrier gas in the range of 25-500 °C by heating 10°C/min.

2.8.8. Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) analysis was done on the Perkin Elmer DSC Instrument (Hosny Hussein, 2015). The thermal properties of EPSs were evaluated using nitrogen as a carrier gas in the range of 25-400 °C by heating 10°C/min.

2.8.9. Antioxidant Activity Measurement

For the antioxidant activity measurement, the method of DPPH activity analysis was employed (Choi et al., 2021). EPSs were dissolved in distilled water to create polysaccharide solutions at different concentrations ranging from 0.5 to 3.0 mg/L. Then, 1 ml EPS solution was mixed with 1 ml of 0.2 mM DPPH reagent and incubated at room temperature for 30 minutes. Ascorbic acid (at concentrations of 50 µg/ml) was used as a positive control, and absorbance values were measured at 517 nm. The scavenging activity of EPSs was calculated according to the following formulation:

$$Scavenging\ activity(\%) = \left[1 - \frac{A1 - A2}{A0}\right] \times 100$$

A0: Absorbance of the control group (using distilled water instead of the sample solution)
A1: Absorbance of the EPS sample solution
A2: Absorbance of the blank group (using ethanol instead of DPPH)

2.8.10. Antibacterial Activity Measurement

The disk diffusion method, which is the most common method for measuring antibacterial activity, was employed. In this method, antibacterial activity was

evaluated based on the inhibited zone around the filter paper disk (Benattouche et al., 2018). Antibacterial activities of EPSs were evaluated against *S. aureus*, *E. coli*, and *B. subtilis* bacteria. Bacteria cultures were seeded onto the LB agar plate at a 1.5×10^8 CFU/ml concentration. Filter paper discs were impregnated with 10 µl of EPS solution at different concentration ranges (1, 2.5, 5, 7.5, and 10 mg/ml) and placed on the agar of bacteria (as seen in Figure 14). During the measurement of the antibacterial activities of EPS, distilled water and well-known antibacterial substances, Ampicillin and Tetracycline, were respectively evaluated as negative and positive control groups.

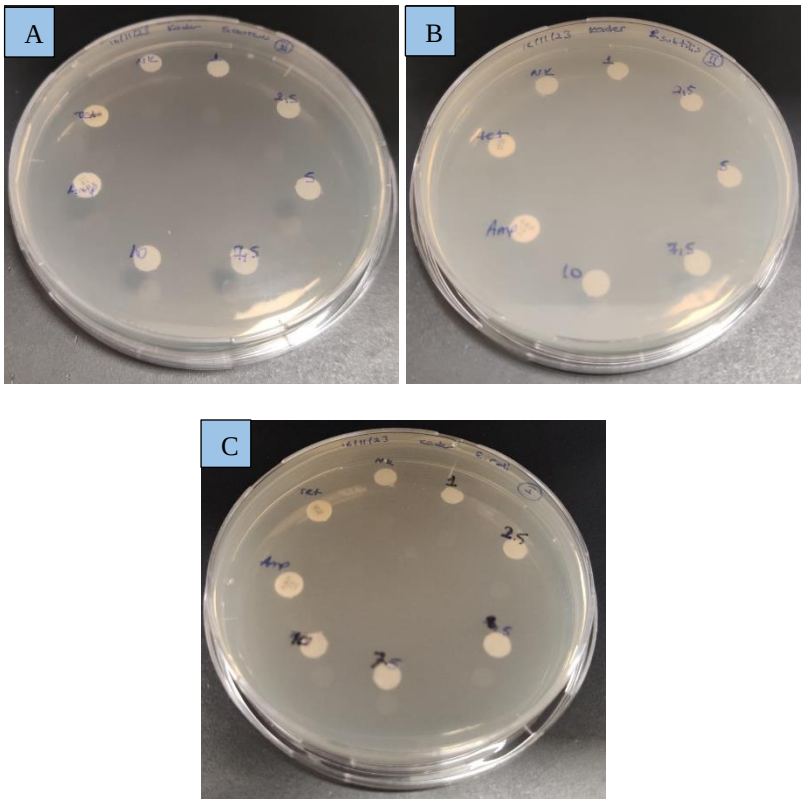


Figure 14. The image of bacterial agar plates before the incubation procedure

After placing the discs, they were incubated at 37°C for 1 day to allow bacterial growth, and subsequently, the inhibition zone around the disk was measured to calculate the antibacterial activities of the EPS.

2.9. Biomaterial Formation from Exopolysaccharides

2.9.1. Film Material Preparation

The solvent casting method was applied to create films from EPS, and optimization studies were conducted at different concentration ranges (Davoodi et al., 2021; Gongi

et al., 2022). Two different additives, polyethylene glycol (PEG) and glycerol, were used for film formation, and their effects on the film structure were evaluated comparatively. Optimization studies were conducted in 10 ml using minimum PEG concentrations to determine the desired EPS concentration for use and tested concentrations are represented in Table 10.

Table 10. Optimization of EPS concentration for film formation

Formulations of film preparation	
0.5% EPS	10% PEG (w/w_{EPS})
1% EPS	
1.5% EPS	

Firstly, EPS solutions at concentrations of 0.5%, 1%, and 1.5% were prepared, and they were kept on a magnetic stirrer for 24 hours to achieve a homogeneous solution. To determine the suitable EPS concentration, 10% PEG (w/w_{EPS}) was added to the solution with the least amount of additive (Figure 15).

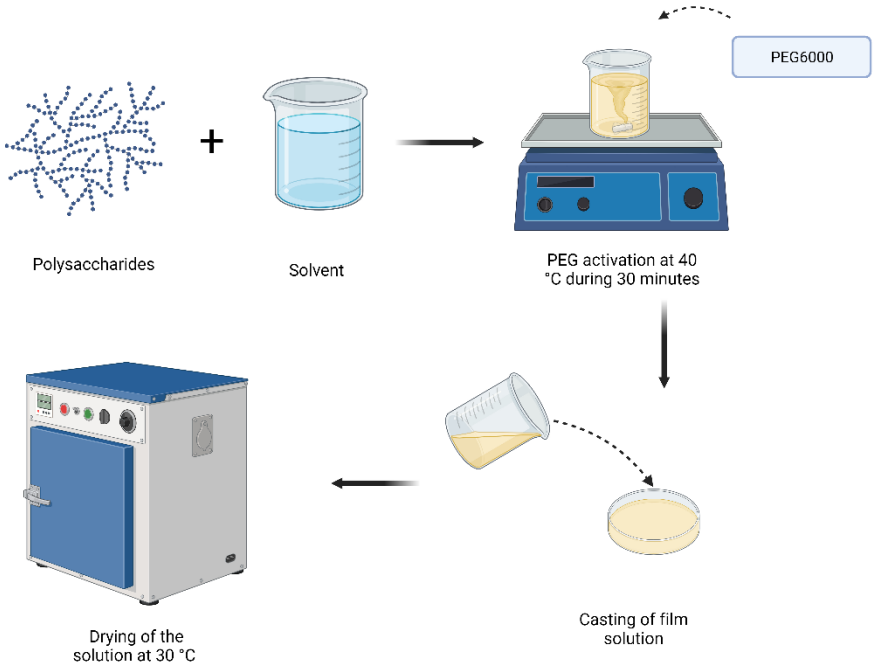


Figure 15. The film preparation procedure

The binding of EPS with PEG was accomplished by incubating at 40 °C for 30 minutes. Film solutions were prepared in 10 ml volumes and poured into 6 cm petri

dishes (Figure 16). Film formation was observed 24-36 hours later in a closed incubator at 30 °C.

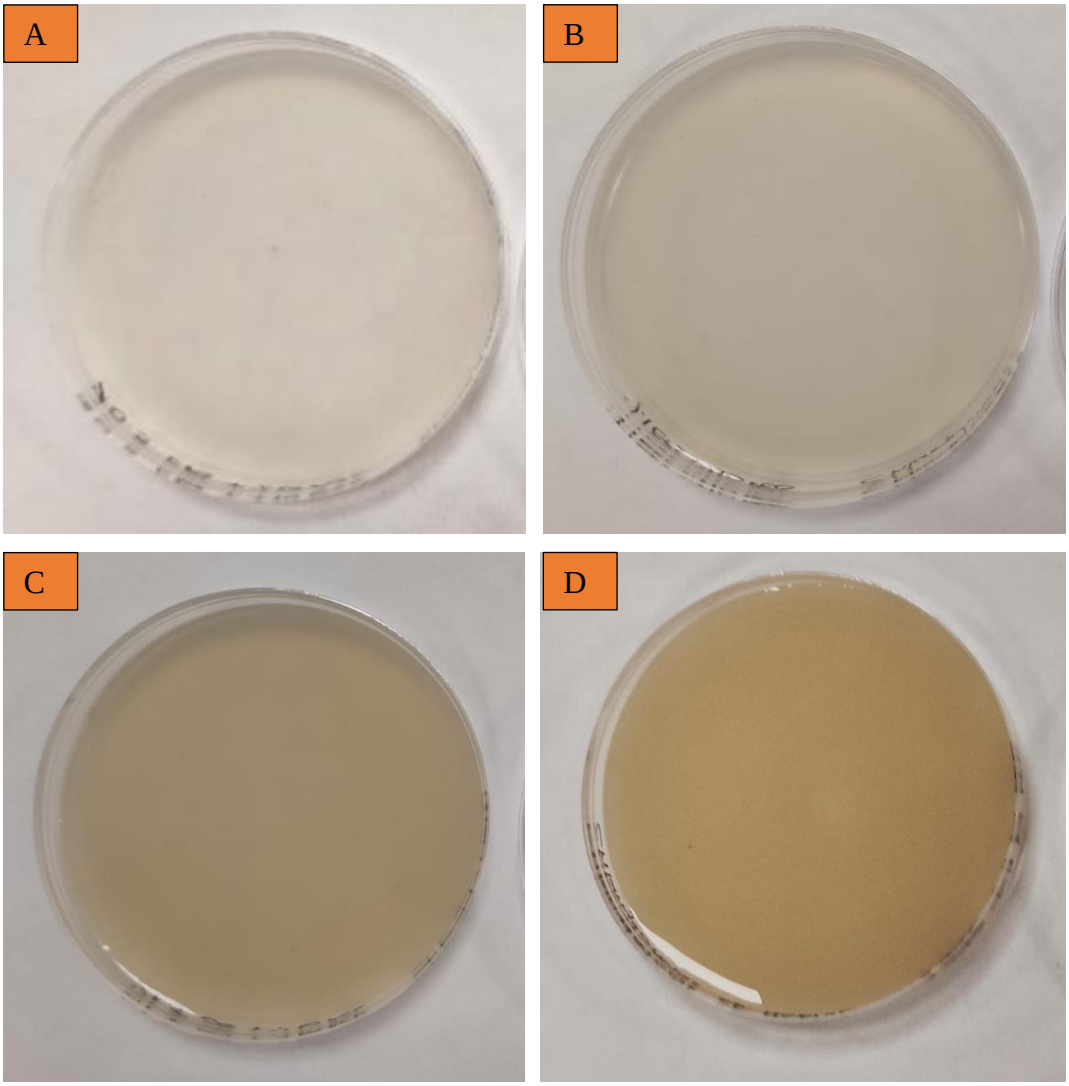


Figure 16. The films were prepared with EPS of *Chlamydomonas* sp. ASYA25 before the incubation (A: 0.5% EPS + 10% PEG (w_{PEG}/w_{EPS}), B: 1% EPS + 10%PEG (w_{PEG}/w_{EPS}), C: 1.5% EPS + 10% PEG (w_{PEG}/w_{EPS}), D: 3% EPS + 10% PEG (w_{PEG}/w_{EPS}))

Upon examining the obtained films, the results showed that the concentration of EPS was insufficient for film formation. Therefore, the EPS concentration was retested at 3% and film formation was monitored again.

2.9.2. Hydrogel Preparation

The physical and chemical crosslinking methods were comparatively evaluated in hydrogel formation studies from EPSs (Figure17). The $CaCI_2$ was used as crosslinkers in the physical crosslinking method to be carried out by ionic interactions while borax ($Na_2B_4O_7.10H_2O$) was used for the chemical cross-linking method with the formation of covalent bonds (Iijima et al., 2002; Thombare et al., 2017). For hydrogel formation by physical crosslinking, EPS solutions were mixed with $CaCI_2$ solutions at final concentrations of 0.2%, 1%, 3%, and 5%.

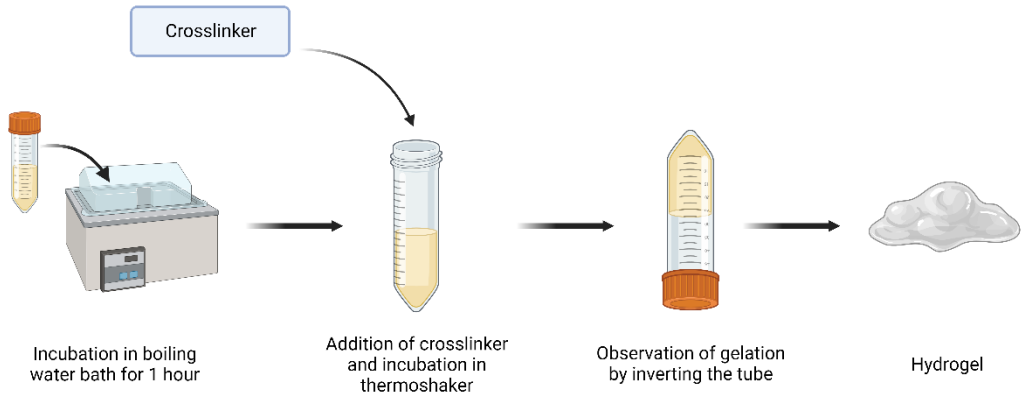


Figure 17. The hydrogel preparation procedure

For hydrogel formation by chemical cross-linking, EPS was prepared by mixing with borax in a ratio of 5%, 10%, 15%, 20%, and 25% by weight. The different concentrations of EPS solutions (0.5, 1, 1.5, and 2 %) were prepared by dissolving them in distilled water.

EPS solutions were incubated in boiling water for 60 minutes to obtain a homogeneous solution. Then, crosslinkers were added to the solution, and after incubation with crosslinker in a thermoshaker, hydrogel formation was evaluated by converting the tubes. The form of EPS solutions is represented in Figure 18 before the incubation.

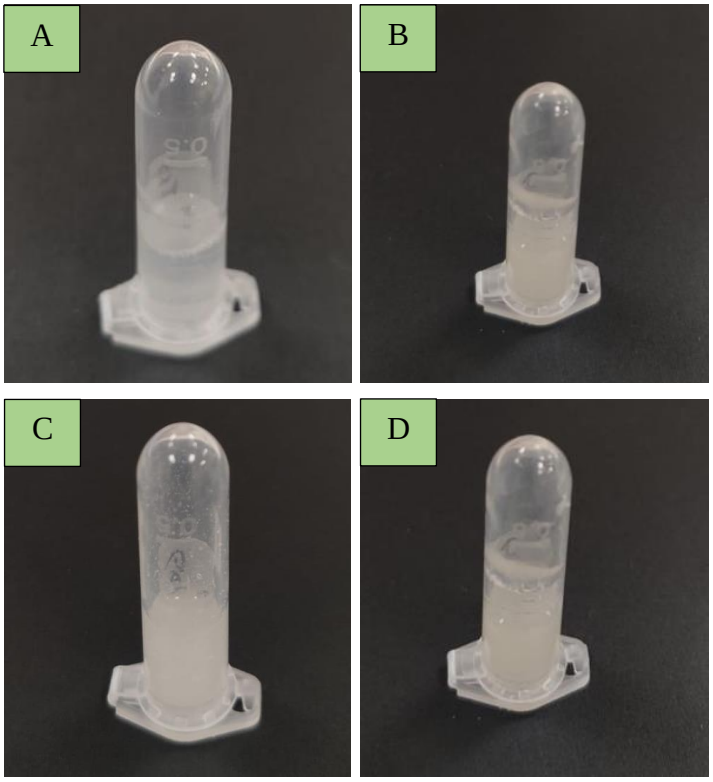


Figure 18. The image of EPS solutions before the incubation process for hydrogel formation (A: 0.5% EPS, B: 1% EPS, C: 1.5% EPS, and D: 2% EPS)

3. RESULTS

3.1. Sampling and Identification

The species used in this thesis was isolated during the 4th Turkish Antarctic Expedition from Horseshoe Island in Antarctica and identified as *Chlamydomonas* sp. ASYA25 (ON723740) according to the result of sequence analysis. For identification, the fragments obtained from DNA extraction were amplified using PCR with the primers specified in the method section. The amplified DNA fragments were loaded into the agarose gel and approximately 3500 bp DNA fragment was obtained (Figure 19).

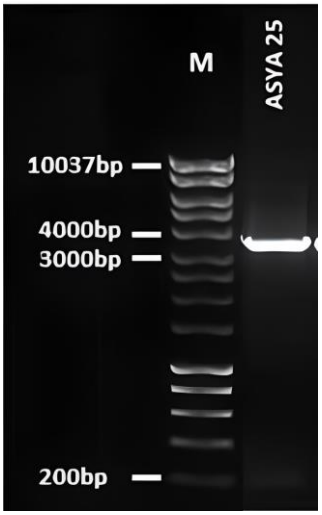
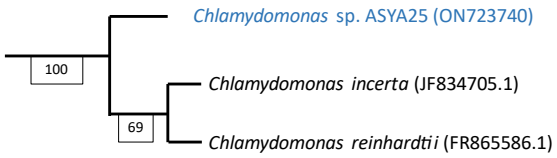


Figure 19. Gel electrophoresis results of amplified DNA with EAF3-ITS055R primer pairs.

After the size determination of DNA fragments, the identification was made with Sanger sequencing. According to the results, the species was identified as *Chlamydomonas* sp. ASYA25 and the phylogenetic tree is represented in Figure 20. The sequence of Chlamydomonas sp. ASY25 shows similarity with *Chlamydomonas incerta* and *Chlamydomonas reinhardtii*.

Figure 20. The phylogenetic tree of *Chlamydomonas* sp. ASYA25



The classification of *Chlamydomonas* sp. ASYA 25 is represented in Table 11.

Table 11. The classification of *Chlamydomonas* sp. ASYA25

Empire	Eukaryota
Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Chlorophyta infrakingdom
Phylum	Chlorophyta
Subphylum	Chlorophytina
Class	Chlorophyceae
Order	Chlamydomonadales
Family	Chlamydomonadaceae
Genus	Chlamydomonas

3.2. Morphological Identification and Growth Characteristics

Growth of Chlamydomonas sp. ASYA25 was comparatively evaluated in TAP, BG-11, and 3N-BBM media at 15 °C under 50 µmol photon light irradiance. According to the results, the most suitable medium for growth was determined as 3N-BBM as seen in Figure 21. The *Chlamydomonas* sp. ASYA25 did not grow well in TAP media when started at the same cell density. When cultured at higher cell density, it did not grow properly and showed contamination.

Figure 21. The growth of *Chlamydomonas* sp. ASYA25 in different media

After determining the appropriate growing medium, growth kinetics were evaluated at different temperatures of 4°C, 15°C, and 25°C. The images of cultures at different temperatures are represented in Figure 22.

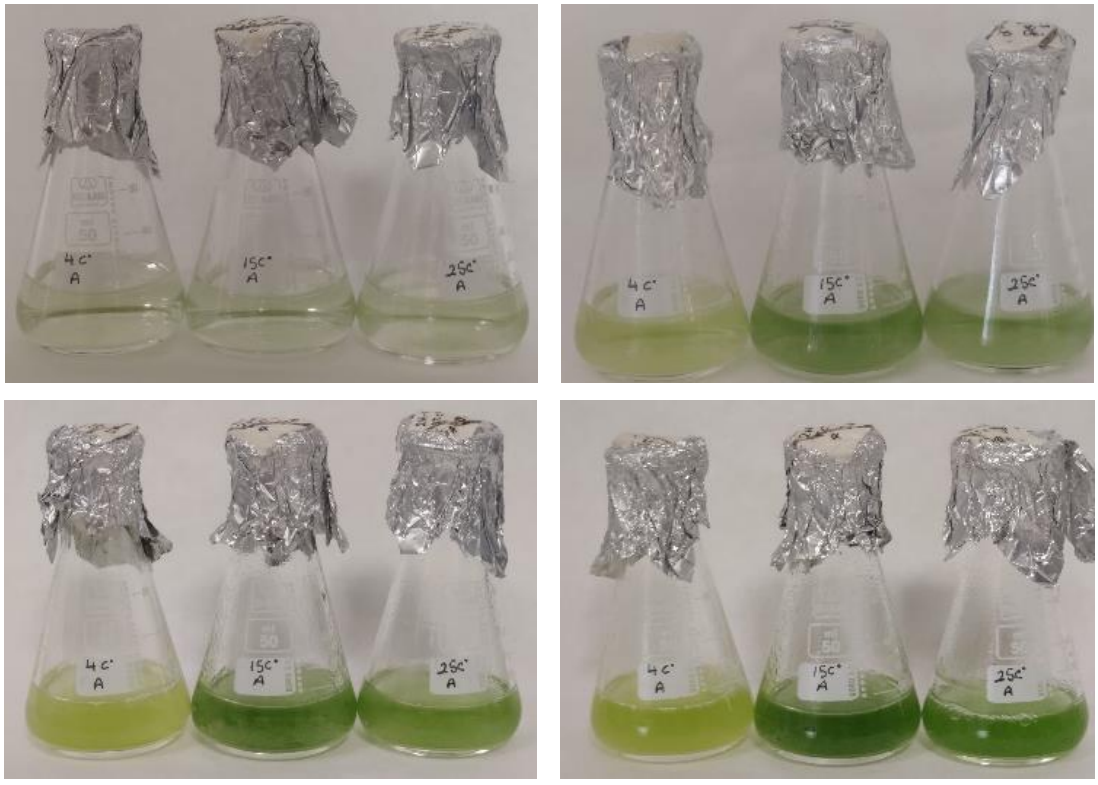


Figure 22. The growth of Chlamydomonas sp. ASYA25 at different temperatures

The growth of Chlamydomonas sp. ASYA25 was followed with spectrophotometric measurements by measuring absorbance at 680 nm and the results are presented with a graphical illustration in Figure 23.

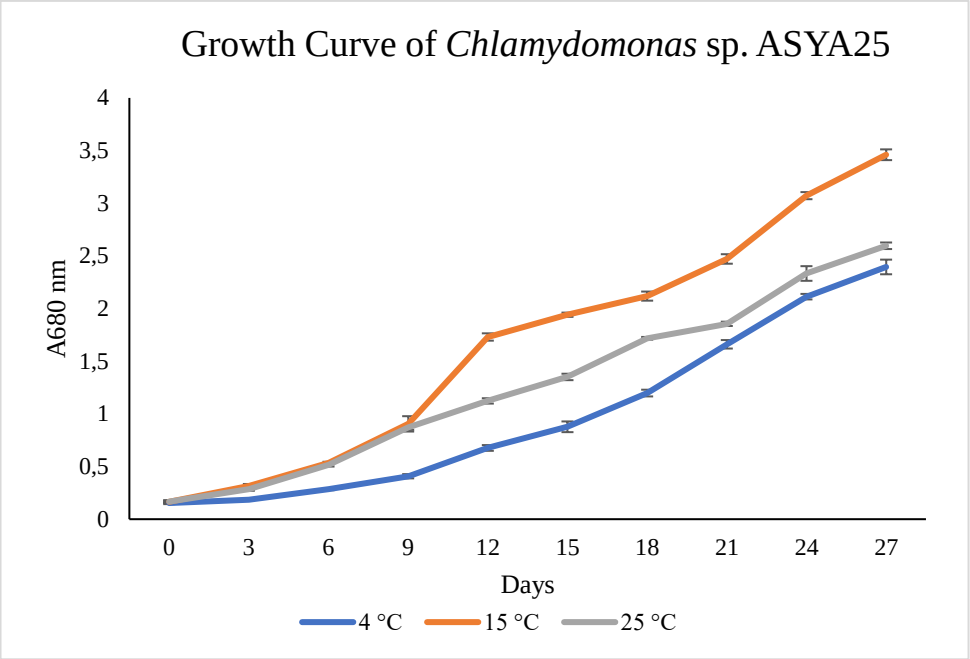


Figure 23. The growth curve of Chlamydomonas sp. ASYA25 at different temperatures

The results show that the optimum temperature for the growth of *Chlamydomonas* sp. ASYA25 is 15 °C. Moreover, morphological characterization with microscopic imaging was performed during the growth following. The microscope images of *Chlamydomonas* sp. ASYA25 taken during growth monitoring is presented in Figure 24.

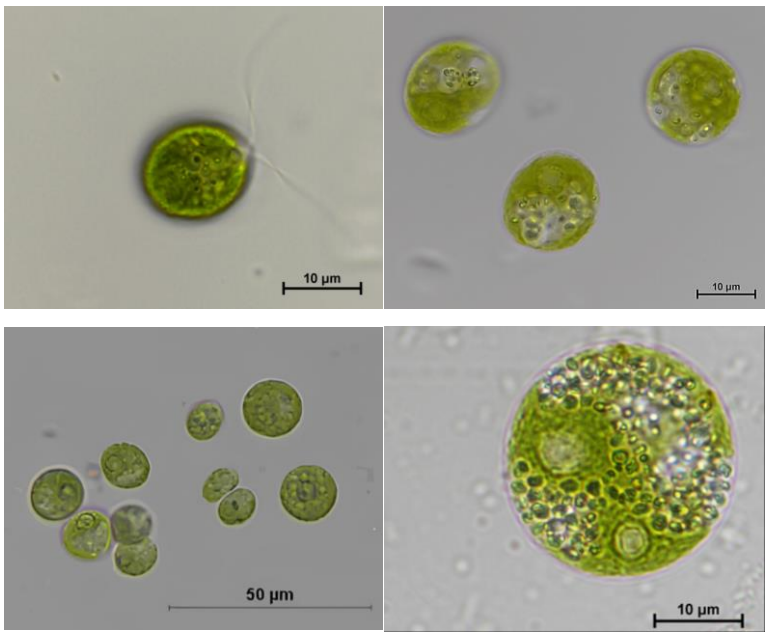


Figure 24. The light microscope images of *Chlamydomonas* sp. ASYA25

The cells are spherical or slightly ellipsoid, and the cell size varies between 9-15 μm. The cells have a cup-shaped chloroplast and a pyrenoid structure in the basal position. In addition, the cells have 2 apical contractile vacuoles and an elliptic eyespot in the anterior part. The cells have flagella at the beginning of growth, but flagella is not seen in the exponential and stationary phases.

3.3.Investigation of Exopolysaccharide Productivity

The culture of *Chlamydomonas* sp. ASYA25 in solid media was observed to have a gel-like structure and this form was associated with EPS productivity. Based on this hypothesis, the EPS productivity was screened among other Antarctic microalgae cultivated in IMU-ASYA culture collections. The results of the screening study showed that the *Chlamydomonas* sp. ASYA25 was able to produce EPS at a higher amount than the other screened microalgae. Then, the EPS productivity of *Chlamydomonas* sp. ASYA25 was compared with the other temperate *Chlamydomonas* cultivated in the IMU-ASYA culture collection. The investigation of EPS production from *Chlamydomonas* sp. ASYA25, *Chlamydomonas moewusii* ARAS123, *Chlamydomonadaceae* ARAS124, and *Chlamydomonas reinhardtii* ARAS125 were carried out by two different methods: examination of petri images and extraction from liquid culture. The petri images of *Chlamydomonas* species are represented in Figure 25.

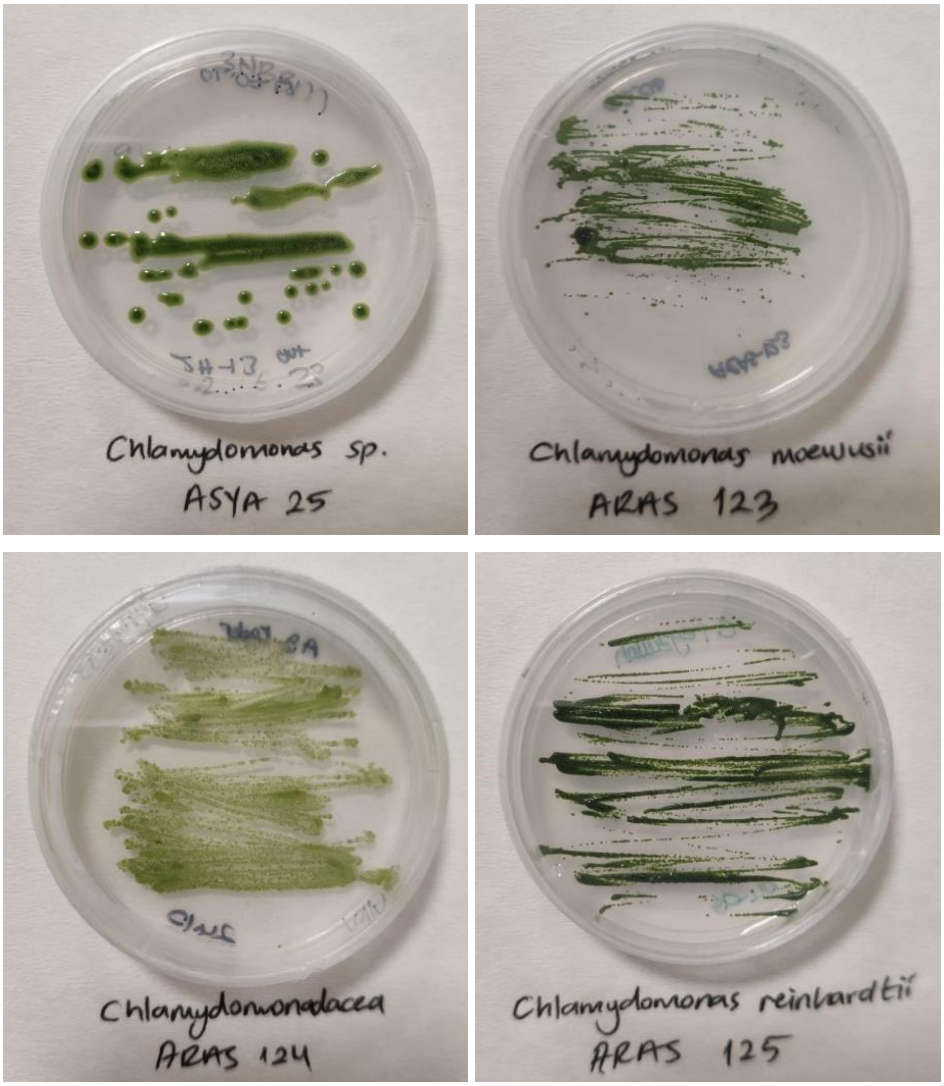


Figure 25. The petri images of different *Chlamydomonas* species for pre-determination of EPS production profile

When we examine the petri images, we can see that *Chlamydomonas* sp. ASYA25 has a more mucous structure than other *Chlamydomonas* species and this mucous structure is formed due to secretion of EPS according to a research study on various *Chlamydomonas* species. This study confirmed the relationship between the mucous structure in the petri image and EPS productivity (Katona et al., 2018). Therefore, we can say that the potential EPS producer is *Chlamydomonas* sp. ASYA25 due to its gelatinous structure in petri. However, to ensure this result, EPS productivity of *Chlamydomonas* species was confirmed with another approach.

In this approach, *Chlamydomonas* species were cultivated in 3N-BBM liquid media and EPS extraction was performed. According to the results, only *Chlamydomonas* sp.

ASYA25 shows significant EPS productivity (Figure 26). The released EPS was obtained as 108 mg/L while the capsular EPS was obtained as 42 mg/L. Therefore, further characterization studies and biomaterial production procedures were applied for EPS of *Chlamydomonas* sp. ASYA25.

Figure 26. EPS extraction yield of *Chlamydomonas* sp. ASYA25 (A: released EPS, B: capsular EPS)

3.4. Optimization of Exopolysaccharide Production

The *Chlamydomonas* sp. ASYA25 produced EPS but a large amount of EPS feedstock is needed to evaluate its potential for biomaterial formation. Therefore, the first aim of this thesis study is to increase EPS productivity by manipulating the culture conditions. Based on preliminary studies, it is known that *Chlamydomonas* sp. ASYA25 produces a significant amount of EPS when cultivated under appropriate conditions. However, as known from literature studies, various cultural (nutrient limitations, salinity stress, pH) and environmental conditions (temperature, light) can influence the production of EPS. In this thesis study, considering the literature information, the effects of nitrogen and phosphate starvation, salt stress, high pH, high light intensity, and high-temperature parameters on EPS production were investigated. For this purpose, 12 different experimental groups created using the Plackett-Burman experimental design method, as presented in the methodology section, were applied to *Chlamydomonas* sp. ASYA25 and results were evaluated comparatively with *Chlamydomonas* sp. ASYA25 cultivated under control conditions. The obtained EPS yield from the

experimental groups are presented in Table 12. According to the results, the maximum EPS yield was obtained when the salinity and high light-intensity stress were applied. In this situation, there is a 48% increase in EPS yield when compared with the control group. Group 7 gives the second-highest EPS yield with a 6% increase in production. The results of other groups show a decrease in EPS yield when compared with the control group. This may be due to the high suppression of growth by the conditions applied. In these groups, the culture conditions like nutrient availability and temperature affect the cell growth and due to the limitation in cell biomass EPS yield also becomes less than the control group.

Table 12. The EPS production yield of experimental groups formed via the Plackett-Burman experimental design

Run	Nitrogen	Phosphate	NaCI	pH	Temperature	Light Intensity	EPS Yield (mg/L)
1	+	-	+	6	25	50	16
2	+	+	-	9	25	50	58
3	-	+	+	6	15	50	131
4	+	-	+	9	25	150	80
5	+	+	-	9	15	50	73
6	+	+	+	6	15	150	222
7	-	+	+	9	25	150	160
8	-	-	+	9	15	50	69
9	-	-	-	9	15	150	64
10	+	-	-	6	15	150	90
11	-	+	-	6	25	150	47
12	-	-	-	6	25	50	11
Control	+	+	-	6	15	50	150

The effect of parameters on EPS productivity was analyzed in Minitab 18 software and salinity (addition of extra NaCI) was determined as the most efficient factor. The relation of salt stress with other factors and the effect of this relation on EPS yield was evaluated with surface plot graphs presented in Figure 27. These surface plots showed that the second efficient factor to increase EPS yield is high light intensity. In addition, the temperature is important, and it should be 15 °C while NaCI and high light intensity conditions are applied. The phosphate should be available in culture media to increase EPS productivity. The pH and nitrogen are not as efficient as the other factors, but the surface plot results showed that nitrogen should be available in culture media and the

pH of the media should be arranged to 6.

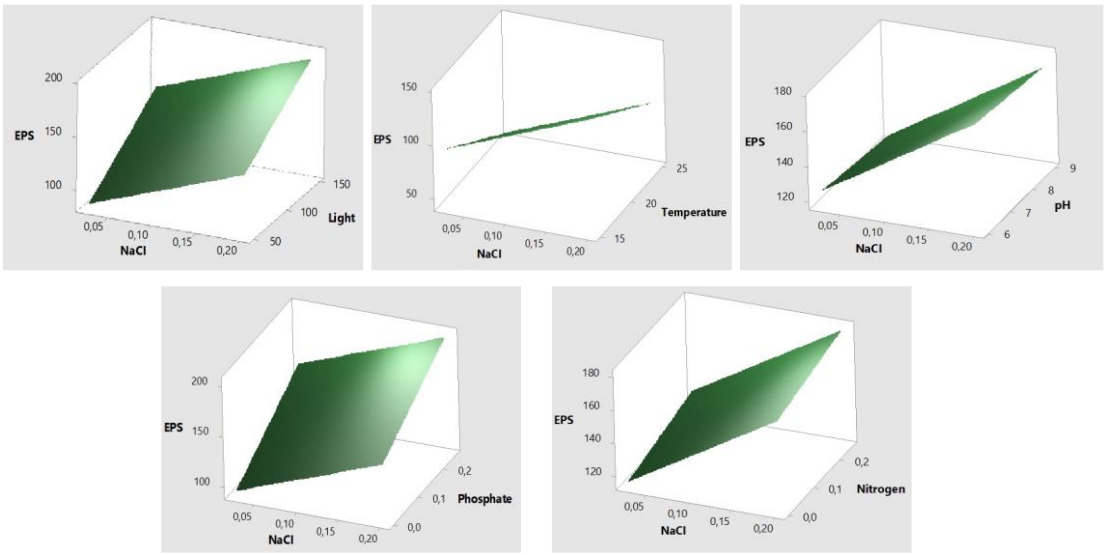


Figure 27. The surface plot obtained from the Minitab18 software

The results obtained from the Plackett-Burman experimental design were analyzed in Minitab18 software and the conditions for maximum EPS yield were optimized (as seen in Table 13). The expected EPS yield is estimated as 202 mg/L under the condition determined as optimum in the system. When the experiment was carried out at the optimized condition, the EPS yield obtained was 197 mg/L. Therefore, we can say that we obtained a consistence result at the end of this optimization study.

Table 13. The optimized condition for maximum EPS yield

Run	Nitrogen	Phosphate	NaCI	pH	Temperature	Light Intensity	Predicted EPS Yield (mg/L)	EPS Yield (mg/L)
Optimized Group	+	+	+	6	15	150	202	197

The images of the EPS fractions obtained from EPS extraction before and after lyophilization are presented in Figure 28 and Figure 29, respectively. The variation in EPS productivity under different culture conditions is visible in these images.

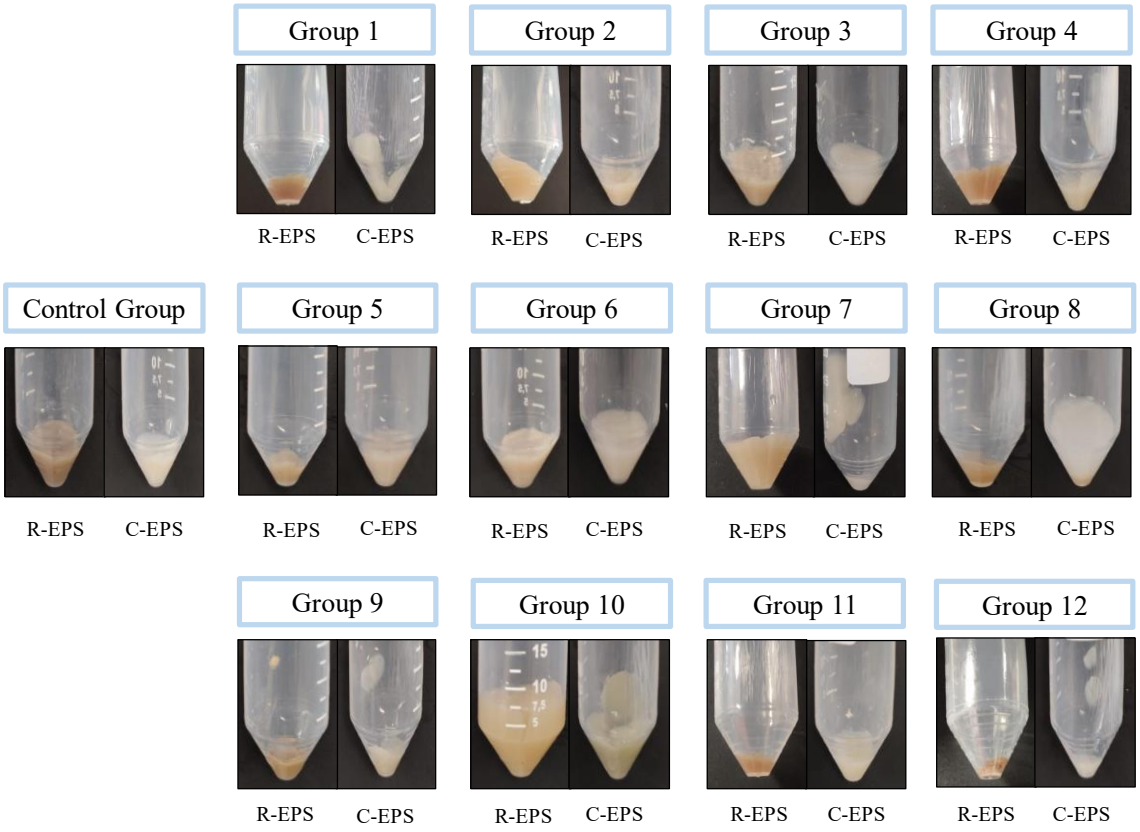


Figure 28. The wet pellets of EPS extracted from different experimental groups (R-EPS: Released EPS, C-EPS: Capsular EPS)

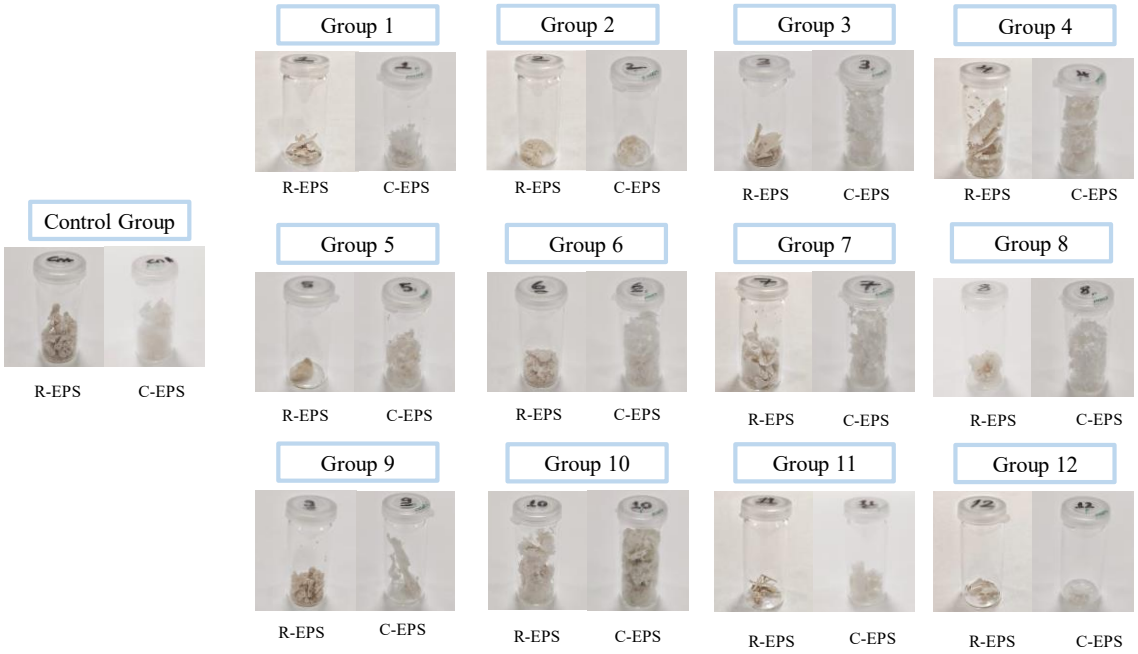


Figure 29. The dry pellets of EPS extracted from different experimental groups (R-EPS: Released EPS, C-EPS: Capsular EPS)

3.5. Characterization of Exopolysaccharides

3.5.1. Total Saccharide, Protein, Sulfate, And Uronic Acid Content of Exopolysaccharide

For the characterization studies, *Chlamydomonas* sp. ASYA25 was grown in the optimized experimental condition and the extraction of released and capsular EPSs was performed. Since both forms of EPS will be mixed and used to form biomaterial, characterization studies were carried out with the stock in which the two forms of EPS were mixed. The total saccharide, protein, sulfate, and uronic acid contents of EPS samples were measured, and the composition of EPS is presented in Table 14. The EPS of *Chlamydomonas* sp. ASYA25 contains 35% total saccharides, 4% protein, 6% uronic acid, and 1.6% sulfate content according to the results.

Table 14. Total composition of EPS extracted from *Chlamydomonas* sp. ASYA25

	Total EPS composition (mg/g)
Carbohydrate	349
Protein	45
Uronic acid	62
Sulphate	16

3.5.2. Monosaccharide Content of Exopolysaccharide

The monosaccharide profile of EPS was analyzed by HPLC analysis, and the chromatography results obtained are presented in Figure 30. According to chromatography results, EPS obtained from *Chlamydomonas* sp. ASYA25 contains mannose, galactose, ribose, arabinose or xylose, and glucose respectively. When we compared it with monosaccharide standard chromatography results, an unknown peak was found in our EPS samples.

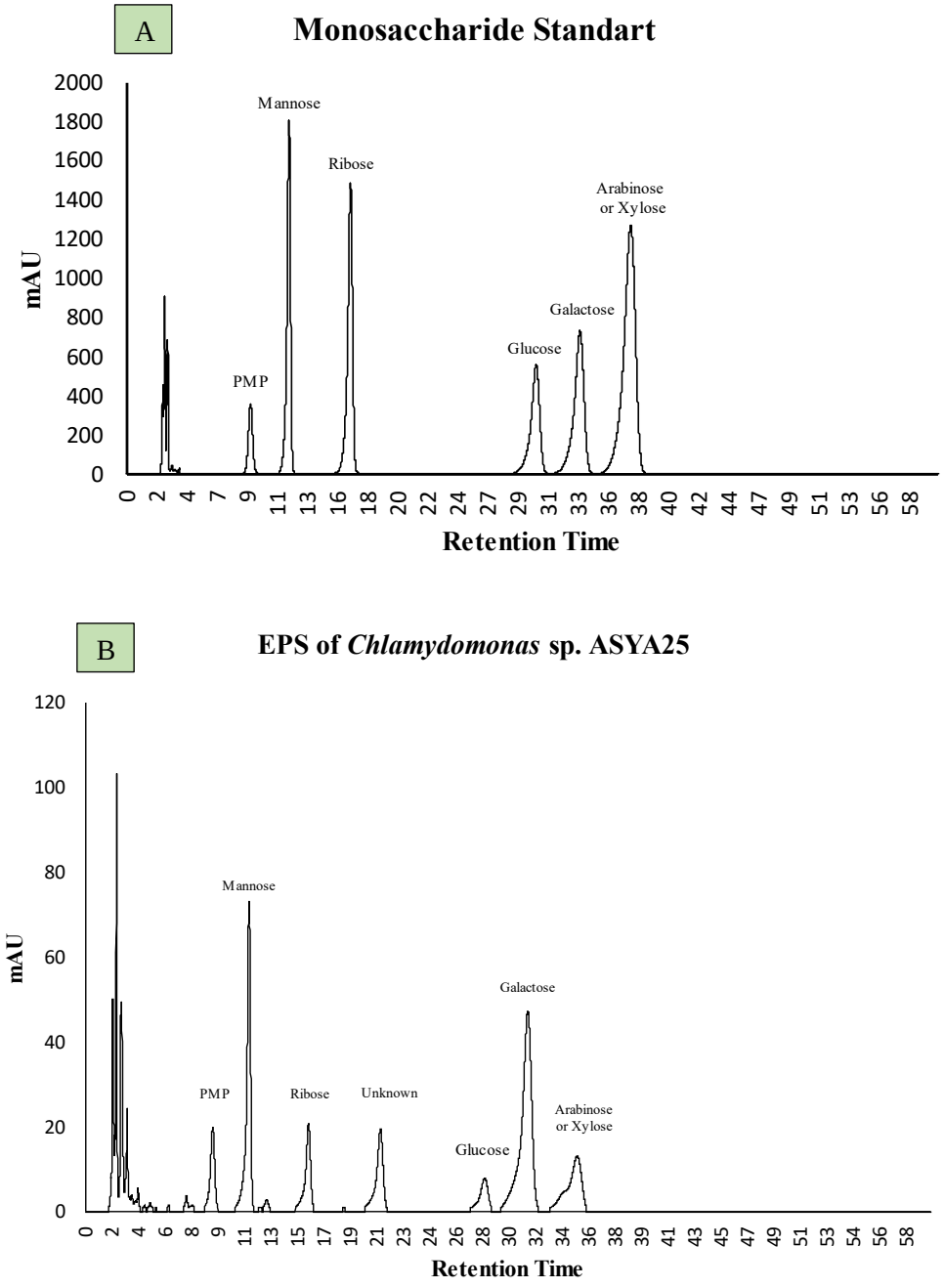


Figure 30. The HPLC results of monosaccharide standards (A) and EPS of *Chlamydomonas* sp. ASYA25 (B)

3.5.3. Fourier Transform Infrared Spectroscopy Analysis of Exopolysaccharide

The functional groups of EPS were analyzed by FTIR spectrophotometer, and the spectrum results are presented in Figure 31. The FTIR spectrum of released and

capsular EPS was evaluated separately. The broad peak at 3329 cm^{-1} represents the stretching vibration of hydroxyl groups in polysaccharide structure. The peak at 2924 cm^{-1} represents the stretching vibration of C-H in CH_3 groups. The region between 1200-800 cm^{-1} is evaluated as a fingerprint region for polysaccharides and gives signals due to the stretching vibration of C-O-C groups. The peak at 890 cm^{-1} is related to sulfated groups but the sulfate groups in the polysaccharide structure can be confirmed with the presence of a strong band in the 1260 cm^{-1} region. The peak at 1640 cm^{-1} is attributed to the C=O stretching vibration while the peak at 1534 cm^{-1} is attributed to Amide II and gives a signal due to the presence of the N-H group.

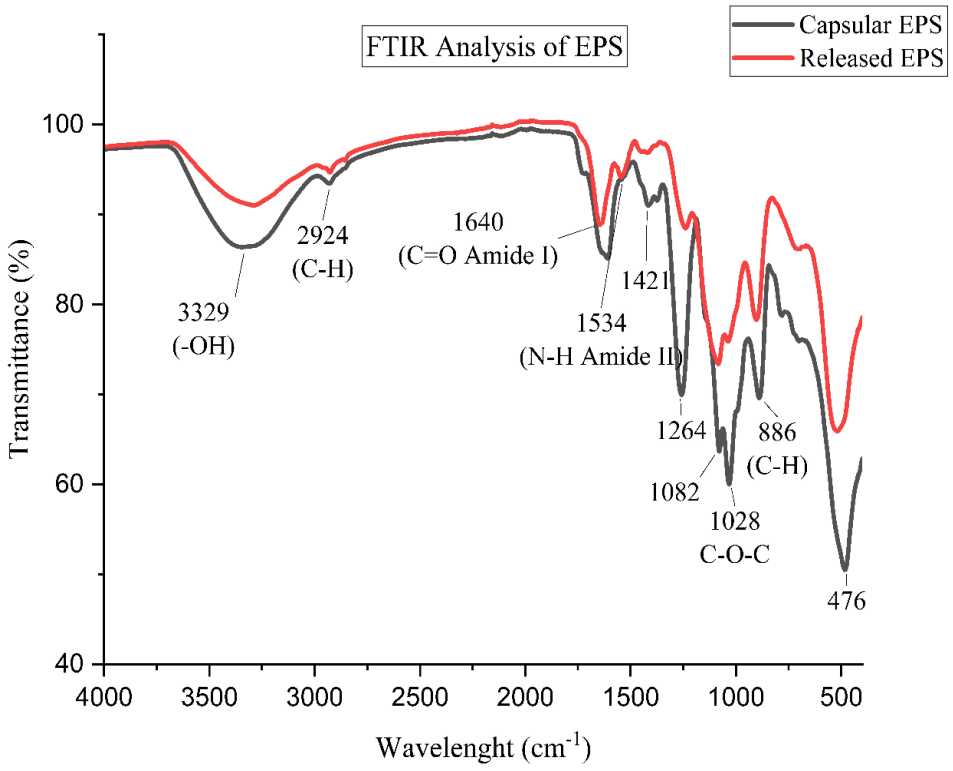


Figure 31. The FTIR spectrum of released and capsular EPS

3.5.4. Thermogravimetric Analysis of Exopolysaccharide

Thermogravimetric analysis of EPSs was performed for determination of weight loss between 25-500 °C. The results showed that EPS degraded in three stages (Figure 32). The first degradation is seen between 25-138 °C with a 10% weight loss due to the removal of water. The second degradation is seen between 138-216 °C with a 2%

weight loss. The main degradation occurs due to the decomposition of EPS between 216-450 °C with a 46% weight loss. Therefore, we can see that the EPS structure can be stable until 216 °C with a total 58% weight loss.

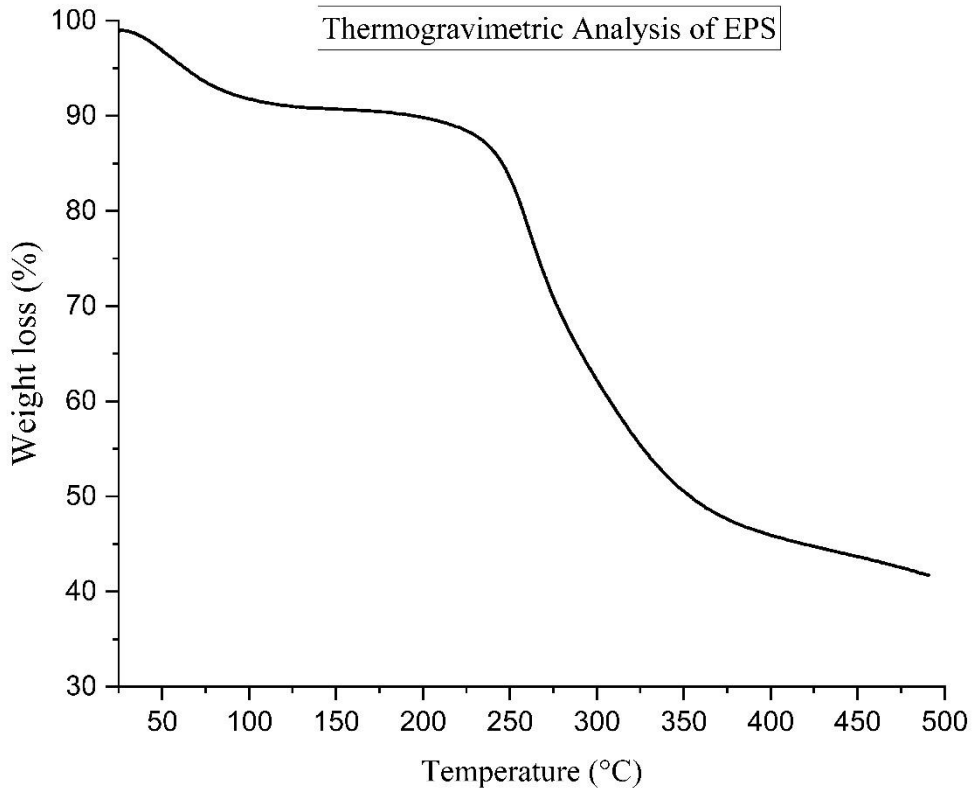


Figure 32. The thermogravimetric analysis of EPS

3.5.5. Differential Scanning Calorimetry Analysis of Exopolysaccharide

DSC analysis was performed to measure the response of EPSs to temperature change. The measurement was done between 25- 400 °C and the result is presented in Figure 33. The DSC thermogram of EPS shows an endothermic peak starting at 100 °C and centered at 255 °C. This peak indicates the occurrence of a degradation process in this temperature range. According to the results, the decomposition range is consistent with the result of TGA analysis.

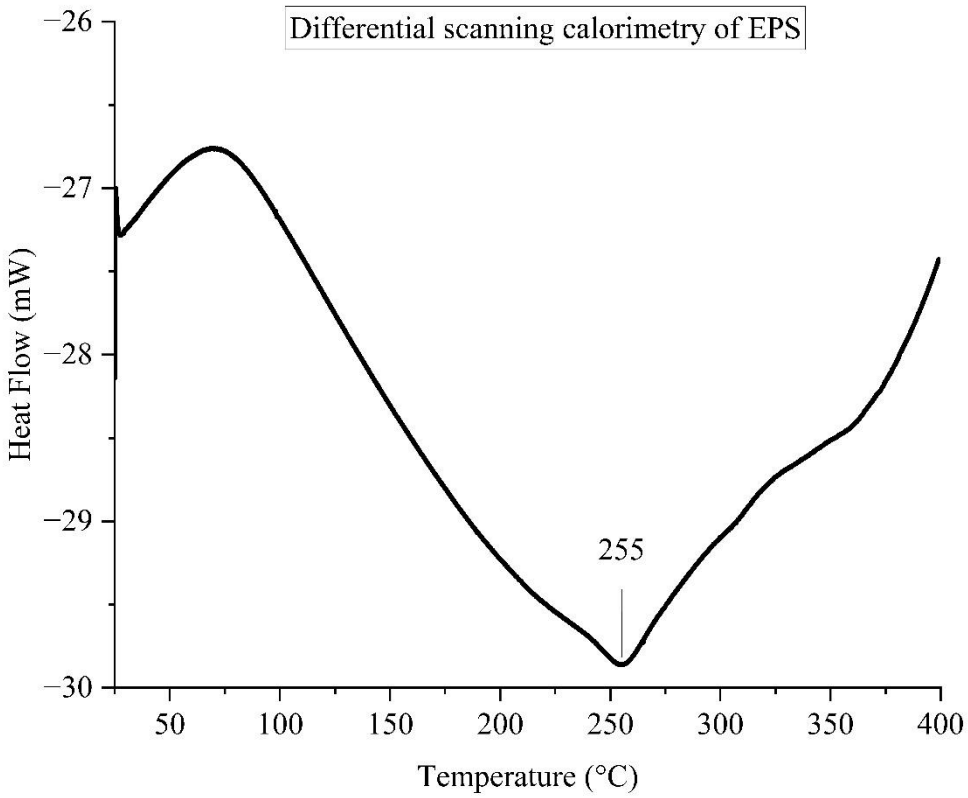


Figure 33. The differential scanning calorimetry analysis of EPS

3.5.6. Antioxidant Activity of Exopolysaccharide

The antioxidant activity of EPS was evaluated according to its DPPH scavenging activity and ascorbic acid was used as a positive control for the confirmation of results (Figure 34). According to the results, ascorbic acid shows 73% scavenging activity at 50 µg/ml concentration. The antioxidant activity of EPSs increases until 2 µg/ml concentration in a dose-dependent manner, but it reaches saturation at this concentration, and scavenging activity decreases at 2.5 and 3 µg/ml concentrations. However, a significant increase in scavenging activity was not recorded at the concentration ranges between 1-2 µg/ml. The maximum scavenging activity was recorded as 70% for 2 µg/ml EPS concentration.

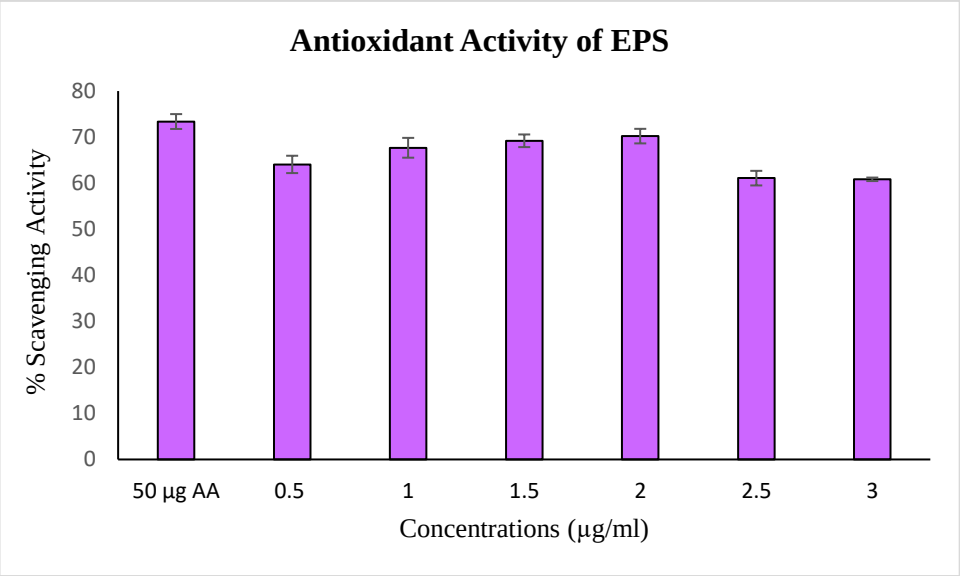


Figure 34. The antioxidant activity of EPS at different concentrations

3.5.7. Antibacterial Activity of Exopolysaccharide

The antibacterial activity of EPS was evaluated with the disc diffusion method against three different bacteria, *S. aureus*, *B. subtilis*, and *E. coli*. Ampicillin (10 µg/ml) and Tetracycline (30 µg/ml) were used as positive control while distilled water was used as a negative control. The results of the antibacterial activity analysis are represented in Table 15 and Figure 35.

Table 15. The inhibition zone results obtained from the antibacterial activity test of EPSs

	Inhibition zone (diameter in mm)		
	***Chlamydomonas* sp. ASYA25**		
Samples	***S.aureus***	***E.coli***	***B. subtilis***
Ampicillin	27	16	16
Tetracycline	18	24	23
10 (mg/ml)	13	9	10
7.5 (mg/ml)	12	8	9
5 (mg/ml)	8	7	8
2.5 (mg/ml)	-	-	-
1 (mg/ml)	-	-	-
NC	-	-	-

The results showed that the antibacterial activity of EPSs is lower than the activity of

Ampicilin (10 µg/ml) and Tetracycline (30 µg/ml). The EPSs show antibacterial activity at 5- 10 mg/ml concentrations. When we compared the results, we can see that EPS shows greater antibacterial activity against *S. aureus* bacteria. The antibacterial activity of EPS against *E. coli* and *B. subtilis* shows similar inhibition zone results.

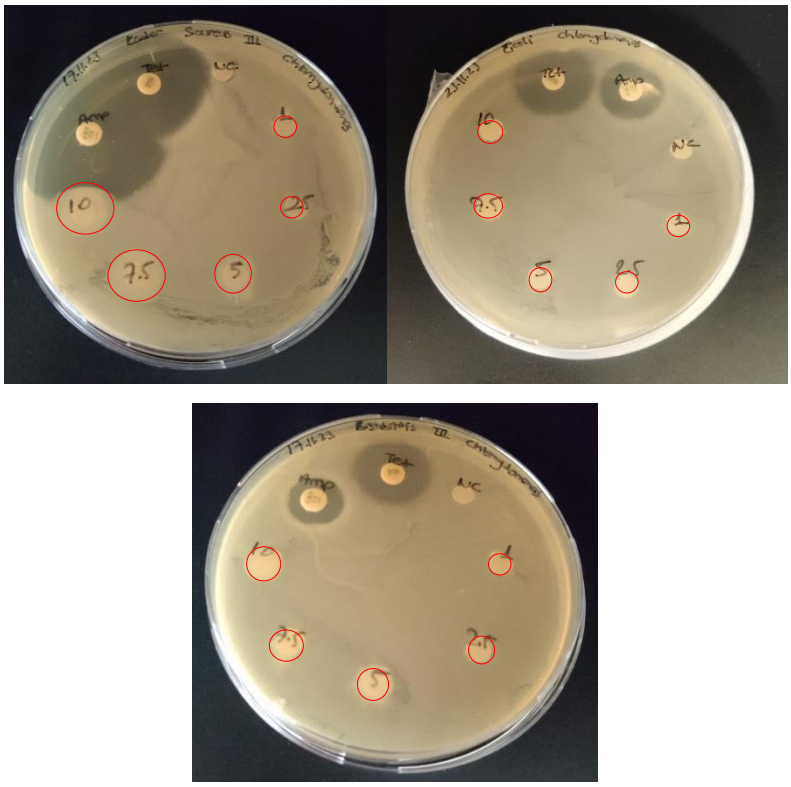


Figure 35. The antibacterial activity analysis of EPS via the disc diffusion method

3.6. Biomaterial Formation

After the characterization studies were completed, the biomaterial formation potential of EPS obtained from *Chlamydomonas* sp. ASYA25 was evaluated. For this purpose, we tried to form film material and hydrogel structure.

3.6.1. Film Material Formation

For the film material formation, different EPS concentrations were evaluated to optimize raw material concentration. In the optimization studies, EPS solutions at 0.5%, 1%, and 1.5% concentrations were prepared, and film formation was followed by adding 10% PEG (w/w_{EPS}). As a result of the optimization study, the structures

shown in Figure 36 were obtained. These results showed that the concentration of raw material was not enough to form a film structure because cracks were present in the structures. The formation of film material is a procedure that depends on the homogeneous dissolution of the raw material used and the interaction of the added crosslinker with this material to form a network. If the raw material concentration becomes low, the remaining materials cannot connect when the solvent is removed during the drying process in the solvent-casting method. The same situation was observed in our results.

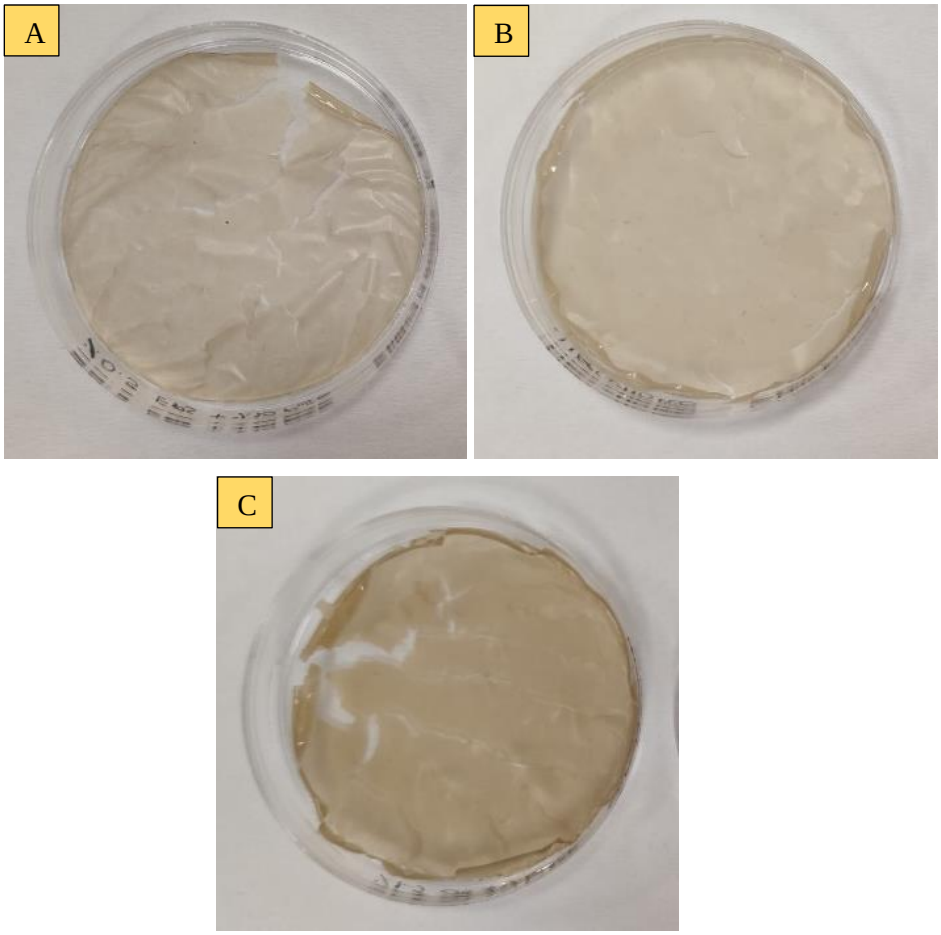


Figure 36. The optimization study of EPS-based film formation (A: 0.5% EPS+10% PEG(w/w_{EPS}), B: 1% EPS+10% PEG(w/w_{EPS}), C: 1.5% EPS+10% PEG(w/w_{EPS}))

Therefore, in the following study, EPS concentration was tried at 3%, and film formation was followed. The 10% PEG (w/w_{EPS}) concentration was used, and results showed that this concentration was suitable for film formation as seen in Figure 37. The film structure formed with a 3% EPS concentration was evaluated

organoleptically and the EPS concentration was optimized as 3%, considering that the EPS concentration was sufficient.

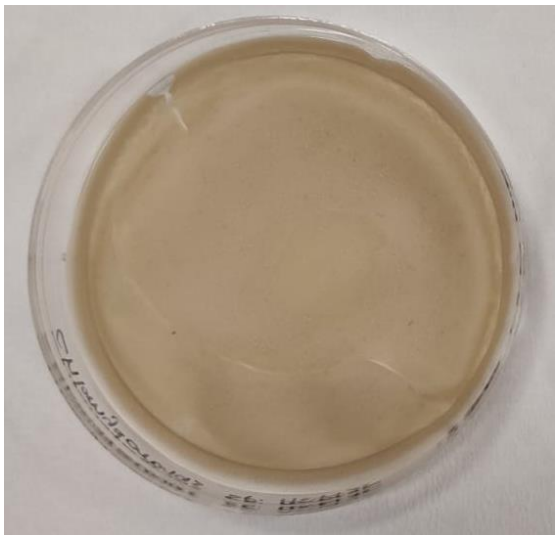


Figure 37. The film structure prepared with 3% EPS +10%PEG (w/w_{EPS})

The form of the film shows a homogenous structure, and the cracks are not seen in the structure. Therefore, we can say that the concentration of raw material is enough to form a network when the solvent is removed. Another proof that this concentration is appropriate is the consistency of the EPS solution before it was poured into the petri dish. The prepared solution was sufficiently viscous and when the concentration is increased further, the solution will reach saturation and the film structure will not be homogeneous as it will not be possible to obtain a homogeneous solution. In further studies, the 20% and 30% PEG ratios can be evaluated to increase the mechanical strength and the elasticity of EPS film according to the results of characterization studies.

3.6.2. Hydrogel Formation

3.6.2.1.Physically Crosslinked Hydrogel

For the evaluation of the hydrogel formation potential of EPS, two different methods were performed. The first method was performed by using $CaCI_2$ as a crosslinker for the physical crosslinking procedure. The crosslinker was added after the homogenization of EPS and later the formation of hydrogel was followed by converting the tube. For the optimization of EPS and $CaCI_2$ concentrations different concentration ranges were evaluated as 0.5%, 1%, 1.5%, 2%, and 0.2%, 1%, 3%, and

5% respectively. The results of this optimization study are presented in Table 16.

Table 16. Physically crosslinked hydrogels via $CaCI_2$ addition

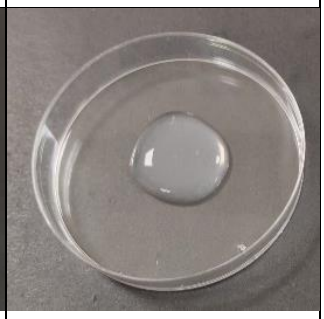
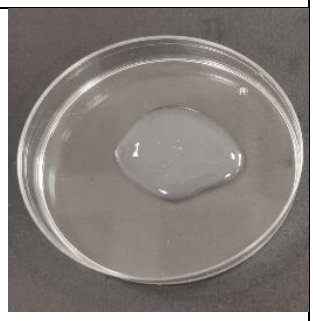
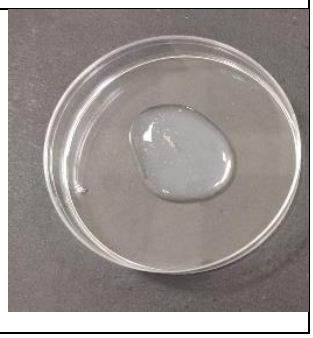
EPS Concentration	$CaCI_2$ Concentration	After heating	After Crosslinker Addition	Image in Petri
0.5 %	0.2 %			
	1%			
	3%			
	5%			

Table 16. continued

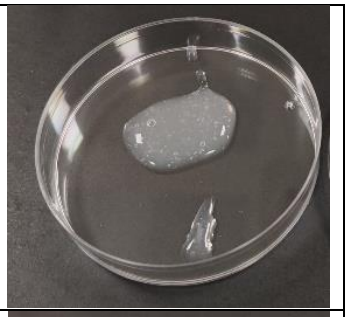
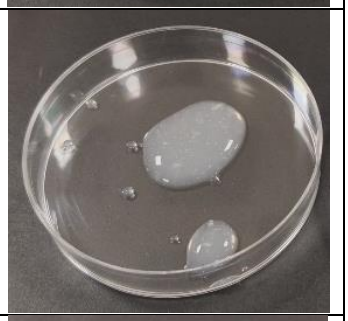
1%	0.2%			
	1%			
	3%			
	5%			

Table 16. continued

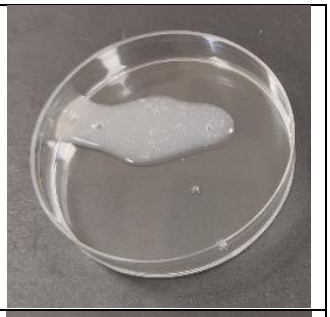
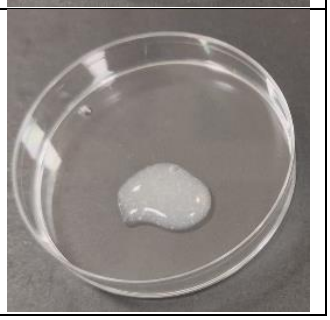
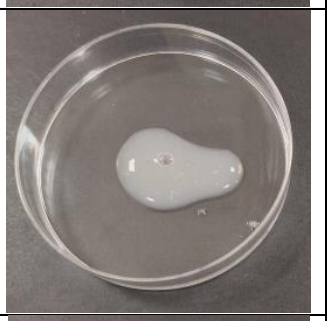
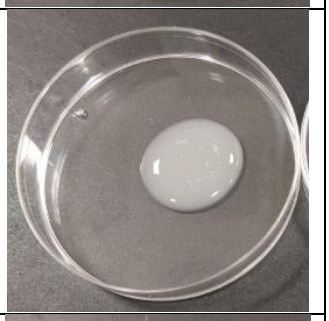
1.5%	0.2%			
	1%			
	3%			
	5%			

Table 16. continued

2%	0.2%			
	1%			
	3%			
	5%			

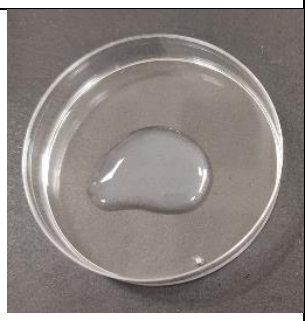
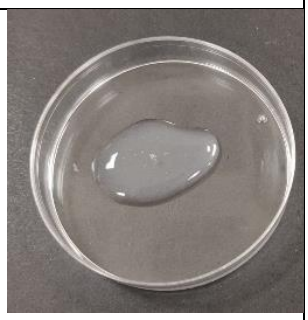
Since $CaCI_2$ is added independently of the EPS concentration, it allows optimization of the appropriate EPS concentration. According to the results, we can see the hydrogel formation when the EPS concentration becomes higher than 1.5%. At the lower EPS concentrations, the viscosity of the solution increases, but the EPS concentration appears to be inadequate as it is fluid when the tube is inverted. The formation of hydrogel and the effect of crosslinker can be seen clearly in petri images. The form of hydrogel enhanced with increasing $CaCI_2$ concentration. For instance, when we look at the 2% EPS concentration group, we can see that the 5% $CaCI_2$ concentration

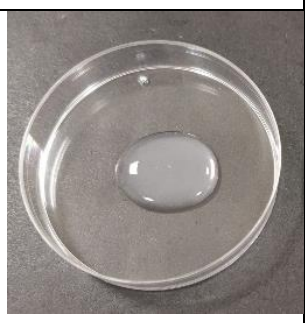
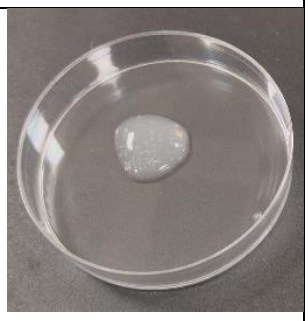
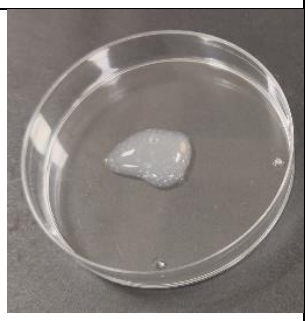
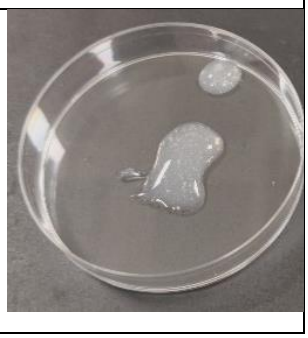
provides a higher viscose form than the 0.2% $CaCI_2$ concentration.

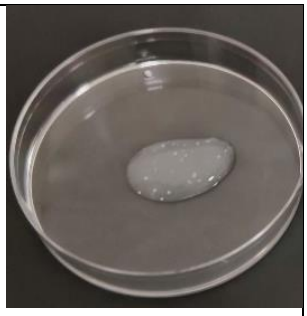
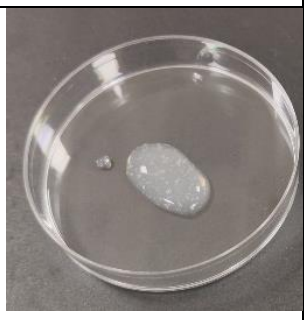
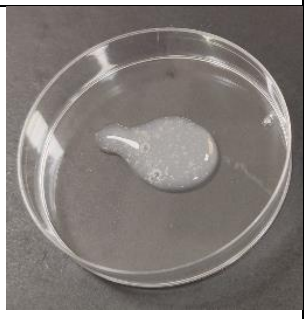
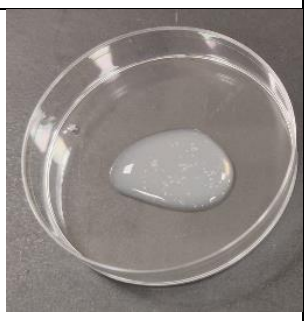
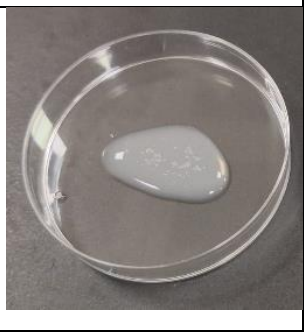
3.6.2.2.Chemically Crosslinked Hydrogel

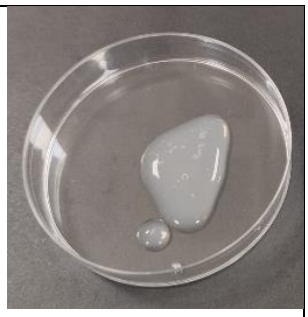
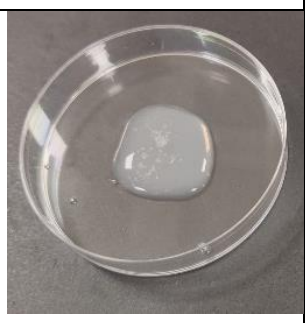
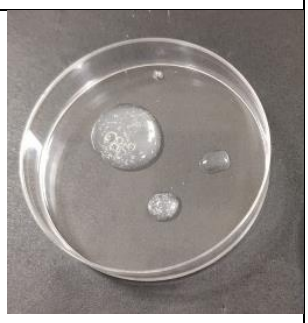
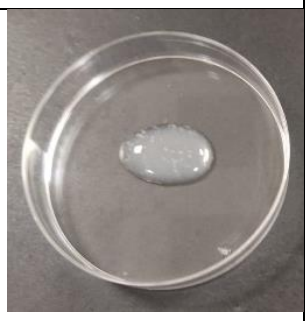
For the chemically crosslinked hydrogel, the borax was used as a crosslinker and added to a homogenous solution prepared at 0.5%, 1%, 1.5%, and 2% EPS concentrations. Borax concentration ranged from 5%, 10%, 15%, 20%, and 25% weight of EPS weight (w/w). Therefore, in this case, the optimum EPS concentration was determined related to the borax concentration. The hydrogels formed with the chemical crosslinking method are presented in Table 17.

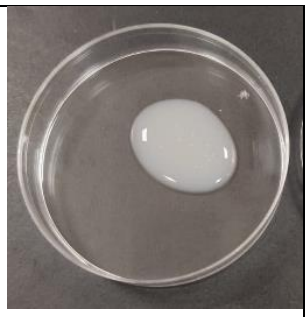
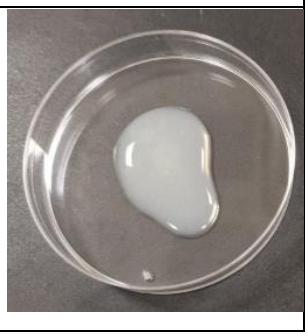
Table 17. Chemically crosslinked hydrogels via borax addition

EPS Concentration	Borax Concentration	After heating	After Crosslinker Addition	Image in Petri
0.5%	5%			
	10%			
	15%			

	20%			
	25%			
1%	5%			
	10%			
	15%			

	20%			
	25%			
1.5%	5%			
	10%			
	15%			

	20%			
	25%			
2%	5%			
	10%			
	15%			

	20%			
	25%			

The results of chemically crosslinked hydrogels showed that hydrogel formation potential increases with increasing EPS concentration as in the case of physically crosslinked hydrogel. The result of 0.5 % EPS (w/w) concentrations shows that the raw material concentration is not sufficient to form hydrogel formation according to the tube inverting test. The hydrogel formation was observed at the EPS concentration higher than 1%. However, the gelation ability of EPS increases with increasing raw material concentrations so the 1.5 %and 2 % EPS concentrations can be evaluated for hydrogel formation with better mechanical stability.

To determine the minimum EPS concentration for hydrogel formation, the prepared hydrogels should be characterized in further studies. In this thesis study, the main aim was to show the hydrogel formation ability of EPS obtained from Chlamydomonas sp. ASYA25 and results showed that it can form a hydrogel in both crosslinking mechanisms. Therefore, we can say that this EPS has the potential to form hydrogel at 1.5% as minimum concentration, and after the determination of the physical and biological properties of this hydrogel via characterization studies, it can be evaluated as a wound dressing or drug carrier system.

4. DISCUSSION

The initial hypothesis of this thesis was that *Chlamydomonas* sp. ASYA25 has a more gel-like culture appearance compared to other Antarctic species. This gelatinous structure led us to evaluate the potential of the species to produce EPS. For this purpose, the EPS productivity of Antarctic microalgae in the culture collection of IMU-ASYA was screened, and *Chlamydomonas* sp. ASYA25 was selected as a potential EPS producer by producing higher EPS than the other species. Then, we hypothesized whether these EPSs could be an alternative source of synthetic polymers for biomaterial formation and evaluated the potential of the characterized EPSs.

The *Chlamydomonas* sp. ASYA25 was isolated from the Horseshoe Island in Antarctica and identified in our laboratory. To molecular identification of *Chlamydomonas* sp. ASYA25, the Sanger sequencing was performed on DNA fragments obtained from the CTAB extraction procedure. According to the sequencing results, the most related species with *Chlamydomonas* sp. ASYA25 is *Chlamydomonas incerta* (JF834705.1), isolated from India. The second related species is *Chlamydomonas reinhardtii* (FR865586.1), isolated from the USA. The *Chlamydomonas reinhardtii* is a model organism and the whole genome sequencing of it is completed. In addition to sequencing results, the morphological characterization of *Chlamydomonas* sp. ASYA25 was performed, and results were evaluated by comparing the morphological properties of other *Chlamydomonas* species.

The morphological imaging results showed that *Chlamydomonas* sp. ASYA25 has a spherical and slightly ellipsoid shape with a diameter of 9-15 μm. The cells have a cup-shaped chloroplast structure with a pyrenoid in the basal position. In the anterior part, two contractile vacuoles are seen, and the eyespot is found in the anterior part. It has flagella at the beginning of its growth. The most related species *Chlamydomonas incerta* has a spherical-broadly ellipsoid shape and a 12-22 μm cell size (Pröschold & Darienko, 2018). It has two apical contractile vacuoles and a cup-shaped chloroplast shape. It contains a medium pyrenoid structure in the basal position. In the anterior position of the cell, there is an elliptic eyespot. The number of zoospores shows variation as 2 or 4. The other most related species is *Chlamydomonas reinhardtii* and it has a round or oval shape at 10 μm size (Jokel, 2018). It has a large cup-shaped chloroplast with a pyrenoid and an eyespot in the inner membrane of the chloroplast.

In the apical part, one contractile vacuole is seen. Another *Chlamydomonas globosa* has a spherical shape with a 5-7.8 μm cell size (Pröschold & Darienko, 2018). It has only one apical contractile vacuole and a cup-shaped chloroplast. The small pyrenoid is seen in the basal position. It has also a dot-like eyespot in the anterior position. All these *Chlamydomonas* species have flagella structures as long as cells. The morphological properties of *Chlamydomonas* sp. ASYA25 was determined as similar with the morphology of other *Chlamydomonas* species.

The growth dynamic of *Chlamydomonas* sp. ASYA25 was analyzed in TAP, 3N-BBM, and BG-11 mediums. The results showed that the suitable growth medium is 3N-BBM. In TAP medium, *Chlamydomonas* sp. ASYA25 did not grow well while growing in 3N-BBM and BG-11 mediums. This result showed that *Chlamydomonas* sp. ASYA25 cannot use ammonium as a nitrogen source because the nitrogen source in TAP media is ammonium chloride (NH_4CI). However, it can use nitrate as a nitrogen source because the nitrogen source in 3N-BBM and BG-11 media is nitrate. When the Antarctic *Chlamydomonas* were screened in literature, all of them grew well in the BBM medium (Cook et al., 2019; Morgan et al., 1998; Pocock et al., 2011).

Various *Chlamydomonas* species have been previously isolated from different regions in Antarctica (Pocock et al., 2004; Raymond et al., 2020; Szyszka-Mroz et al., 2022; X. Zhang et al., 2021; Z. Zhang et al., 2018). These *Chlamydomonas* are known especially for their cold and UV-tolerant activities. The polar *Chlamydomonas* can adapt to these unfavorable conditions by changing their metabolite profile. The increase in polyunsaturated fatty acid profile is one of these strategies. Another strategy enhanced for cold adaptation is a high polysaccharide and protein containing extracellular polymeric substances that are referred to as EPSs. The cryoprotective role of EPSs in polar microalgae was confirmed by a study conducted on *Coccomyxa subellipsoidea* (Blanc et al., 2012). Some types of algae can become palmelloid under a certain environmental condition or over a certain period, under the influence of several factors that allow the cells to come together to form a colony. This structure may arise as a defense mechanism of algae against environmental stresses, or it may serve to store accumulated nutrients when feeding conditions are unfavorable. Some of the Antarctic *Chlamydomonas* species show pallmelloid formation due to unsuitable environmental conditions.

Salinity is one of the factors affecting the growth of photosynthetic organisms. Microalgae can survive under salinity stress with the mechanisms they have developed in the face of this stress. In a valuable review, the response of *Chlamydomonas* species to salinity stress was evaluated (Bazzani et al., 2021). According to information in this article, several *Chlamydomonas* have been used in salinity stress studies. The *Chlamydomonas* sp. UWO241, *Chlamydomonas nivalis*, and *Chlamydomonas* sp. ICE-L are the most studied polar microalgae, and these microalgae adapt to extreme cold temperatures and salinity. The response of different *Chlamydomonas* to salinity stress shows variation. The most known example of these responses is palmelloids formation of *Chlamydomonas reinhardtii*. In the palmelloids stage, cells come together and remove their flagella. In addition, the EPS secretion was observed, and cells were covered with a single membrane that is found embedded in EPS. In a research study, pallmelloid formation was observed for *Chlamydomonas reinhardtii* at 50 mM NaCI concentration (Neelam & Subramanyam, 2013). In another study, the 100-150 mM NaCI concentration caused pallmelloid formation in *Chlamydomonas reinhardtii* (Khona, Shirolikar, Gawde, & Hom, 2016).

In a valuable study, the cold stress effect on snow algae *Chlamydomonas nivalis* was evaluated and the results showed that the EPS production increased when cold stress was applied (Peng et al., 2021). In our results, the temperature did not show a significant effect on EPS production but the 15 °C is selected more suitable because the suitable growth temperature for *Chlamydomonas* sp. ASYA25 is determined as 15 °C.

The literature studies showed that EPS productivity can be increased by manipulating the culture conditions. The EPS production yield varies depending on the species and culture conditions. In a study on *Chlamydomonas reinhardtii*, the effect of culture medium content on EPS yield was investigated using Plackett-Burman experimental design and Response Surface Methodology (RSM) design methods (Bafana, 2013). As a result, it was observed that the EPS yield varied between 96-409 mg/L according to the content of the culture medium. After determining that the amount of $CaCI_2$ and $NaNO_3$ had the highest effect, EPS yield could be increased to 628 mg/L by optimizing the culture conditions for maximum EPS yield using RSM. In another study conducted with *Chlamydomonas mexicana*, the EPS yield showed an increase in the stationary

phase due to the depletion of nitrogen and phosphorous.

Therefore, in this thesis project, we used the Plackett-Burman experimental design method to determine the cultivation conditions that provide the highest EPS yield. For this purpose, the nutrient limitations, salinity, high temperature, basic pH, and high light intensity were applied to the culture of *Chlamydomonas* sp. ASYA25. The results showed that salinity is the most efficient factor that affects the EPS yield. This is an expected result because the pallmelloid formation response of *Chlamydomonas* species under salinity conditions is known. Then, the culture conditions were optimized in Minitab 18 software according to the findings of optimization studies. The results showed that if *Chlamydomonas* sp. ASYA25 was cultivated under salinity and high light intensity, the maximum EPS yield can be obtained as 202 mg/L. When these cultural conditions were applied, the EPS yield was obtained as 197 mg/L and this confirms the reliability of results.

The EPSs are mainly composed of polysaccharides and may contain small amounts of protein as well as very low amounts of lipids and nucleic acids. Therefore, in the characterization studies of EPSs, the total saccharide and protein content are measured but the lipid and nucleic acid contents are ignored. In addition, EPSs can contain some non-sugar groups like sulfate, and uronic acid. The physical and biological activities of EPSs vary based on their contents. The composition of EPS shows variation from one species to another and is also affected by cultural conditions. In this thesis study, the total saccharide, protein, uronic acid, and sulfate content were measured, and results showed that EPS of *Chlamydomonas* sp. ASYA25 has 349 mg/g total saccharide, 45 mg/g protein, 62 mg/g uronic acid, and 16 mg/g sulfate contents. In a research study, the total saccharide content of EPS obtained from *Chlorella vulgaris* was measured as 495 mg/g EPS (El-Naggar et al., 2020). In another study, the EPS obtained from green microalgae *Graesiella* sp. showed 520 mg/g EPS total saccharide content (Trabelsi et al., 2016). In a research study, the EPS compositions of two species, *Porphyridium cruentum* and *Porphyridium purpureum*, were analyzed and evaluated comparatively (W. N. Wang, Li, Zhang, et al., 2021). According to the results obtained, EPS obtained from *Porphyridium cruentum* contained 420 mg/g total saccharide while the EPS obtained from Porphyridium purpureum contained 460 mg/g total saccharides.

The EPS of *Botryococcus braunii* showed 420 mg/g EPS total saccharide content (W. N. Wang et al., 2022). In another research study, the total saccharide content of EPSs extracted from various marine microalgae shows variation between 67-103 mg/g EPS. The EPS of *Chlorella* sp., *Chlorella sorokiniana*, and *Picochlorum* sp. was detected as 103 mg/g, 95 mg/g, and 67 mg/g, respectively (Mousavian et al., 2022). In another study conducted with *Chlamydomonas mexicana*, the EPS yield showed an increase in the stationary phase due to the depletion of nitrogen and phosphorous (Kroen & Rayburn, 1984). In another study, the EPS of *Chlamydomonas reinhardtii* was analyzed due to its bio-flocculant activity (C. Zhu et al., 2012). When the composition of this EPS was analyzed, the total saccharide content was found as 483 mg/g EPS. When we compared our findings with the literature information, we can see that the EPS of *Chlamydomonas* sp. ASYA25 has an average saccharide content.

The second main component of EPS is known as protein, but it generally comprises a small part of EPS. The EPS from *Chlamydomonas reinhardtii* was found to have an exceptional protein content of 483 mg/g EPS (C. Zhu et al., 2012). The protein content of EPS extracted from *Graesiella* sp. was found as 120 mg/g EPS (Trabelsi et al., 2016). The Spirulina is a model organism that is known for EPS productivity and the EPS of Spirulina has 110 mg/g protein content (Chentir et al., 2017). The EPS of these species has higher protein content when compared to other EPS-producing microalgae because in general, the protein content is less than the 10 % of EPS. For instance, the EPS of *Rhodella* sp. has 60 mg/g EPS (De Jesus Raposo et al., 2013). In another study conducted with *Botryococcus braunii,* the EPS had 40 mg/g EPS protein content. The EPS of *Nostoc* sp. produced EPS that contained approximately 30 mg/g EPS protein (Alvarez et al., 2021). In this thesis study, the results showed that EPS of *Chlamydomonas* sp. ASYA25 contains 45 mg/g EPS protein content, and it shows a consistent result according to the literature.

The sulfate is a non-sugar group that is found in EPS at different ratios and affects the biological activity of EPS. The sulfate content is found in mostly marine microalgae and the EPS of this microorganism is sometimes referred to as sulfated polysaccharides due to their high sulfate content. Some species can produce EPS with a high content of sulfate, while the EPS of other species may contain very low or no sulfate. In this project, the EPS of *Chlamydomonas* sp. ASYA25 has 16 mg/g sulfate content. In a

valuable study, the sulfate content of EPS obtained from different marine microalgae was analyzed and the marine microalgae are known for their high sulfate-containing EPSs. In this study, *Chlorella* sp., *Chlorella sorokiniana*, and *Picochlorum* sp. were used, and the sulfate content of their EPSs showed variation between 35-93 mg/g EPS. When the sulfate content of EPS obtained from *Chlorella sorokiniana* and *Chlorella* sp. species was analyzed, it was determined as 93 and 76 mg/g dry weight, respectively (Mousavian et al., 2022). In another study, the EPSs of several red microalgae were evaluated, and the sulfate contents showed variability between 16-264 mg/g EPS (Borjas Esqueda et al., 2022). The *Erythrolobus coxiae* shows the lowest sulfate content at 16 mg/g EPS while *Chroothece richteriana* shows the highest sulfate content at 264 mg/g EPS. The EPS of *Botryococcus braunii* has a 15-16 mg/g sulfate content (W. N. Wang et al., 2022). Therefore, we can see that the sulfate content of *Chlamydomonas* sp. ASYA25 is low when compared to literature findings.

The uronic acid is one of the most pronounced non-sugar groups in EPS structure and gives the anionic nature to EPS. The ratio of uronic acid in EPS shows variation according to species and cultivation conditions as for other components in EPS structure. The EPS of Chlamydomonas sp. ASYA25 contains 62 mg/g EPS uronic acid content. In a study, the EPS compositions of two species, *Porphyridium cruentum* and *Porphyridium purpureum*, were analyzed and evaluated comparatively (W. N. Wang, Li, Zhang, et al., 2021). The EPS obtained from *Porphyridium purpureum* contained 54 mg/g EPS uronic acids, while the EPS obtained from Porphyridium purpureum contained 42 mg/g EPS uronic acids. In another research study, the EPS of *Graesiella* sp. showed 230 mg/g EPS uronic acid (Trabelsi et al., 2016). When the EPS content of *Porphyridium sordidum* species was examined, results showed that it had 202 mg/g EPS uronic acid content (Esqueda et al., 2022). One of the prevalent microalgae for EPS studies *Chlorella vulgaris* produces EPS that contains 170 mg/g EPS uronic acid (El-Naggar et al., 2020). The EPS of *Botryococcus braunii* which contains 74 mg/g EPS uronic acid shows similar results with our findings (W. N. Wang et al., 2022).

The monosaccharide content of EPS is specific to species and shows variation with changing environmental conditions. Even if the monosaccharide types remain the same, the ratio of monosaccharides shows variations. When the monosaccharide profile of EPS extracted from *Chlamydomonas* sp. ASYA25, the results showed that

it contains mannose, galactose, ribose, arabinose or xylose, and glucose, respectively. When the monosaccharide profile of EPS obtained from *Botryococcus braunii* species is examined, it is seen that it contains galactose, glucose, and arabinose, respectively (W. N. Wang et al., 2022). The EPS of *Chlamydomonas reinhardtii* has six monosaccharide types and these are arabinose, rhamnose, galactose, glucose, xylose, and ribose (Bafana, 2013). The EPSs of *Chlamydomonas mexicana* and *Chlamydomonas sajao* have the same monosaccharide profile containing arabinose, rhamnose, galactose, glucose, xylose, mannose, fucose, and ribose (Barclay & Lewin, 1985). In our HPLC results, there is an unknown peak at 21 minutes and this peak can be rhamnose or fucose when compared with the monosaccharide profile of other *Chlamydomonas* species. The monosaccharide profile of EPS affects its biological activity. For instance, the presence of galactose at a high percentage is associated with anti-inflammatory activity of EPS on macrophages (Y. C. Chen et al., 2019).

The antioxidant activity of *Chlamydomonas* sp. ASYA25 EPS was evaluated according to its DPPH scavenging activity and the results showed that the maximum scavenging activity was obtained as 70% at 2 μg/ml EPS concentration. Ascorbic acid was used at 50 μg/ml concentration as positive control, and it shows 73% DPPH scavenging activity. This result is consistent with the reference article that is used for methodology (Choi et al., 2021). The EPS of *Chlamydomonas* sp. ASYA25 shows similar antioxidant activity with 50 μg/ml ascorbic acid. When we compared the results with literature information, the results showed that it has a high antioxidant activity. A study evaluated the antioxidant activity of EPS fractions obtained from *Chlorella pyrenoidosa*, *Scenedesmus* sp., and *Chlorococcum* sp. species by examining DPPH scavenging activity (J. Zhang et al., 2019). According to the results obtained, *Chlorella pyrenoidosa and Scenedesmus* sp. showed maximum DPPH scavenging activity of 55.2% and 68.2% at 2 mg/ml EPS concentration, respectively. When the antioxidant activities of EPSs obtained from three different *Chlorella* sp. and *Chlorella sorokiniana* species were evaluated according to DPPH scavenging activities, it was observed that the maximum activity was approximately 30% at a maximum concentration of 2 mg/ml (Mousavian et al., 2022). In another study, the EPSs of *Chlorella zofingiensis* and *Chlorella vulgaris* show 71% and 59% DPPH scavenging activity at 3 mg/ml concentration. When we compare our results with those

studies, we can see that the EPS of *Chlamydomonas* gains prominence with its antioxidant activity. The antioxidant activity of polysaccharides may vary depending on factors such as the chemical composition, structural properties, purity, and type of source from which the polymer under study was obtained. For example, some types of polysaccharides, especially beta-glucans or alginates, can have strong antioxidant properties. Antioxidant activity may be due to phenolic compounds, flavonoids, proteins, vitamins, or other substances with antioxidant properties present in polysaccharides. These can reduce oxidative damage by neutralizing free radicals. The sulfate content of EPS influences its scavenging activity. In a research study, the EPS of *Lactobacillus plantarum* showed higher scavenging activity when it was sulfated (Z. Zhang et al., 2016). However, polysaccharides can often have lower antioxidant capacity compared to other antioxidant compounds. Therefore, the higher antioxidant activity of *Chlamydomonas* sp. ASYA25 EPS has the potential to be used in different applications.

To evaluate the antibacterial activity of EPSs disc diffusion procedure was performed at different EPS concentrations ranging from 1 mg/ml to 10 mg/ml. The antibacterial activity was evaluated against *S. aureus*, *E. coli*, and *B. subtillis* bacteria. While the Ampicillin (10 µg/ml) and Tetracycline (30 µg/ml) were used as a positive control, the distilled water was used as a negative control. The results showed that EPS shows the highest antibacterial activity against *S. aureus* while similar antibacterial activity was observed for *E. coli* and *B. subtillis*. The EPS showed antibacterial activity against *S. aureus* with 13 mm, 12 mm, and 8 mm inhibition zones at the 10 mg/ml, 7.5 mg/ml, and 5 mg/ml concentrations respectively. The EPS showed activity against *B. subtillis* with 10 mm, 9 mm, and 8 mm inhibition zones at concentrations of 10 mg/ml, 7.5 mg/ml, and 5 mg/ml concentrations respectively. The EPS showed activity against *E. coli* with 9 mm, 8 mm, and 7 mm inhibition zones at concentrations of 10 mg/ml, 7.5 mg/ml, and 5 mg/ml concentrations respectively. In a research study, the EPS obtained from bacteria showed antibacterial activity against *S. aureus* with a 10 mm inhibition zone at 1 mg/ml concentration (Raho Ghalem, 2017). In the same study, the EPS showed activity against *E. coli* with a 13 mm inhibition zone at 1 mg/ml concentration. In another study, the antibacterial activity of 2.5 mg/ml, 5 mg/ml, and 7.5 mg/ml bacterial EPS concentrations for different EPS fractions were evaluated against *E. coli*

and *B. subtilis*. The results showed that one of the EPS fractions showed activity against *E. coli* with a 12 mm inhibition zone at 5 mg/ml and 7.5 mg/ml concentrations. The same EPS fraction shows activity against *B. subtillis* with an 8 mm inhibition zone at 7.5 mg/ml concentration. The other EPS fraction shows activity against *B. subtillis* with a 15 mm inhibition zone at 7.5 mg/ml concentration while showing activity against *E. coli* with a 10 mm inhibition zone at 7.5 mg/ml concentration. When we compare our results with the literature, we can see that EPS of *Chlamydomonas* sp. ASYA25 shows significant antibacterial activity at 10 mg/ml concentration, especially against the *S. aureus* bacteria. Therefore, this EPS can be evaluated with its antibacterial activity in different application areas from cosmetic to food industries.

The thermal properties of EPS were evaluated with TGA and DSC analysis. The TGA results showed that when the EPS is exposed to increasing temperatures between 25-500 °C. According to the TGA result of EPS, weight loss occurs in three steps. The first step takes place between 25-138 °C with a 10% weight loss while the second step takes place between 138-216 °C with a 2% weight loss. The weight loss in these steps occurs due to loss of moisture and degradation of carboxyl groups. The third step occurs between 216-450 °C with a 46% weight loss and this step indicates the degradation of EPS. Total weight loss is determined as 58% and the main degradation occurs between 216-450 °C. The DSC analysis that performed between 25-400 °C and the result showed an endothermic peak starting from 100 °C and giving a sharp peak at 255 °C. This peak represents the decomposition of EPS, and this result is consistent with the TGA result. The TGA analysis of EPS extracted from *Chlorella sorokiniana* and *Chlorella minutissima* showed a 63% weight loss when heated from 20 to 450 °C (Koçer et al., 2021). In another research study, EPS of *Lactobacillus plantarum* showed 60% weight loss between 20-450 °C(Ismail & Nampoothiri, 2010). According to the results and literature information, the EPS showed good thermal stability until 216 °C and it can be used in industrial applications that performed under this temperature range.

The structure and functional groups of EPS were investigated with FTIR analysis by scanning 4000-400 cm^{-1}. The spectrum of released and cell-bound EPS shows similar results. The characteristic peaks of polysaccharides are seen between 1200-900 cm^{-1} and the FTIR results of EPS extracted from *Chlamydomonas* sp. ASYA25 gives a peak

at these wavelengths. The hydroxyl group gives a peak at 3329 cm^{-1} due to the moisture of EPS.

In recent years, EPSs have been evaluated as natural polymer sources for biomaterial formation due to their non-toxic and biodegradable natures. In addition to this, EPSs can be obtained from renewable sources like microalgae and cyanobacteria. Synthetic polymers derived from petrochemical sources are used in various applications like food packaging and biomedical. The materials produced from these synthetic polymers show good mechanical stability and can be obtained easily. However, their non-biodegradable natures and toxicity are seen as disadvantageous due to their harmful effects on nature and human health.

At this point, the natural alternative polymer sources have gained attention in recent years. The biomaterial formation has been studied for bacterial EPSs but the studies containing microalgae EPS are restricted. The cause of this may be the higher EPS production capacity of the bacteria because the formation of biomaterial needs a high raw material amount. There are a few studies that evaluate the biomaterial production from microalgal EPSs. One of these studies is the biodegradable film preparation studies by using EPS of *Graesiella* sp. (Gongi et al., 2022). In this research study, a biodegradable film was formed by mixing 0.9% EPS with 0.1% polyethylene glycol, and the obtained film was evaluated for meat packaging. In this thesis study, the EPS extracted from psychrotolerant microalgae *Chlamydomonas* sp. ASYA25 was evaluated for film material formation. Different EPS concentrations were investigated to optimize raw material concentration and results showed that the 3% EPS concentration was enough to form a film structure when it was mixed with 10% PEG. In the lower EPS concentration, the structure becomes weak, and many cracks occur in the structure. When the EPS composition of *Chlamydomonas* sp. ASYA25 was compared with *Graesiella* sp. EPS, we can see that *Graesiella* sp. EPS has a higher polysaccharide and protein content than Chlamydomonas sp. ASYA25 EPS. The EPS of Chlamydomonas sp. ASYA25 has 349 mg/g polysaccharide and 45 mg/g protein contents while the EPS of *Graesiella* sp. has 800 mg/g polysaccharide and 140 mg/g protein contents. The cause of optimum EPS concentration differences for film formation between two microalgae species may be their polysaccharide content differences. Due to the highest polysaccharide content of *Graesiella* sp. EPS, the film

can be formed at low concentrations. The viscosity also is an important parameter for biomaterial formation because the form of biomaterial shows variation according to the gelation capability of EPS. The result of this thesis study showed that EPS of *Chlamydomonas* sp. ASYA25 can form a homogenous structure at 3% EPS concentration. Despite the mechanical properties and disadvantages of EPS films compared to synthetic films, the non-toxic, biodegradable, antioxidant, and antibacterial nature of EPS provides advantages to EPS film. The mechanical properties of EPS films can be enhanced in further studies by modifying polysaccharide structure or adding plasticizer. The structure of the film can be enhanced by increasing the PEG concentration to 30% or another plasticizer like glycerol can be added to increase the elasticity of the film structure. If the films can be obtained with good mechanical strength, they can be evaluated for food packaging activity. The natural antibacterial and antioxidant nature of film will increase the value of film because it does not need any additives to provide these activities. Based on this thesis study, EPS of *Chlamydomonas* sp. ASYA25 could be evaluated as a new film-forming material. In addition, another option is to consider the addition of EPSs as a side material in other film structures due to their antioxidant and antibacterial nature.

Another biomaterial potential that can be formed by using EPS is hydrogel. The hydrogels are 3D polymeric networks prepared by crosslinking of polymers and can absorb large amounts of water or body fluids with excellent swelling characteristics. The EPS shows naturally gelatinous characteristics, so they are evaluated as potential polymer sources for hydrogel production. In this thesis project, hydrogel formation was evaluated by two different approaches physical and chemical crosslinking. In physical crosslinking, $CaCI_2$ was used while borax was used in chemical crosslinking. The result of physical crosslinking showed that the 1.5% and 2% EPS concentrations are suitable for hydrogel formation according to organoleptic analysis by converting test tubes. However, as understood from petri images the 2% EPS is more effective than the 1.5% concentration. The gelling ability of EPS increases with increasing $CaCI_2$ concentrations. In a research study, the $CaCI_2$ was evaluated for hydrogel formation by mixing the EPS of *Navicula* sp., but any gelation was not observed (Fimbres et al., 2016). Therefore, the $FeCI_3$ addition was applied instead of $CaCI_2$, and significant gelation was observed for 1% EPS and 0.8% $FeCI_3$ at that time. In another

study conducted with *Nostoc* sp. EPS, the hydrogel formation was recorded for 1% EPS and 0.4% $FeCI_3$ concentrations (Alvarez et al., 2021). Based on this thesis study, the hydrogel formation can perform at 1.5% or 2% EPS concentrations with $CaCI_2$ additions. In further studies, the hydrogel formation can be evaluated with $FeCI_3$ and so the need for raw material may be decreased.

In chemical crosslinking, the borax was added to the solution based on the weight of EPS. The gelation was followed organoleptically, and the results showed that gelation occurred at 1-2 % EPS concentrations and increased with increasing borax concentration. The gelation character of chemically crosslinked hydrogel is better than that of physically crosslinked hydrogel. The cause of this situation is due to the mechanism of crosslinking because in chemical crosslinking, the covalent bond formation occurs between the polymers while ionic interactions or hydrogen bonding occur between polymers in physical crosslinking. The chemical crosslinking provides a more stable structure but the toxicity of hydrogel due to the chemical crosslinker limits the usage of these hydrogels. However, borax has the lowest toxicity when compared to other chemical crosslinkers like glutaraldehyde. Borax also has advantages due to its water-soluble and low-cost properties (Tanpichai et al., 2022). In a valuable study, guar gum was evaluated for hydrogel formation by crosslinking with borax (Thombare et al., 2017). The guar gum is an EPS used in various application areas like food and biomedical industries. For the hydrogel formation, 0.7% guar gum was cross-linked with 5, 10, 15, 20, and 25% of the weight of guar gum and the results showed that hydrogel formation occurred at the lowest borax concentrations. The same borax concentrations were evaluated in this thesis project and the suitable gelation was observed at a 1% concentration of EPS while showing an increasing gelation with increasing EPS concentration. In summary, the results showed that EPS of *Chlamydomonas* sp. ASYA25 shows gelation at 1.5-2% concentrations for physical crosslinking and 1-2% concentrations for chemical crosslinking. In further studies, the characterization of EPS hydrogels can be made, and they evaluated for wound healing or drug delivery systems.

CONCLUSION

In conclusion, EPSs isolated from *Chlamydomonas* sp. ASYA25 showed antioxidant and antibacterial activity and the potential to be used as an alternative polymer source in forming biomaterials such as films and hydrogels. These materials are usually made of synthetic polymers, as they provide stronger mechanical strength and are easier to manufacture. However, they are seen as disadvantageous due to their toxic and non-biodegradable nature and their potential to harm both human health and the environment. Moreover, the production of synthetic polymers is often derived from petrochemical products, which can lead to environmental impacts such as the use of fossil fuels and greenhouse gas emissions. Thus, interest in biodegradable, non-toxic polymers produced from renewable resources has been growing rapidly in recent years. Although there are many review and research articles evaluating the use of bacterial EPS for biomaterial formation, there is not a substantial amount of literature available regarding microalgal EPS. Therefore, this thesis project can be considered as a novel contribution to literature in this field. Moreover, the significant antioxidant and antibacterial activity of EPS obtained from *Chlamydomonas* sp. ASYA25 will enhance the value of biomaterial derived from this EPS.

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