**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL**

# RECOMBINANT PRODUCTION AND CHARACTERIZATION OF CHITINASE ENZYME FROM *Pseudomonas mandelii* KGI_MA19

**M.Sc. THESIS**

**Emine Tuğçe SARAÇ CEBECİ**

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**ISTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ**

# *Pseudomanas mandelii* KGI_MA19 ORGANIZMASINA AİT KİTİNAZ ENZİMİNİN REKOMBİNANT ÜRETİMİ VE KARAKTERİZASYONU

**YÜKSEK LİSANS TEZİ**

**Emine Tuğçe SARAÇ CEBECİ**
**521201111**

**Moleküler Biyoloji-Genetik ve Biyoteknoloji Anabilim Dalı**

**Moleküler Biyoloji-Genetik ve Biyoteknoloji Programı**

**Tez Danışmanı: Prof. Dr. Nevin GÜL KARAGÜLER**

**HAZİRAN 2022**

Emine Tuğçe SARAÇ CEBECİ, a M.Sc. student of ITU Graduate School student ID 521201111, successfully defended the thesis entitled "RECOMBINANT PRODUCTION AND CHARACTERIZATION OF CHITINASE ENZYME FROM *Pseudomonas mandelii* KGI_MA19", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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|---|---|---|
| **Thesis Advisor:** | **Prof. Dr. Nevin GÜL KARAGÜLER**<br>Istanbul Technical University | ..... |
| **Jury Members :** | **Prof. Dr. Zeynep Petek ÇAKAR**<br>Istanbul Technical University | ..... |
| | **Dr. Günseli KURT GÜR**<br>Yıldız Technical University | ..... |

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***To my beloved husband Anıl, my lovely family and Elly…***

## FOREWORD

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June 2022

Emine Tuğçe SARAÇ CEBECİ
(Molecular Biologist)

**TABLE OF CONTENTS**

**Page**

## ABBREVIATIONS

**bp** **:** Base Pair
**$CaCl_2$** **:** Calcium Chloride
**$dH_2O$** **:** Distilled Water
**FGSP** **:** Forward Gene Specific Primer
**HCl** **:** Hydrochloric Acid
**His-tag** **:** Histidine tag
**DNA** **:** Deoxyribonucleic Acid
**DNS** **:** Dinitro Salicylic Acid
**dNTP** **:** Deoxynucleotide Triphosphate
***E. coli*** **:** *Escherichia coli*
**kb** **:** Kilo base
**kDa** **:** Kilo Dalton
**$MgCl_2$** **:** Magnesium Chloride
**min** : Minute
**ml** **:** Milliliter
**mM** **:** Millimolar
**PCR** **:** Polymerase Chain Reaction
**RGSP** **:** Reverse Gene Specific Primer
***P. mandelii*** **:** *Pseudomonas mandelii*
**rpm** **:** Revolutions per minute
**sec** : Second
**SDS-PAGE** **:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
**SDS** **:** Sodium Dodecyl Sulfate
**TAE** **:** Tris Acetate EDTA Buffer
**TBS-T** **:** Tris Buffered Saline - Tween20
**UV** **:** Ultraviolet
**µg** **:** Microgram
**µl** **:** Microliter

**LIST OF TABLES**

**Page**

**LIST OF FIGURES**

## RECOMBINANT PRODUCTION AND CHARACTERIZATION OF CHITINASE ENZYME FROM *Pseudomonas mandelii* KGI_MA19

## SUMMARY

Biological catalysts, generally found in protein structure, that catalyze the biochemical reactions necessary for life are called enzymes. They increase the reaction rate by lowering the activation energy of the reaction catalysed. In recent years, enzymes have gained a large share in different industrial areas, like food, agriculture, pharmacy, cosmetics, waste removal, leather processing, detergent, and medical applications. Enzymes can minimize the formation of unwanted by-products, they are environmentally friendly and cheap, and have biodegradable properties. They are also considered safe for the cleaning, health, and food industries. In addition to their preferred properties, enzymes that remain active at low/high temperatures, in the presence of organic solvents, at different salt concentrations and pH values, and with an affinity for different substrates are attracting the attention of the industry. Meeting the increasing demand for the use of biomolecules with different properties in the industry is possible with the development of the physical and biochemical properties of the biocatalysts defined today with the help of protein engineering, metagenomics, advanced DNA technologies, nanotechnology, and finally, the discovery of new biocatalysts. Scientific research has been increasing in this direction, especially because enzymes from living things that live in extreme conditions can be used in a wide range of industries.

Chitin $(C_8H_{13}O_5N)_n$ is an inelastic, hard, white, non-elastic biopolymer formed by the bonding of N-Acetyl-D-glucosamine monomers (Glc-NAc) with β-1,4 glycosidic bonds, which ranks second after cellulose among the most abundant biopolymers in nature. It is a nitrogen-containing polysaccharide. Chitin is found in the cell wall of fungi, the outer shells of insects, the shells of sea creatures such as lobster, crab, shrimp, and the mouth areas of cephalopods such as cuttlefish and octopus which have a high annual production in the world. According to the Food and Agriculture Organization (FAO, 2019) data, 10.5 million chitin-rich shellfish (12.3%) are grown in aquaculture. The need for converting chitin wastes, which are rising in quantity by the day, into biologically useful products develops. In recent years, the employment of biocatalysts in the removal of chitin wastes instead of chemical methods, which are poisonous to the environment and expensive, has allowed for a safe conversion for the environment.

Chitinase enzymes (EC 3.2.1.14), in the hydrolase class, catalyse the destruction of β-1-4 glycosidic bonds in chitin $(C_8H_{13}O_5N)_n$ and separate N-acetyl-D-glucosamine molecules from the polysaccharide chain. The chitinase enzyme, which is one of the most common hydrolase enzymes in nature, is found in insects, plants, fungi and viruses. It is also commonly found in different bacterial genera such as *Aeromonas, Arthrobacter, Bacillus, Chromobacterium, Flavobacterium, Pseudomonas, Sanguibacter, Serratia,* and *Streptomyces*. With the increase in green and

environmentally friendly technology in recent years, the interest in chitinases is increasing day by day. Chitinase enzymes, which have agricultural importance, replace chemicals and pesticides used in agriculture with their ability to be used as biocontrol agents. It creates a positive effect on the marine ecosystem by recycling marine waste. Due to their pharmaceutically antifungal and antibacterial properties, they are used in the production of anti-inflammatory and anti-fungal drugs, anti-cancer and immune-enhancing agents, wound dressings, contact lenses, and surgical sutures. It is a bioprotective additive used to increase shelf life in the food industry.

Antarctica with the highest elevation and lowest temperature has recently become a popular research area. 70 % of the freshwater resources on the Antarctic continent, which has not been touched by human hands until lately, are in the form of ice. It also features powerful winds, extremely cold temperatures, and is exposed to low temperatures in the winter and high UV rays in the summer. It has a natural and distinct habitat as a result of these factors. Because of the harsh circumstances, it is unavoidable to uncover new species and enzymes and genes. Extremophiles are living systems that can thrive in harsh environments. Biocatalysts derived from extremophilic organisms and demonstrating catalytic activity even under adverse circumstances have been dubbed extremozymes. Psychrophiles and psychrotolerants are extremophilic microorganisms that can live at 0-20 °C and 0-30 °C, respectively. Because of their low energy needs, flexibility, and high catalytic activity, cold-compatible enzymes derived from psychrophilic and psychrotolerant organisms are crucial for commercial applications. Biotechnological approaches help the identification of novel and diverse extremophiles and extremozymes from Antarctica that has not been described in the literature yet.

In the study, which was carried out using Antarctic sediment samples collected within the scope of the 2nd National Antarctic Science Expedition (TAE-2), 12 sediment samples taken from 8 different regions were cultured and enriched. Subsequently, freeze-thaw stress was applied to each cultured sample. After the applied freeze-thaw stress, it was observed that five cultures were resistant to stress. Two morphologically different colonies were selected from each stress-resistant sample, DNA isolations were made and 16S rRNA analyzes were carried out. The sample with the lowest similarity rate according to 16S rRNA analysis was sent for whole-genome analysis and according to the results of the analysis, a new strain of *Pseudomonas mandelii*, *Pseudomonas mandelii* KGI_MA19, was identified. Phenotypic and biochemical characterizations of the identified KGI_MA19 strain were performed.

The next step was the recombinant production and the characterization of the chitinase enzyme of the psychrotolerant *Pseudomonas mandelii* KGI_MA19. The gene region of the chitinase enzyme of *Pseudomonas mandelii* KGI_MA19 was amplified by polymerase chain reaction (PCR) by designing gene-specific primers containing *Sac*I and *Not*I restriction sites. The amplified gene region of interest and the pET-28a (+) vector were cut with *Sac*I and *Not*I restriction enzymes. T4 DNA ligase enzyme was used for ligation of the cut PCR product and expression vector. Plasmid pET-28a (+) containing the PCR product was transformed into *Escherichia coli* BL21 (DE3) competent cells. The colony PCR method was applied to determine the colonies containing the relevant gene as a result of the transformation, and plasmid isolation of two colonies selected from among the colonies thought to be positive was performed. Then, to understand whether the colonies contain the gene product or not, the plasmid was cut with the *Eco*RV endonuclease enzyme, which has a fast-cutting feature. The DNA sequencing results showed that the chitinase gene-specific PCR product was

correctly inserted into the pET-28a (+) plasmid. The Magic Media$^{TM}$ (Invitrogen), which is commercially available and used in the expression of *E. coli* cells, was used to monitor the expression level of the chitinase gene in *E. coli* BL21 cells. At the end of Magic Media incubation, cells were treated with lysis (0.1M Tris-HCl, pH 8.0, 0.3M NaCl) buffer, and homogenization was performed by ultrasonication using the M73 probe. A high amount of produced chitinase enzyme was purified by the His-Tag affinity chromatography method using the His-Trap column. The activity of the chitinase enzyme obtained in high purity was determined by the 3, 5 - Dinitrosalicylic acid (DNS) method which allows one to determine the amount of reducing sugars expected to be released as a result of the reaction performed by the usage of colloidal chitin as a substrate. Biochemical characterization including optimum pH, pH stability, and optimum temperature, thermal stability has been completed.

A new psychrotolerant strain, *Pseudomonas mandelii* KGI_MA19, was identified from sediment samples from the Antarctic King George Island, and its molecular, phenotypic, morphological, and biochemical characterization was completed. In addition, the chitinase enzyme which has an increasing impact in the industrial field has been successfully produced and biochemically characterized by using recombinant DNA methods. The cold-adaptive chitinase enzyme of the *Pseudomonas mandelii* KGI_MA19 strain, which was obtained for the first time within the scope of this thesis, is thought to be a promising potential biocatalyst for industrial applications.

# *Pseudomonas mandelii* KGI_MA19 ORGANİZMASINA AİT KİTİNAZ ENZİMİNİN REKOMBİNANT ÜRETİMİ VE KARAKTERİZASYONU

## ÖZET


Canlılık için gerekli olan biyokimyasal reaksiyonları katalizleyen genellikle protein yapıda bulunan biyolojik katalizörlere enzim adı verilmektedir. Enzimler, katalizledikleri reaksiyonların aktivasyon enerjisini düşürerek reaksiyonun oluşum hızını arttırırlar. Son yıllarda, gıda, tarım, eczacılık, kozmetik, atık giderimi, deri işleme, deterjan ve tıbbi uygulamalar için farklı endüstriyel alanlarda, çevre dostu, ucuz, istenmeyen yan ürünlerin oluşumunu en aza indirgemeleri ve biyobozunur özelliğe sahip olmaları sebebiyle büyük bir paya sahiplerdir. Ayrıca temizlik, sağlık ve gıda endüstrileri için de güvenli kabul edilmektedirler. Tercih edildikleri özelliklere ek olarak, düşük/yüksek sıcaklıklarda, organik çözücülerin varlığında, farklı tuz konsantrasyonları ve pH değerlerinde aktif kalan ve farklı substratlara afinitesi olan enzimler, endüstrinin ilgisini çekmektedir. Sanayide farklı özelliklere sahip biyomoleküllerin kullanımı için artan talebin karşılanabilmesi, günümüzde tanımlanmış biyokatalizörlerin fiziksel ve biyokimyasal özelliklerinin protein mühendisliği, metagenomik, ileri DNA teknolojileri ve nanoteknoloji yardımıyla geliştirilmesi ve /veya yeni biyokatalizörlerin keşfi ile mümkündür. Özellikle, ekstrem koşullar altında yaşamını sürdüren canlılardan elde edilen enzimlerin, farklı endüstriyel alanlarda kullanılabilirlikleri nedeniyle, bilimsel araştırmalar da bu yönde hız kazanmıştır.

Kitin $(C_8H_{13}O_5N)_n$, doğada en çok bulunan biyopolimerler içinde selülozdan sonra ikinci sırada yer alan, β-1,4 glikozidik bağlarla N-Asetil-D-glukozamin monomerlerinin (Glc-NAc) bağlanması ile oluşan elastikiyeti olmayan, sert, beyaz, azot içeren bir polisakkarittir. Kitin özellikle dünya üzerinde yıllık üretimi fazla olan, mantarların hücre duvarında, böceklerin dış kabuklarında, ıstakoz, yengeç, karides gibi deniz canlılarının kabuklarında, mürekkep balığı ve ahtapot gibi kafadan bacaklı canlıların ağız bölgelerinde bulunmaktadır. Gıda ve Tarım Örgütü (Food and Agriculture Organization (FAO), 2019), verilerine göre de su ürünleri yetiştiriciliğinde, 10,5 milyon kitince zengin kabuklu deniz canlısı (%12,3) yetiştirilmektedir. Her geçen gün artan miktarda oluşan kitin atıklarının, biyolojik olarak değerli ürünlere dönüştürülmesi gerekliliği ortaya çıkmaktadır. Son yıllarda, kitin atıklarının giderilmesinde çevre için toksik ve maliyet açısından da yüksek olan kimyasal proseslerin yerine biyokatalizörlerin kullanılmaya başlanması, çevre için güvenli bir dönüşümün sağlanmasına olanak tanımaktadır.

Hidrolazlar sınıfında bulunan kitinaz enzimleri (EC 3.2.1.14), kitin $(C_8H_{13}O_5N)_n$ içerinde bulunan β-1-4 glikozidik bağlarının yıkımını katalizler ve N-asetil-D-glukozamin moleküllerini polisakkarit zincirinden ayırırlar. Doğada yaygın bulunan kitinaz enzimi, böceklerde, bitkilerde, mantarlarda, virüslerde bulunmaktadır. Ayrıca,

*Aeromonas, Arthrobacter, Bacillus, Chromobacterium, Flavobacterium, Pseudomonas, Sanguibacter, Serratia* ve *Streptomyces* gibi farklı bakteri cinslerinde de yaygın olarak bulunmaktadır. Son yıllarda artan yeşil ve çevre dostu teknoloji ile kitinazlara olan ilgi de günden güne artış göstermektedir. Tarımsal öneme sahip olan kitinaz enzimleri, biyokontrol maddesi olarak kullanılabilmesi özelliği ile tarımda kullanılan kimyasalların ve pestisitlerin yerini almaktadır. Deniz atıklarının geri dönüşümünü sağlayarak deniz ekosisteminin üzerinde olumlu bir etki yaratmaktadır. Farmasötik açıdan antifungal ve antibakteriyel özellikleri sebebiyle iltihap ve mantar önleyici ilaç, anti kanser ve bağışıklık güçlendirici ajan, yara örtüleri, kontakt lensler ve cerrahi dikişlerin üretilmesinde kullanılmaktadırlar. Gıda sanayinde ise raf ömrünün arttırılmasına yönelik olarak kullanılan biyokoruyucu katkı maddesi olarak karşımıza çıkmaktadır.

Yeryüzünde en son keşfedilen, en yüksek rakıma ve en düşük sıcaklığa sahip olan Antarktika kıtası son dönemlerde popüler bir araştırma alanı olmuştur. Yakın bir geçmişe kadar insan elinin değmediği Antarktika kıtasında tatlı su kaynaklarının %70'i buz halinde bulunur. Ayrıca kuvvetli rüzgarlara, çok düşük sıcaklıklara sahiptir ve kışın düşük, yazın ise yoğun UV radyasyonuna maruz kalmaktadır. Bu nedenlerden dolayı doğal ve eşsiz bir yaşam habitatına sahiptir. Ekstrem koşulları sayesinde yeni türlerin bulunması, çeşitli enzimlerin ve genlerin keşfedilmesi kaçınılmazdır. Ekstrem koşullarda canlılıklarını sürdürebilen canlılar ekstremofiller olarak adlandırılmaktadır. Ekstremofilik canlılardan elde edilen, zor koşullar altında dahi katalitik aktivitelerini gösteren biyokatalizörler, ekstremozimler olarak literatürde yerini almıştır. Psikrofiller 0-20 °C'de, psikrotolerantlar ise 0-30 °C'de yaşamlarını sürdürebilen ekstremofilik mikroorganizmalardır. Psikrofil ve psikrotolerant canlılardan elde edilen soğuğa uyumlu enzimler ise endüstriyel uygulamalarda düşük enerji ihtiyaçları, esneklikleri ve yüksek katalitik aktiviteye sahip olmaları sebebiyle tercih edilmektedir. Biyoteknolojik araştırmalar sayesinde, Antarktika'ya ait literatürde henüz bulunmayan yeni ve çeşitli ekstremofillerin ve ekstromozimlerin keşfi devam etmektedir.

2. Ulusal Antarktika Bilim Seferi (TAE-2) kapsamında toplanan Antarktika sediment örnekleri kullanılarak gerçekleştirilen çalışmada, 8 farklı bölgeden alınmış, 12 adet sediment örneğinin kültüre edilerek zenginleştirilmesi sağlanmıştır. Ardından her bir kültüre edilmiş örneğe don-çöz stresi uygulanmıştır. Uygulanan don-çöz stresi sonrasında beş adet kültürün strese dirençli olduğu gözlemlenmiştir. Strese dirençli her bir örnekten morfolojik olarak farlılık gösteren iki adet koloni seçilerek, DNA izolasyonları yapılmış ve 16S rRNA analizleri gerçekleştirilmiştir. 16S rRNA analizine göre benzerlik oranı en düşük olan örnek tüm genom analizine gönderilmiş ve analiz sonucuna göre *Pseudomonas mandelii* türüne ait yeni bir suş olan *Pseudomonas mandelii* KGI_MA19 tanımlanmıştır. Tanımlanan KGI_MA19 suşunun fenotipik ve biyokimyasal karakterizasyonu gerçekleştirilmiştir.

Psikrotolerant bir organizma olan *Pseudomonas mandelii* türüne ait hidrolitik kitinaz enziminin üretimi ve biyokimyasal karakterizasyonuna yönelik çalışma literatürde henüz mevcut değildir. *Pseudomonas mandelii* KGI_MA19'a ait kitinaz enziminin gen bölgesi, *Sac*I ve *Not*I kesim bölgeleri içeren gen spesifik primerler tasarlanarak polimeraz zincir reaksiyonu (PZR) ile çoğaltılmıştır. Çoğaltılan ilgili gen bölgesi ve pET-28a (+) vektörü, *Sac*I ve *Not*I restriksiyon enzimleriyle kesilmiştir. Kesimi yapılmış PZR ürünü ve ekspresyon vektörünün ligasyonu için T4 DNA ligaz enzimi kullanılmıştır. PZR ürününü içeren pET-28a (+) plazmidi, *Eshericia coli* BL21 (DE3) kompetant hücrelerine transforme edilmiştir. Transformasyon sonucunda ilgili geni içeren kolonilerin belirlenebilmesi için koloni PZR yöntemi uygulanmış ve pozitif

olduğu düşünülen koloniler arasından seçilen iki adet koloninin plazmit izolasyonu gerçekleştirilmiştir. Ardından kolonilerin gen ürününü içerip içermediğini anlayabilmek için hızlı kesim yapma özelliğine sahip *EcoR*V endonükleaz enzimiyle plazmidin kesimi gerçekleştirilmiştir. DNA dizileme sonuçları kitinaz genine spesifik PZR ürününün pET-28a (+) plazmidi içerisine doğru bir şekilde eklendiğini göstermiştir. Ticari olarak satılan *E.coli* hücrelerinin ekspresyonlarında kullanılan Magic Media™ (Invitrogen), *E.coli* BL21 hücreleri içerisinde bulunan kitinaz geninin ekspresyon seviyesinin gözlemlenmesi için kullanılmıştır. Magic Media inkübasyonu sonunda hücreler lizis (0.1M Tris-HCl, pH 8,0, 0,3M NaCl) tamponu ile muamele edildikten sonra M73 probu kullanılarak ultrasonikasyon ile homojenizasyon gerçekleştirilmiştir. Yüksek miktarda üretilen kitinaz enzimi His-Trap kolonu kullanılarak His-Tag afinite kromatografisi yöntemi ile saflaştırılmıştır. Yüksek saflıkta elde edilen kitinaz enziminin aktivitesi, kolloidal kitinin substrat olarak kullanılması ile gerçekleştirilen reaksiyon sonucunda açığa çıkacağı beklenen indirgen şekerlerin miktarını belirlemeyi sağlayan 3, 5-Dinitrosalisilik asit (DNS) yöntemi ile belirlenmiştir. Optimum pH ve optimum sıcaklık, pH stabilitesi ve termal stabilitesini içeren biyokimyasal karakterizasyonu tamamlanmıştır.

Bu tez çalışması kapsamında, Antarktika King George adasından alınan sediment örneklerinden yeni, psikrotolerant bir suş olan *Pseudomonas mandelii* KGI_MA19 suşu tanımlanmış ve moleküler, fenotipik, morfolojik ve biyokimyasal karakterizasyonu tamamlanmıştır. Ayrıca endüstriyel alanda artan öneme sahip kitinaz enzimi, rekombinant DNA yöntemleri kullanılarak başarılı bir şekilde üretilmiş ve biyokimyasal karakterizasyonu gerçekleştirilmiştir. İlk kez bu tez kapsamında elde edilen *Pseudomonas mandelii* KGI_MA19 suşuna ait soğuğa uyumlu kitinaz enziminin, endüstriyel uygulamalar için umut vadeden potansiyel bir biyokatalizör olduğu düşünülmektedir.

# 1. INTRODUCTION

## 1.1 Summary of Enzymes

Enzymes are biological catalysts mostly in protein structures that accelerate metabolic reactions. Considering their structural properties, it is known that enzymes are protein-structured except for a small part of catalytic RNA (Roskoski et al., 2015). Enzymes operate as extremely effective catalysts in biological processes, accelerating reactions without changing the equilibrium constant by offering an alternate reaction route with lower activation energy (Robinson et al., 2015). Biocatalysts have several benefits, including the fact that they are biodegradable, non-toxic, and reusable, as well as having high specificity, selectivity, and activity.

Due to these properties, the interest in the use of enzymes in industrial areas has increased. They are widely used in industrial applications, particularly because the processes they catalyse produce environmentally friendly products while creating no environmental pollution (Garske et al., 2017). Industrial enzyme research is becoming more important in terms of biotechnological development as a result of advancements in enzyme technology (Chapman et al., 2018).

### 1.1.1 Enzyme structure and function

Protein-based enzymes exhibit catalytic activity thanks to their primary, secondary, tertiary, and quaternary structures. To catalyse processes, enzymes require organic complexes called coenzymes or cofactors, which are metal ions. The protein part of the enzyme without a cofactor is called the apoenzyme. A holoenzyme is a name given to the enzyme containing coenzyme and prosthetic group. The part that regulates the catalytic activities of enzymes is the part of the apoenzyme that is affected by the amino acid sequences. When apoenzymes are together with their coenzymes or prosthetic groups, they show catalytic activity (Hartl et al., 2017).

The catalytic mechanism of enzymes is to form products by reacting with their substrates. Many models have been identified for how enzyme reactions take place.

The first of these is the model in which the catalytic domain of the enzyme, known as the Key-Lock model, binds to the substrate in an appropriate form (Fischer et al., 1894). The other model is the induced conformation, in which the enzyme is in free form when the substrate is not in the medium, and when the substrate is added, it shows conformational changes such that the enzyme binds to the substrate (Koshland et al., 1958). The last model is a conformational selection model based on the assumption that free enzymes exist in multiple conformational states and that the substrate selects the higher energy conformation to form the enzyme-substrate complex. For the past few decades, this model has been the accepted model (Figure 1.1) (Chen et al., 2015).

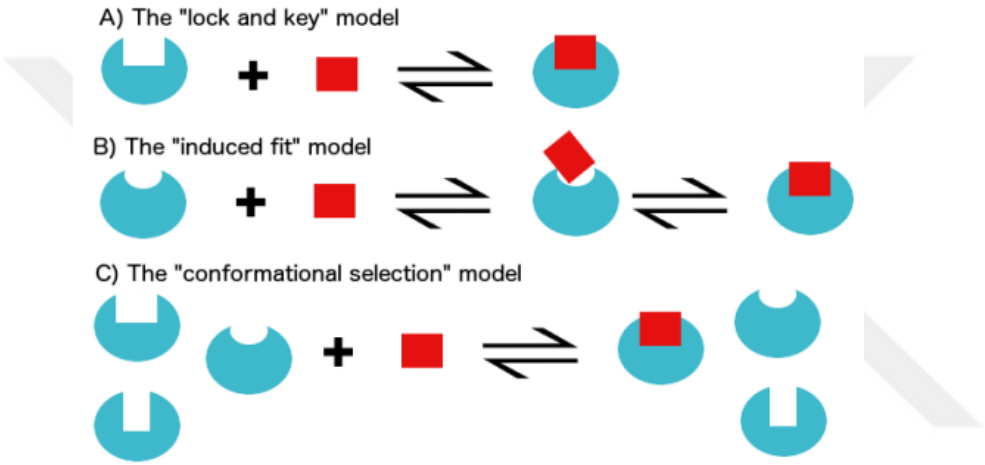


**Figure 1. 1** Models of enzyme catalysis (A) The lock-and-key model, (B) Induced-fit model, and (C) conformational-selection model (Chen et al., 2015).

### 1.1.2 Classification of enzymes

An Enzyme Commission (EC) was formed as a consequence of talks between the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry and Molecular Biology (IUBMB), and the systematic identification and categorization of enzymes were carried out (Khan, et al., 2021). The Enzyme Commission categorizes enzymes into six types based on the processes they catalyze. According to the most recent investigations, the seventh enzyme class was introduced to the literature in 2018 (Balaji et al., 2021).

Oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases, and translocases are the main classes of enzymes in the enzyme categorization (Table 1.1). Enzymes are classified using four digits (McDonald et al., 2014). The first of them

defines the main category to which the enzyme belongs. The second number denotes the enzyme's subgroup. The third and fourth digits represent the sub-subgroup and sub-sub-sub-subgroup, respectively.

**Table 1. 1** Enzyme classification and functions determined by the Enzyme Commission (McDonald et al., 2021; Tipton et al., 2018).

| Enzyme Commission Number | Enzyme Classification | Mechanism of Catalysis |
|---|---|---|
| EC 1 | Oxidoreductases | Catalysis of redox reactions between substrates. |
| EC 2 | Transferases | Catalysis of the transfer of groups other than $H^+$ in molecules. |
| EC 3 | Hydrolases | Catalysis of the hydrolysis of ester, ether, peptide, glycoside, acid anhydride, C-C, C-halide or P-N bonds with the incorporation of a water molecule. |
| EC 4 | Lyases | Catalysis of reactions generated by double bonds and removal of groups from substrates by processes other than hydrolysis. |
| EC 5 | İsomerases | Catalysis of the interconversion of geometric, optical, or structural isomers. |
| EC 6 | Ligases | Catalysis of the bonding reactions of two compounds with the help of the energy released as a result of the cleavage of the phosphate bond by high-energy phosphate compounds such as ATP or GTP. |
| EC 7 | Translocases | Catalysis of the movement of ions or molecules across the cell membrane or their separation within the membrane. |

### 1.1.3 Industrial applications enzymes

Due to the rapid increase in the world population, there is an increasing interest in the industrial market for the use of biomolecules that are environmentally friendly, inexpensive, easy to produce and ecologically degradable. Enzymes have an important share in the industry as biological catalysts due to their easy production, substrate specificity, stability and high efficiency. Among the factors affecting the activities of enzymes are pH, temperature and substrate concentration (Chapmanet al., 2018). Enzymes are biomolecules specific to the reactions they catalyze, and a wide variety

of reactions can be catalyzed by different enzymes (Table 1.2). In addition, thanks to protein engineering approaches, enzymes can gain the ability to catalyze different substrates at different temperatures and pH conditions (Cao et al., 2021).

**Table 1. 2** Enzyme applications in industry.

| Industries | Enzymes | Applications of enzymes |
|---|---|---|
| Food industry | Amylase, papain, amylopullulanase, trypsin, pectinase | Starch to glucose conversion, high fructose corn syrup manufacturing, prebiotics synthesis, fruit juice debittering (Badgujar et al., 2016; Kapoor et al., 2017). |
| Pharmaceutical industry | Transaminase, lipase, nitrile hydratase, polyphenol oxidase, chitosanase | Intermediate synthesis of the manufacture of pharmacologically active components (Goncalves et al., 2018; Marouf et al., 2018) |
| Paper and Pulp industry | Laccase, xylanase, cellulase, lipase | Elimination of lignin to enhance bleaching, increasing fiber qualities (Choi et al., 2015; Dewan et al., 2017) |
| Detergents | Cellulase, lipase, protease, amylase | Elimination of stains, elimination of fats and oils, and colour permanence (Dewan et al., 2017; Singh et al., 2016) |
| Waste treatment | Laccase, manganese peroxidase, lignin peroxidase and tyrosinase, monooxygenase | Toxic organic compound detoxification, breakdown of halogenated organic materials containing contaminants, and waste recycling for reuse (Pandey et al., 2011; Le Roes-Hill et al., 2016) |
| Biofuel | Xylanase, cellulase, lipase | Degradation of lignocellulosic materials for bioethanol synthesis, formation of fatty acid methyl esters (Lima et al., 2017; Olofsson et al., 2017) |

For many years, people have used enzymes for their purposes. Enzymes employed in the industry are of microbial origin because they can rapidly replicate, and their production can be easily regulated. Biocatalysts produced from microorganisms can be employed to provide various products in industrial areas. At the beginning of their use in industry, they were in food, medicine, agriculture, cosmetics, paper, pharmaceuticals, textiles, leather processing industry, and bioremediation (Figure 1.2) (Singh et al., 2016).


**Figure 1. 2** Applications of enzymes (Singh et al., 2016).

## 1.2 Extremozymes

In the development of industrial enzymes, genetic engineering methods have been applied. The techniques utilized may increase enzyme features such as substrate specificity, solvent tolerance, selectivity, and stability (Elleuche et al., 2014). Simultaneously, extremozymes generated by extremophilic microorganisms have lately emerged as biotechnologically fascinating biomolecules. Extremozymes are enzymes that execute catalytic activities in high and low temperatures, extreme pressure, alkaline and acidic environments, high salt concentrations, and high UV conditions (Davids et al., 2013). Extremozymes have industrially desirable enzyme capabilities without the use of protein engineering techniques. As a result, there has been a surge in interest in extremozymes in recent years. Extremozymes are named based on their characteristics. It is referred to as a "thermozyme" if it is derived from thermophilic organisms that live in very hot conditions (Mahejibin et al., 2017). It is referred to as a "psychrophilic enzyme" if it is derived from a psychrophilic or psychrotolerant organism in a cold environment (Feller et al., 2003). The properties of the enzymes produced by extremophiles are largely determined by the circumstances in which they exist. Among the enzymes with specialized qualities, those with halophilic properties can catalyze at low water concentrations, as they can maintain stability at high salt concentrations. In the presence of proteases and detergents, thermozymes remain active, while psychrophilic enzymes are utilised to produce heat-affected products. In the presence of proteases and detergents, thermozymes remain

active, while psychrophilic enzymes are utilised to produce heat-affected products (Demirjian et al., 2001). Extremozymes are enzymes that catalyse processes in an ecologically beneficial way without the need for additional energy consumption under diverse circumstances. New extremozymes are being introduced to the literature every day. The growing number of extremozymes has attracted the attention of the industry.

### 1.2.1 Psychrophilic enzymes

Antarctica is renowned as the world's coldest area. The living circumstances in the area, which is covered with glaciers for 70% of the year, are equally as difficult as the weather. Food shortages are a major concern, especially in a cold and radiation-rich environment. Psychrophiles are microorganisms that have adapted to these circumstances and live in the region. "Psychros" means cold in Greek, while "Philos" signifies loving. Psychrophile, on the other hand, means that loves the cold. Psychrophiles are also known as "rigophiles" and "cryophiles". Psychrophilic bacteria are classified into two types (Stokes et al., 1963). The earliest members of this group must be psychrophiles, with an optimal temperature of 20 °C. The other kind of bacterium is psychrophilic (psychrotolerant), which has an optimal development temperature above 20 °C and is obligatory facultative. As a consequence, since their membranes contain more unsaturated fatty acids, psychrophilic microbes may live in very cold environments (Al-Maqtari et al., 2019).

Biocatalysts produced by psychrophilic organisms are known as psychrophilic enzymes and may also be referred to as cold-adapted enzymes (Feller et al., 2013). In catalytic reactions, the primary factor determining the reaction rate is the decrease in temperature. Because they aim to keep the process moving at a constant rate, cold-adaptive enzymes encounter a major challenge (Somero et al., 1995). It has been discovered that cold-adapted enzymes have ten times the activity of their mesophilic homologues. This mechanism is recognized to be a low-temperature adaption gained by generating heat-labile enzymes. High activity in a cold environment is accomplished by keeping the enzyme's active region flexible. Although the active site of the enzyme remains active, it is assumed that the removal of proteins that cannot remain active at low temperatures also plays a role in selectivity and activity (Feller et al., 2003).

Cold-adapted enzymes have greater catalytic activity under cold conditions than their mesophilic homologues. This is due to alterations that occur in their active sites, which enable them to quickly attach to their substrates. Some features of psychrophilic enzymes are distinguished when compared to their thermophilic and mesophilic homologues. Among these characteristics are reduced electrostatic interaction and disulfide bonds, less proline amino acid content and greater glycine content, and increased surface and decreased core hydrophobicity (Gerday et al., 2000). Because of their minimal energy requirements, their economic usage is considerably less expensive (Cavic-chioli et al., 2011). It is widely used in various food, textile, medicine and detergent. Because of the many advantages they provide, psychrophilic enzymes have a high potential for industrial usage and have received increased attention in recent years.

### 1.3 Extremophiles

It has been revealed as a result of the discoveries and scientific research made in the last century that life can be sustained not only in certain situations but also in a variety of harsh conditions. While severe environments are defined as circumstances in which humans cannot survive, these settings appear to be ones in which microbes can survive. Microorganisms can live metabolically and chemically adapted, surviving under extreme environmental stress. Creatures that can live in extremely harsh conditions are called extremophiles (Obulisamy et al., 2021). Considering the places where extremophilic organisms live, high temperatures, salt concentration, pressure, osmotic compound content, heavy metal content, radiation, acidic or basic pH, extreme dryness, and extreme cold can be given as examples (von Hegner et al., 2020).

Extremophiles are classified according to their environment: psychrophiles (cold habitats), thermophiles and hyperthermophiles (hot and very hot habitats, respectively), alkaliphiles (alkaline habitats at high pH), acidophiles (acidic habitats at low pH), barophiles (high-pressure habitats), halophiles (hypersaline habitats with high salt concentration), toxic-tolerant (habitats with toxic substances, chemicals, and heavy metals) and radiophiles (high radiation habitats) (Table 1.3). It is also known that most of these creatures are polyextremophiles which have evolved to exist in environments with extreme values of many physicochemical parameters (Dumorné et al., 2017).

**Table 1. 3**Extremophiles and their living conditions (Merino et al., 2019).

| Nomenclature | Extreme Environment |
|---|---|
| **Acidophiles** | Low-pH microorganisms (pH 3.0-4.0) |
| **Alkaliphiles** | High-pH microorganisms (≥ pH 10.0) |
| **Halophiles** | High salt concentration microorganisms (≥ 1M salt) |
| **Hyperthermophiles** | High temperature microorganisms (≥ 80 °C) |
| **Thermophiles** | High temperature microorganisms (65 °C-80 °C) |
| **Psychrophiles** | Low temperature microorganisms (<10 °C and 20 °C≤) |
| **Piezophiles** | High pressure microorganisms (≥ 40 MPa) |
| **Metalotolerant** | High concentration of heavy metals microorganisms (arsenic, zinc, copper) |
| **Xerophiles** | Low water activity and resistant to high desiccation microorganisms |
| **Radioresistant** | High levels of ionizing radiation microorganisms |
| **Oligotroph** | Nutritionally deplete microorganisms |
| **Toxitolerant** | High levels of damaging agents microorganisms (organic solvents) |

Due to their biodegradability and high stability, extremozymes, which are biomolecules derived from extremophilic organisms, have come to the fore in the usage of industrial applications. Extremozymes offer enormous promise in various industrial processes because of their tolerance to extreme conditions and high activity while retaining viable, such as food, agriculture, medicine, pharmaceuticals, textiles, paper and pulp, detergents, and leather (Figure 1.3). With the advancement of biotechnology, extremozymes and secondary metabolites derived from extremophiles have played a significant role in fulfilling the industry's rising need for the discovery of novel biomolecules. As a result, biomolecules derived from extremophiles have industrial significance (Jourquera et al., 2019).

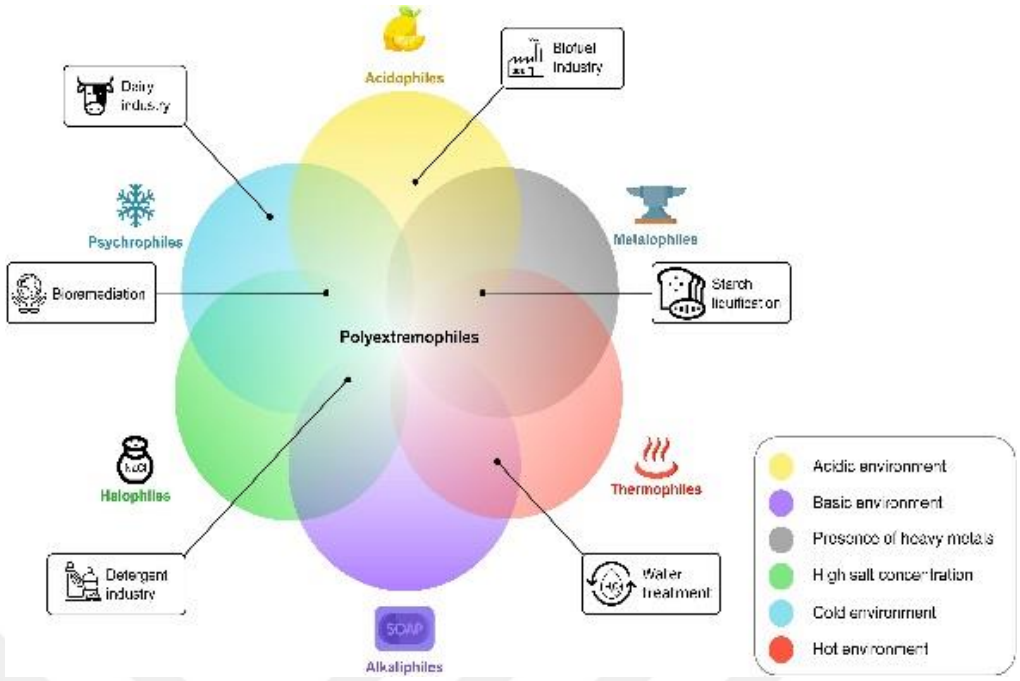


**Figure 1. 3** Extremophiles and applications (Syosoev et al., 2021).

## 1.4 Antarctica

Antarctica, the world's fifth-largest continent, is found in the southern region and includes the South Pole (Waat et al., 2021). Antarctica, which has never been touched by humankind, possesses 70% of its aquatic resources in the form of glaciers. Severe storms, incredibly low temperatures, and sensitivity to low UV radiation in the winter and severe UV radiation in the summer define this region (Cowan et al., 2004). The coldest surface temperature in Antarctica, the world's coldest, driest, and windiest continent, was measured at -89°C, and its winds were found to be stronger than hurricanes. The Antarctic continent is classified as a desert because ice covers 99% of its surface area (Figure 1.4). Aside from these characteristics, the continent plays a significant role in determining climate change and the climate problem. The melting of glaciers on the continent as a result of global warming alters the climate and poses a serious threat to humankind. As a result of these occurrences, it has developed a unique and natural environment (David et al., 2013).

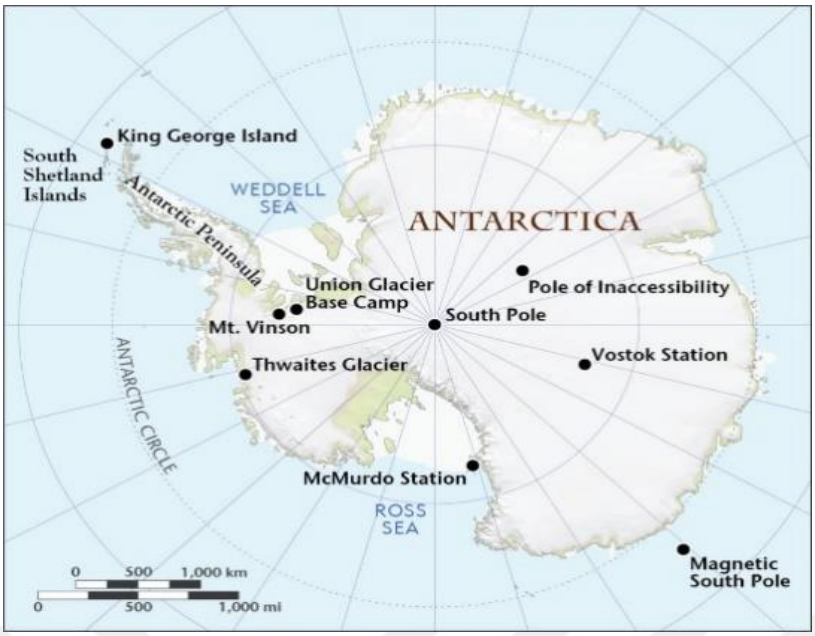


**Figure 1. 4** Continent of Antarctica (URL1).

The Antarctic Peninsula, which has the world's most difficult conditions, is recognized to have a high level of biodiversity. Vertebrates that live on the continent include seals, penguins, whales, and indigenous bird species. In addition, every year, the biological variety of the marine ecosystem is noted to rise. Furthermore, microbial colonies, which make up a large portion of the biomass that makes up the continent, are among the creatures that have survived the extreme environment. Microorganisms play an important role in the functioning of the marine food web and biogeochemical cycles on the continent. In addition to microbial variety, new biochemical pathways, biomolecules, and genes are predicted to be found in the field of biotechnology as a result of data gathered from species living in the region (David et al., 2013).

The Antarctic Peninsula, which has the highest elevation and the coldest temperature in the world, has recently become a popular study destination. Due to the harsh conditions, the discovery of numerous enzymes and genes for research, as well as the discovery of new species such as psychrophiles and polyextremophiles living on this continent, is inevitable. Biotechnological research is also encouraging the discovery of previously unknown Antarctic species and biomolecules (David et al., 2013).

King George Island is the biggest of the South Shetland Islands, separated by the Bransfield Strait near the northernmost extremity of the Antarctic Peninsula (Rakusa et al., 2002). The island has the mildest climate in the region, with low wind speeds, high temperatures, and significant precipitation, making it excellent for study.

Maxwell Bay, Admiralty Bay, and King George Bay are the three bays of King George Island. Admiralty Bay, with its gulfs and fjords, typifies the physiological organization of the South Shetland Islands. Admiralty Bay is the mildest bay on King George Island (Rakusa et al., 2002). The freezing and thawing cycle occurs on a regularly, providing chemicals in the water to be transported to the soil, particularly during the summer months. The biological activity in the flora, soil, and area can be identified as a result of organic molecules dissolved in the environment (Roser et al., 1993). For all these reasons, Admiralty Bay on King George Island is a prominent venue for scientists.

### 1.5 Chitin

The chitin molecule $(C_8H_{13}O_5N)_n$, discovered in fungus by Henri Braconnot in 1811, is a biopolymer. It is the second most prevalent polysaccharide in nature after cellulose and has an insoluble structure. It is a nitrogen-containing inelastic, rigid, white polysaccharide generated by the bonding of N-Acetyl-D-glucosamine monomers (Glc-NAc) with β-1,4 glycosidic linkages. Chitin is present in the cell walls of fungus, algae, yeast, insects, shrimp, octopuses, crabs, and lobsters and provides these species with resilience to external conditions. Chitin, which is employed as a nitrogen and carbon source, may also be considered a significant food supply for microbial life.

The synthesis of chitin is catalyzed by the enzyme chitin synthase, which uses UDP-N-acetylglucosamine monomers as the substrate. This enzyme is active only in the presence of divalent cations such as $Mg^{+2}$, $Mn^{+2}$ and $Ca^{+2}$ (Merzendorfer et al., 2003). The determination of its activity enzymatically, was suggested by Candy and Kilby in 1962, that the formation of chitin in glucose began through biosynthesis and was supported by radiolabeled precursor studies. Genetic and biochemical studies of chitin biosynthesis in insects have been completed (Merzendorfer et al., 2003). Chitin forms biochemically via the hexosamine pathway (Figure 1.5). In the hexosamine pathway, fructose-6-phosphate is converted to UDP-N-acetylglucosamine, and UDP-N-acetylglucosamine is polymerized to form chitin by the chitin synthase enzyme. Glucosamine-6-phosphate N-acetyltransferase (GNA) [EC: 2.3.1.4] is one of the enzymes in the hexosamine pathway and the transfer of acetyl groups taken from Acetyl-CoA to the first amine group of glucosamine-6-phosphate is also carried out (Kramer et al., 1986).

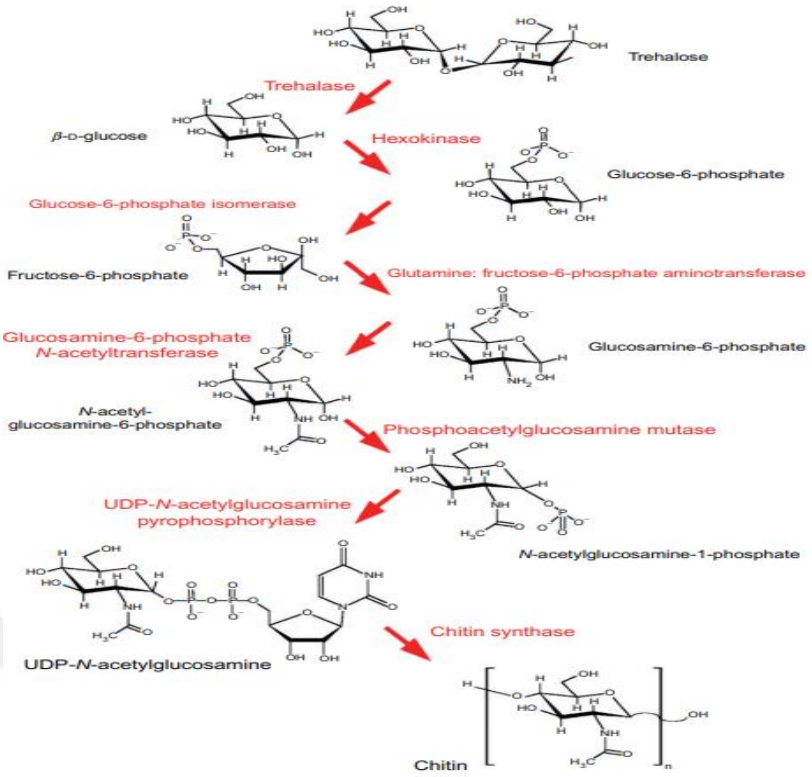


**Figure 1. 5** Biosynthesis of chitin molecule (Daraghmeh et al., 2011).

When the crystal structure of chitin is considered, it has been observed that there are 3 forms of chitin in nature: α-chitin (mostly), β-chitin and γ-chitin (Figure 1.6). It is known that these forms are caused by the polarity properties of the chitin molecule and the way it is packaged. When the structure of α-chitin is examined, it appears to be the most common form of chitin in nature. It is known to be insoluble and does not become swollen in a solvent. Since the (Glc-NAc) chains are located in antiparallel form, they are strongly packed. α-chitin is generally found in shrimp shells, cell walls of fungi, tendons, and shells of shrimp and crabs. β-chitin, which has weak intermolecular bonds, has a less stable structure than α-chitin. It can transform into the α-chitin form by dissolving and swelling in the solvent, but not the other way around. It is found in cuttlefish, some worm species, and seaweed. The γ-chitin form, which is a mixture of parallel and anti-parallel (Glc-NAc) chains, has both α and β-chitin structures. The γ-chitin form is found in the cocoons of insects (Rinaudo et al., 2006).

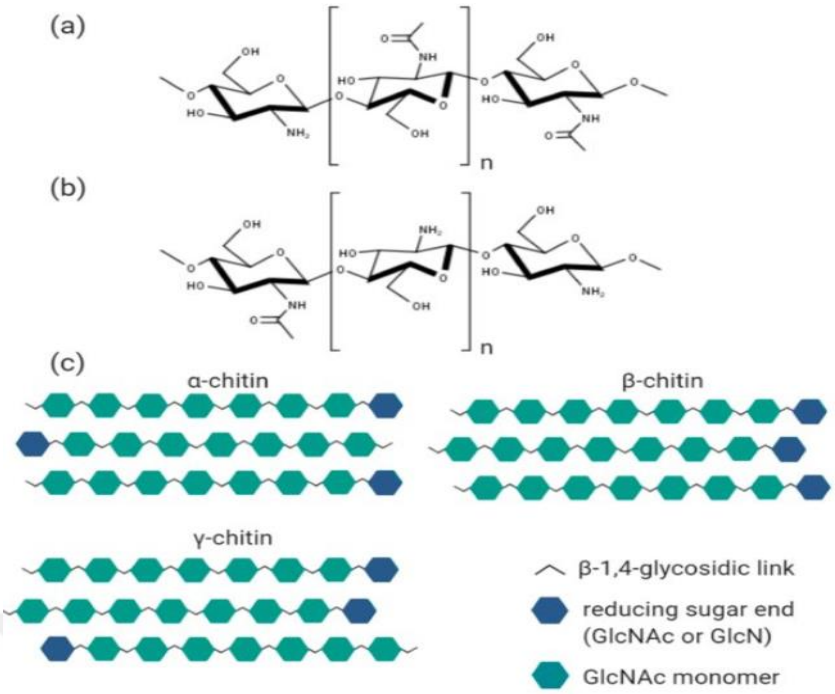


**Figure 1. 6** Crystal structure of chitin (Arnold et al., 2020).

### 1.5.1 Application of chitin and chitosan

Chitin is found in the outer shells of creatures such as insects, crabs, and shrimp. Chitosan, a biopolymer with a polycationic feature, is formed by partially or completely deacetylating chitin, which is formed by the combination of N-acetyl glucose amine monomers in an alkaline medium (Peter et al., 2021). Chitosans are also advantageous because they are non-toxic, biocompatible, and biodegradable like chitin. Chitosans, like chitin, can be used in a variety of industries, including pharmacy, agriculture, food, cosmetics, textiles, and agriculture (Islam et al., 2017).

Since 1980, chitosans have been used, especially in agriculture, as biopesticides, biofertilizers, and coating materials for seeds. Also, their use in pharmaceutical, medical, and cosmetic fields is supported due to their antifungal, antiviral, and antibacterial properties. Considering the antiviral and immune system-supporting effects of chitin and chitosan products in the recent COVID-19 pandemic, research has also been carried out in the field of health and pharmacy against COVID-19It has been observed that their use in the clinic against COVID-19 is promising (Safarzadeh et al., 2021). Chitin and chitosan products worth $2,900 million increased by 14.8% in the global market in 2017. By 2024, this growth is expected to be $63 billion (Joseph et al., 2021).

Chitin chitosan products are used for different purposes in the industrial field. Some of the areas it`s used are the biomedical, textile, food, and pharmaceutical industries (Figure 1.7). The purposes of use in these industries can be listed as follows:


**Figure 1. 7** Chitin and chitosan applications (Joseph et al., 2021).

Chitin and chitosans have biomedical uses such as the development of coagulation by allowing platelets to be active (Wang et al., 2018), being used as a soft tissue adhesive by generating a hydrogel and being used as a wound dressing for wound healing by impregnating fibroblast growth factors on chitosan films (Rasweefali et al., 2022). Because of their polycationic, non-toxic, and biodegradable qualities, chitin and chitosans can be utilized in wastewater treatment (No et al., 2000). Many wastewater contaminants, such as magnetite, bentonite, and palm oil ash, can be absorbed by chitosan. It is also used to remove industrial wastewater of colors, pesticides, oil, and oil contaminants (Nejhati et al., 2017).

Chitosans show antimicrobial properties against fungi, yeast, and mold bacteria due to the cationic groups they contain (Friedman et al., 2010). In addition to their features such as biodegradability and non-toxicity, they are preferred in the industry because they are cheap and easily available (Barikani et al., 2014). It has the feature of forming a film with membranes that are semi-permeable to gases and coating materials with phased properties. These formed films can be used for coating foodstuffs such as vegetables, fruits, and meats (Aider et al., 2010).

Since synthetic textile fibers used in the textile business are non-biodegradable, the damage they cause to the environment increased every year (Zhu et al., 2019). As a result, other options have been researched. Natural biopolymers such as chitin, chitosan, and cellulose have recently gained prominence in the textile industry (Duan et al., 2018). These goods have grown in popularity as a result of their applications in

sectors such as odour control, antibacterial capabilities, and maintaining sanitary conditions (Kenawy et al., 2007).

### 1.6 Chitinase

Enzymes carry out metabolic processes because of their specificity, speed, and efficiency. Enzymes are classified into seven families by the EC. The EC3 hydrolase family plays a vital role in industry. The chitinase enzyme, which belongs to the hydrolase family, is involved in the breakdown of β-1-4 glycosidic linkages in chitin and separates the N-Acetyl Glucosamine polymers that constitute chitin (Oyelele et al., 2018). Chitinases are essential for chitin metabolism and environmental nutrient cycling. Chitinases can generally be used for plant diseases and insect control in agriculture (Patil et al., 2000), for the production of chitooligosaccharides (COS) in food and medicine (Yang et al., 2016), for the removal of chitin wastes in bioremediation, and also for biofuel production (Ramirez et al., 2016). Chitinase enzyme is found in several different genera, including *Aeromonas, Arthrobacter, Bacillus, Chromobacterium, Flavobacterium, Pseudomonas, Sanguibacter, Serratia,* and *Streptomyces* (Santa-cruz et al., 2021).

According to the Food and Agriculture Organization (FAO, 2019), 10.5 million chitin-rich shellfish (12.3 percent) are farmed in aquaculture (FAO, 2021). The need for converting chitin wastes, which are rising in quantity by the day into biologically useful products is becoming apparent (Hamed et al., 2016). In recent years, the use of biocatalysts (Kumar et al., 2018) instead of chemical methods, which are hazardous to the environment and expensive, in the removal of chitin wastes has allowed for a safe conversion to the environment.

Chitinases are classified into six classes (Class I-IV) based on their gene sequences, two groups (endo-exo chitinase) based on their hydrolysis capabilities, and three families (GH18, GH19, GH20) based on their amino acid sequences (Beier et al., 2013, Javed et al., 2013).

Endo-chitinase and exo-chitinase are the two main groups, distinguished by their hydrolysis characteristics. Endo-chitinases cleave β-1–4 glycosidic linkages from the inner regions to generate glucosamine (GlnAc) oligomers such as chitotetrose, chitotriose, and chitobiose. Exo-chitinases, on the other hand, are divided into two

subgroups: chitobiosidases and β-1,4-N-acetylglucosaminidases. Chitobiosidases generate di-acetylchitobiose by acting on the non-reducing end of chitin. On the other hand, 1,4-N-acetylglucosaminidases are enzymes that convert oligomeric di-acetylchitobiose, chitotriose, and chitotetroses into N-acetylglucosamine (GlcNAc) monomers (Figure 1.8) (Singh et al., 2021).

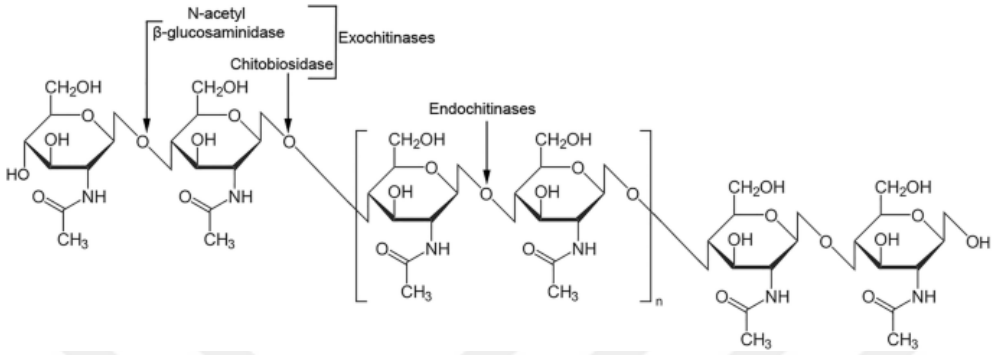


**Figure 1. 8** Catalytic mechanism of endochitinase / Exochitinase (Mathew et al., 2020).

Chitinolytic enzymes are classified according to the CAZY database (Lombard et al., 2013). Chitinases are classified as Glycosyl Hydrolase (GH) 18,19,20 families. As a result of biochemical analysis, it has been observed that these families, which differ in their catalytic activities, amino acid sequences, and therefore their three-dimensional structures, have chitinolytic enzyme properties (Oyeleye et al., 2018). The GH18 and GH19 families are considered chitinases because they catalyze the degradation of chitin polymers (Martinez- Zavala et al., 2020). The GH20 family includes chitobiose and β-N-acetylhexosaminidase enzymes that cleave terminal N-acetylglucosamine, glucosamine or chitobiose dimers from glycoconjugates (Vaaje-Kolstad et al., 2013) (Figure 1.9).

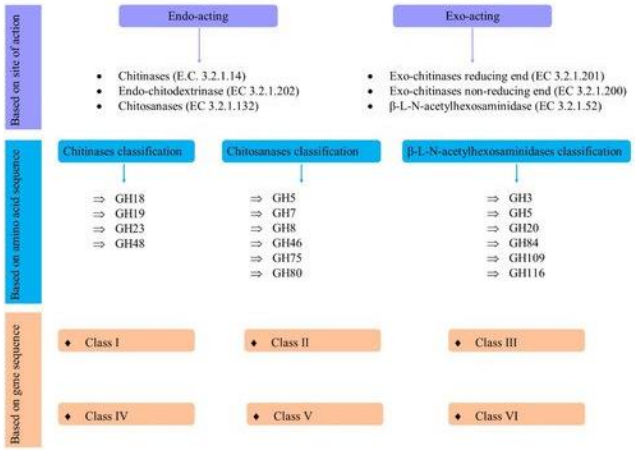


**Figure 1. 9** Classification of chitinase (Poria et al., 2021).

Chitinases of the GH18 family are enzymes with eight α-helices, eight β-strands, and eight TIM-barrel domains in their secondary structure (Khan et al., 2015). Chitinases belonging to the GH18 family are commonly found in plants, bacteria, fungi, viruses, animals, and insects. It plays an active role in physiological processes such as tissue deterioration and the defense of immunity (Huang et al., 2011). Although GH18 chitinases are used to break down endogenous chitin in chitin-containing organisms, most microorganisms can produce chitinases to use exogenous chitin. Recently, studies on the phenotypic and biochemical properties of the chitinase GH18 family have increased due to the increasing need for specific biomolecules, especially in the fields of medicine and agriculture (Chen et al., 2020).

GH18 family of bacterial chitinases has 3 different subgroups (Figure 1.10). These subgroups are named A, B and C. Subgroup A is separated from groups B and C by the α+β insertion domain it contains (Li et al., 2010). Since the subgroups are named according to CAZY, they are not involved in the naming of individual enzymes. Chitinases with different nomenclature may be included in different subgroups. The diversity of chitinases' domains determines which subgroup they belong to (Chen et al., 2020). In addition to the catalytic domain, the GH18 subfamily contains fibronectin type III-like (FnIII) and carbohydrate-binding module (CBM) domains (Larsbrink et al., 2016).

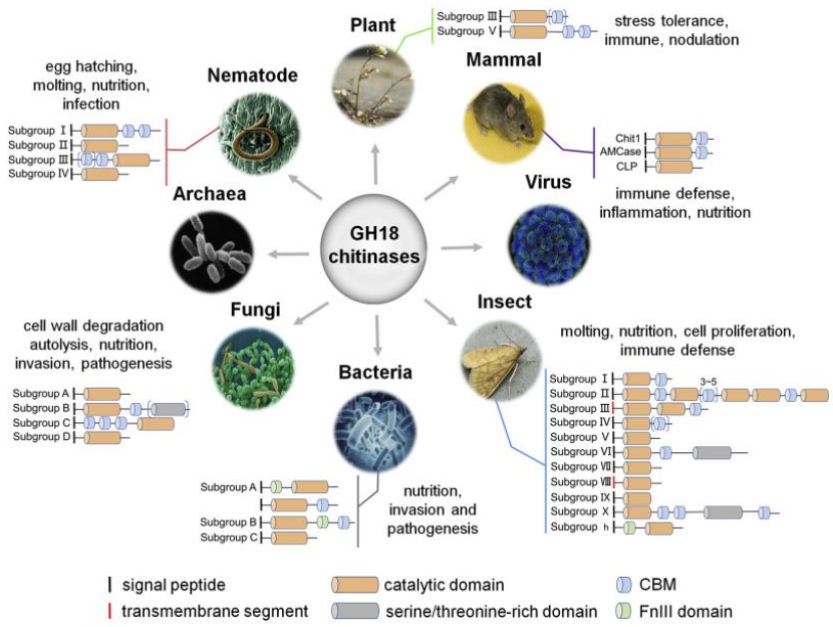


**Figure 1. 10** GH18 family chitinase origins, structure, and domains (Chen et al., 2020).

In general, GH18 chitinases serve three purposes. Their primary function is to produce chitinase for digestion when feeding on or hosting chitin-containing species. The second function is produced during the growth of creatures with a tough chitin layer, as well as during the chitin layer's disintegration, and reconstruction. The third function is that hosts that are infected with chitin-containing organisms create chitinase, which damages the chitin layers of these pathogens. In addition to these qualities, it is known that chitinases are employed in bacteria for nutrition and pathogen protection, fungi for cell wall fragmentation, autolysis, nutrition and pathogen protection, and mammals for immunological defense, inflammation, and nutrition (Chen et al., 2020).

Chitinase enzymes from the GH19 family are found in plants, several viruses, and a few fungal and bacterial species (Han et al., 2015). It has also been detected in non-green sulphur and purple bacteria, as well as actinobacteria, either directly or indirectly via horizontal gene transfer, according to the literature (Udaya et al., 2010). The GH19 family of chitinases is thought to be a part of the defensive system against fungal infections (Ohnuma et al., 2011).

Other than arabinose, glucose, pentose, and hexose sugars, as well as +2 valence cations including $Hg^{+2}$, $Cu^{+2}$, $Co^{+2}$, and $Mn^{+2}$, act as chitin inhibitors (Saito et al., 1998, Xiayun et al., 2012). Activators were identified as cations such as $Mg^{2+}$, $Ba^{2+}$, and $Ca^{2+}$ in the literature (Xiayun et al., 2012, Veliz et al., 2017).

### 1.6.1 Chitinase applications

Chitinases have a variety of industrial uses, including biocontrol agents, the synthesis of single cell proteins and chitooligosaccharide (COS), biomedical applications, as well as waste management.

#### 1.6.1.1 Phytopathogenic fungal control and plant defense

Diseases produced by the fungus, bacteria, and insects on crops are one of the leading causes of crop loss in agricultural areas. Fungal diseases, in particular, are the primary cause of plant disease. Pathogenic fungi with chitin polymer in their cell walls include *Fusarium oxysporum, Fusarium graminearum, Puccinia spp., Botrytis cincerea, Ustilago maydis, Magnaporthe oryzae,* and *Mycosphaerella graminicola* (Burgess et al., 2009). The antifungal activity of chitinase can be used to suppress the germination of fungal spores and mycelial growth. Plants can also acquire tolerance to abiotic stress

factors, which are environmental stress factors (Nagpure et al., 2014). Furthermore, COS molecules generated by chitinases improve plant defences against phytopathogens (Khan & Bisetty et al., 2015). In addition, pathogenic insects have chitin in their exoskeletons to protect them from harsh environmental conditions. Chitinases can be used to combat phytopathogenic fungi and insects in agriculture. According to certain research, chitinases, which enhance the mortality of insects during their larval and pupal phases, can be utilized in biological control (Patil et al., 2015).

#### 1.6.1.2 Food preservation

In food preservation, chemical procedures or bioprotective compounds can be used. Biomolecules derived from microorganisms are favoured as preservatives because they are biocompatible, cost-effective, and can be supplied quickly (Sharma et al., 2009). Chitinase protein, which has many diverse characteristics and is derived from microorganisms, has been proven in studies to have antifungal effects against fungal diseases (Castillo et al., 2016; Le et al., 2018; Deng et al., 2019).

Chitinases are beneficial in food storage because they inhibit the growth of fungal spores, break cell walls, and so prevent food spoilage. Chitinases are considered as promising biomolecules which are used in food preservation (Goma et al., 2021).

#### 1.6.1.3 Production of single cell proteins

Chitinases and chitins are used to make SCPs, which are a source of high-value proteins known as single-cell protein (SCP). SCPs may be produced from fish flesh and soybean meal, respectively. Some scientific research has indicated that the SCPs formed by chitin and chitinases have a relatively high protein valence (Patil et al., 2014, Le et al., 2019).

#### 1.6.1.4 Medical applications

Chitinases are biomolecules that are becoming more prominent in medicine. The conjugation of chitinase with different components or microorganisms has enhanced its antibacterial activity. Chitinases have been employed in the treatment of fungal infections, with positive outcomes (Allonsius et al., 2010). Furthermore, chitinases are employed in the majority of ophthalmic solutions because of their antibacterial, antifungal, and antiviral characteristics (Halder et al., 2019). Chitinases, which are

expected to be utilized in cancer treatment, have been tested on cancerous mice, and it has been shown that their cytotoxic impact destroys cancer cells (Abu-Tahon et al., 2019). More research is currently required before all these findings can be applied to human therapy. It has been discovered that the utilization of chitinases, particularly in allergic inflammations such as asthma, activates macrophages and provides a response to allergen threats (Wu et al., 2010).

#### 1.6.1.5 Biotechnological applications

The biotechnological importance of chitinases stems from their capacity to convert chitin into chito-oligomers, the compounds formed during chitin degradation. One of the most significant products is chitooligomers (COS), which are created as a consequence of the catalytic mechanism of chitinase (Fu et al., 2020). COSs are created by decreasing the molecular weight of chitin and chitosans after they have been degraded. COSs can be produced using a variety of chemical and enzymatic processes. Because of its ecologically favorable features, the usage of the chitinase enzyme, one of the enzymes employed in enzymatic procedures, has recently gained popularity (Gomaa et al., 2021).

COSs have been identified as potential prebiotics and valuable components such as short-chain fatty acids. They are produced after COSs are broken down by some beneficial bacteria in the human gut (Selenius et al., 2018). COS was added to yoghurt, milk, and various fruit juices in one study, and the status of probiotics in the colon was assessed. An increase in the population of probiotics such as *Lactobacillus spp.* and *Bifidobacterium spp.* was seen towards the conclusion of the trial (Kong et al., 2014).

Research on the use of COS in agricultural production has also been considerable (Liang et al., 2018). Based on the data acquired, it has been shown that they are employed in agriculture not only for pathogen protection but also to increase the development of plants under stress. For example, when COS was applied to the leaf sections of *Brassica rapa*, it was discovered that the leaves' cadmium tolerance rose and plant development improved (Zong et al., 2017).

#### 1.6.1.6 Waste Management

Chitin wastes generated by seafood consumption are increasing daily. Chitinase enzymes assist in the conversion of chitin wastes into industrially valuable chemicals (Kidibule et al., 2018). These substances have an important role in medicine,

agriculture, food, and medicine. It is generated in single-cell proteins, which are employed as nitrogen and carbon sources in biomass, following chitinase treatment with chitin (Dukariya et al., 2020). Various chitinase enzymes have been utilized in investigations on the breakdown of chitin wastes, and it has been reported that chitin waste is destroyed and biologically transformed into N-acetyl-D-glucosamine (Kumar et al., 2018; Das et al., 2019; Liu et al., 2020).

### 1.7 *Pseudomonas* Species

*Pseudomonas* species have been known since their discovery in 1894 (Migula, 1894). *Pseudomonas*, which is increasing in number with each day, currently has 254 identified genera (Gupta et al., 2020). Considering its morphological features, it is gram-negative, rod-shaped, catalase-active, and non-spore-forming. Because it has one or more peritrichous and polar campers, it is a motile bacterium (Pavlov et al., 2020). *Pseudomonas* may be found in a variety of habitats including marine environments, mammalian tissues, and plant rhizospheres (Silby et al., 2011).

They have improved adaptability and survival abilities because they can utilize organic substances as energy sources, withstand various sorts of injury, and produce a wide range of metabolites against other microorganisms (Moradali et al., 2017). *Pseudomonas* (proteobacteria) is divided into two species: *P. aeruginosa* and *P. fluorescens*. Within the first cluster, there are six groups. *Pseudomonas fluorescens* group (18 species), *Pseudomonas syringae* group (12 species), *Pseudomonas putida* group (12 species), *Pseudomonas aeruginosa* group (11 species), *Pseudomonas chlororaphis* group (5 species), and *Pseudomonas stutzeri* group (3 species). *Pseudomonas pertucinogena* (2 species) is the only member of the second group (Reddy et al., 2004).

The capacity of *Pseudomonas* species to endure various stress conditions is one of its most notable characteristics. There are an abundance of *Pseudomonas* psychrotolerant and psychrophilic strains that have been isolated from Antarctic and Arctic regions. (Kristoffersen et al., 2018). According to the microbial diversity analyses conducted in the region, *Pseudomonas* spp. was shown to be the predominant species (Romaniuk et al., 2019). Some of the *Pseudomonas* species identified in Antarctica have been classified as *Pseudoalteromonas arctica, Pseudoalteromonas haloplanktis sp., Pseudoalteromonas sp., Pseudomonas antarctica, Pseudomonas grimontii,*

*Pseudomonas jessenii, Pseudomonas lini, Pseudomonas meridiana, Pseudomonas psychrophile, Pseudomonas trivialis, Pseudomonas mandelii, Pseudomonas brenneri, Pseudomonas fildesensis, Pseudomonas fragi, and Pseudomonas frederiksbergensis* in the literature (Núñez-Montero et al., 2018; Silva, et al., 2018; Lo et al., 2020; Pavlov et al., 2020).

### 1.7.1 *Pseudomonas mandelii*

*Pseudomonas mandelii* is a fluorescent gram-negative bacterium that can survive at temperatures as low as 4 °C but not at 37 °C, can produce extracellular enzymes and has non-halophilic properties (Jang et al., 2012). This species relates to the *Pseudomonas fluorescence* family photogenically. It is prevalent in agricultural regions and mineral springs (Anzai et al., 2000). According to recent research, many various strain of *Pseudomonas mandelii* are situated on the Antarctic continent. *P.mandelii* PD30, *P.mandelii* JR1, *P.mandelii* B2/38-Q-III, *P.mandelii* YF10, *P.mandelii* 6A1, *P.mandelii* LMG 21607, *P.mandelii* 36MFCvi1.1, *P.mandelii* NBRC 103147 were currently defined *P.mandelii* strains (https://www.ncbi.nlm.nih.gov/data-hub/taxonomy/75612/). Numerous research is now being conducted on *P. mandelii* to conduct phenotypic and genetic investigations.

For example, in investigations on the JR1 strain, genome announcement was performed initially, followed by recombinant production, purification, and characterization of NAD-dependent sorbitol dehydrogenase, esterase, and lipase enzymes, all of which are regarded to be biotechnologically essential (Hong et al., 2012; Jang et al., 2012; Justung et al., 2013; Dang Thu et al., 2021).

The effects of pH and temperature on denitrification enzymes were studied in research on the PD30 strain after genome announcement, (Formusa et al., 2014) and it was discovered that denitrification reduced at pH 5.0 and was affected by temperature (Saleh-Lakha et al., 2009).

The synthesis of polyhydroxy butyrate (PHB) by the CBS1 strain at low temperatures was examined. As a consequence, it has been shown that PHB generated by CBS1 is of significant industrial value when compared to PHB produced by other strains (Li et al., 2013).

The formation of silver nanoparticles (AgNPs) at low temperatures was investigated in research using the psychrotolerant SR1 strain. So, it was discovered that the

synthesis of AgNP at low temperatures reduced as the exopolysaccharide (EPS) concentration increased (Mageswari et al., 2015).

The best investigated strain of *Pseudomonas mandelii* in terms of genomic and phenotypic characteristics is 6A1. The bacteria were first discovered from marine sediment samples collected in Fildes Bay, Antarctica, using 16S rRNA sequencing and multilocus sequence analysis (MLSA) (Vásquez-Ponceetal et al., 2018). The capacity to produce biofilms using alginate synthesized at low temperatures was examined next, and it was shown that increasing the alginate produced at low temperatures improved the ability to form biofilms (Vásquez-Ponce et al., 2017). Finally, the carbon sources used, and morphological parameters of the strain were established to perform phenotypic characterization (Higuera-Llantén et al., 2018). As the first known isolate of *Pseudomonas mandelii* from Antarctica, this particular strain was published in the scientific literature (Vásquez-Ponce et al., 2018).

### 1.8 Aim of The Study

Antarctica is the last discovered area in the world due to its extreme conditions, and it is an independent region with a scientific study base. In line with the studies carried out to understand the life cycle in the region, many species and biomolecules have been discovered. The studies not only provide an opportunity to understand the life of the region, but also to illuminate the unknown evolutionary process on earth. The majority of the studies accomplished today are the discovery of new species and biomolecules. Each discovered biomolecule has a distinctive industrial and biotechnological importance.

Within the scope of this study, de novo whole genome analysis was performed by identifying a new strain of *Pseudomonas mandelii*, *Pseudomonas mandelii* KGI_MA19, from sediment samples collected during the 2nd National Antarctic Science Expedition (TAE-2). The phenotypic and biochemical properties of the organism were characterized. According to the analysis performed in the Protein Data Bank tool, it was determined that the industrially important cold adaptive chitinase enzyme of the KGI_MA19 strain showed 84.7% similarity to the chitinase enzyme of the *Pseudomonas* sp BG5 strain. Specific primers have been designed suitable for the recombinant production of the enzyme. Finally, the psychrophilic chitinase enzyme was purified and biochemical characterization was performed.

# 2. MATERIAL AND METHODS

## 2.1 Material

### 2.1.1 Laboratory equipment

The equipment used in this study are given in Appendix A.

### 2.1.2 Culture medium

The compositions of culture medium are given in Appendix B.

### 2.1.3 Buffer, gel and solution

The compositions of buffers, gels and solutions are given in Appendix C.

### 2.1.4 Chemicals, enzymes and kits

The chemicals, enzymes and kits used in the experiments are given in Appendix D.

### 2.1.5 Bacterial strains and vectors

In the host *Escherichia coli* BL21 (DE3), the pET-28a (+) bacterial expression vector (Figure 2.1) was employed to obtain recombinant chitinase enzyme. The pET-28a (+) vector has a length of 5369 base pairs. T7 lac promoter, thrombin cleavage site, internal T7 epitope tag, and restriction enzyme cleavage sites for *Hind*III, *Not*I, *Xho*I, *Sal*I, *EcoR*I, *Sac*I, *BamH*I, *Nde*I, *Nhe*I, *EcoR*V, *Nco*I, and *Bmt*I are all included in the vector. In addition, the pET-28a (+) vector has a lac operon, N- and C-terminal Histidine tags for protein purification, and a kanamycin resistance gene ($Kan^R$).

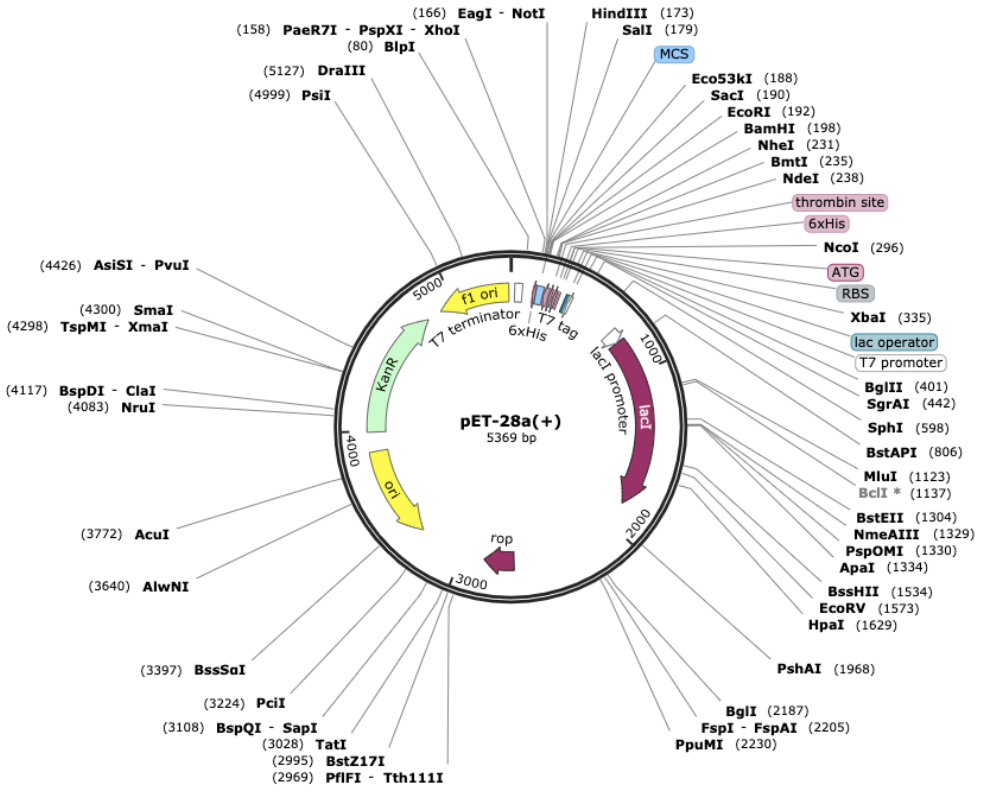


**Figure 2. 1** Genomic map of pET-28a (+) expression vector.

## 2.2 Methods

### 2.2.1 Identification of *Pseudomonas mandelii* KGI_MA19

#### 2.2.1.1 Obtaining environmental samples

In this study, Prof Dr Nurgül Çelik Balcı kindly shared 12 different sediment and soil samples collected from certain regions of the Antarctic continent as part of the 2nd National Antarctic Science Expedition (TAE-2).

#### 2.2.1.2 Enrichment culture

Twelve samples taken from 8 different regions of King George Island were named as 18, 19, 20, 21, 22, 24, 25, 26, 27, 29, 30, 31 and each sediment sample was incubated in 15 ml TGY medium at 4 °C for 96 h with shaking at 270 rpm. At the end of the incubation, the samples were centrifuged at 3000 *xg* for 20 min at 4 °C to remove the sediment parts. DNA isolation was performed from the supernatant obtained at the end of centrifugation by applying the Gram-positive DNA isolation protocol of the GeneJET Genomic DNA Purification Kit (Thermo Scientific™). The result of DNA isolation was checked on a 0.7 % agarose gel under 120V for 20 min. In order to

determine the microbial diversity of each location, samples 18, 20, 21, 24, 26, 27 and 30 were sent for 16s rDNA analysis.16S rRNA analysis from the obtained DNAs was performed by Sanger Sequencing. (Genometry, Izmir, Turkey). A stock of 15 % glycerol was prepared at -80 °C for each sample. Finally, both 12 sediment samples were inoculated onto TGY agar medium and incubated at +4 °C for 96 h, and for contamination control, they were inoculated into TGY medium and incubated at 37 °C for 18 h.

**Agarose gel electrophoresis**

Firstly, 0.28 g agarose was weighed for gel preparation, combined with 40 mL of 1X TAE buffer, then agarose melted and boiled in a microwave oven until a homogenous solution was created. The solution was then put into the electrophoresis tank, followed by the comb. At room temperature, agarose gel was incubated until it hardened. After adding 1X TAE buffer to the tank, the comb was eliminated.

For UV visulation, 5 L of each genomik DNA was combined with 1 L of 6X DNA loading dye (EZ vision, #N472-1ML). As a marker, GeneRuler 1 kb Plus (Thermo Scientific, #SM1331) was employed. The gel was visualized under UV light after running it for 20 minutes at 120 volts with 1X TAE buffer. The 16S rRNA analysis of the obtained DNA samples was performed by Sanger Sequencing (Genometry, Izmir, Turkey). For each sample, a 15% glycerol stock was prepared at -80 °C.

**2.2.1.3 Freeze-thaw stress**

Twelve different samples incubated at 15 °C for 18 h were exposed to freeze-thaw stress at -20 °C and 4 °C for 96 h. After stress, the cells were precipitated at 9000*xg* for 30 min at 4°C. The pellet was dissolved in 250 µl of TGY medium and incubated on TGY agar at 4°C. After incubation, 10 colonies, one large and one small, from 5 sediment samples resistant to freeze-thaw stress, were selected according to their morphological differences. Selected colonies were incubated in 3 ml of TGY medium for 96 h at 4 °C. At the end of the incubation, the culture was precipitated at 9000*xg* for 30 min at 4 °C and DNA isolation was performed by application of the Gram-positive DNA isolation protocol of the GeneJET Genomic DNA Purification Kit (Thermo Scientific™). The result of DNA isolation was checked on a 0.7 % agarose gel under 120V, 20 min conditions. The 16S rRNA analysis of the obtained DNA

samples was performed by Sanger Sequencing (Genometry, Izmir, Turkey). For each sample, a 15 % glycerol stock was prepared for the storage at -80 °C.

#### 2.2.1.4 Whole genome analysis

Among the samples, 1 µl was taken from the stock at -80℃ of sample 19/2, inoculated on TGY agar and incubated at 4 ℃ for 96 h. At the end of 4 days of incubation, it was incubated again in 3ml of FDY medium at +4 ℃ for 96 h with shaking at 270 rpm. At the end of the incubation, the culture was centrifuged at 1300 *xg* for 5 min and DNA isolation was performed by applying the GeneJET Genomic DNA Purification Kit (Thermo Scientific™) gram positive DNA isolation protocol. The result of DNA isolation was checked on a 0.7% agarose gel under 120V, 20 min conditions. The amount of DNA obtained was measured by nanodrop. Samples with sufficient amount of DNA were sent for de novo whole genome analysis.

### 2.2.2 Characterization of *Pseudomonas mandelii* KGI_MA19

#### 2.2.2.1 Phenotypic characterization

**Growth curve**

**Determination of optimum temperature**

15 µl of bacterial sample from sample 19/2 with glycerol stock at -80 °C was inoculated into 5 ml of TGY fresh medium and incubated at 18 °C for 16 h with shaking 200 rpm to prepare a pre-culture. Cells were transferred from the preculture to 100ml fresh TGY medium ($OD_{600}$: 0.1) and incubated at 200 rpm for 24 h at six different temperatures (4 °C, 10 °C, 15 °C, 20 °C, 30 °C). Cell density was measured at 600 nm every three hours, and the measurement was taken for 24 h and then the growth curve was plotted.

**Determination of optimal pH and optimum salinity**

A pre-culture was prepared by incubating at 18 °C for 16 hours and 200 rpm. Preculture was transferred to 15 ml of TGY medium ($OD_{600}$:0.1) with different pH values (3, 4, 5, 6, 7, 8, 9, 10) and ten different concentrations of NaCl (0 - 0.5 - 1 - 1.5 - 2 - 2.5 - 3 - 4 - 5 - 6 %) and incubated at 20 °C for 24 h. The graphs of pH and NaCl effects were drawn by taking measurements at 600 nm wavelength every 3 h.

#### 2.2.2.2 Biochemical characterization

The detailed biochemical characterization of the *Pseudomonas mandelii* KGI_MA19 strain was accomplished by determining the strain's carbon source utilization, observing acid generation from carbonhydrates (API20E), and determining the strain's oxidase and catalase activities. To evaluate the novel *Pseudomonas mandelii* KGI_MA19 strain's phenotypic features, the cells were cultured on TGY agar at a temperature of 20 °C for 18 h and single colonies were collected for each of the experimental characterisation procedures. To determine whether a certain bacterial isolate is capable of producing catalase, a single colony of *Pseudomonas mandelii* KGI_MA19 was combined with a 3% hydrogen peroxide solution. The isolate's oxidase activity was determined using the Bioanalyse Oxidase Test Kit. A single colony was collected and put on the oxidase test strip for the oxidase activity test. M9 minimum media with agar ($Na_2HPO_4$, $KH_2PO_4$, NaCl, $NH_4Cl$) was used to assess the usage of various carbon sources (11 various amino acids, 3 monosaccharides, and 15 disaccharides) (1M $MgSO_4$, 1M $CaCl_2$, 0.5% Thiamine). Each carbon source was adjusted to a final concentration of 100mM, and each media was inoculated with a 10 µl cell sample dissolved in 0.85% NaCl. The growth on the solid culture was examined after 24 h.

**Antibiotic resistance**

The disk diffusion technique was used to assess the antibiotic resistance of *Pseudomonas mandelii* KGI_MA19. To begin, the sample was spread over TGY agar media. Used antibiotics to evaluate the antibiotic resistance were Novobiocin (5 g), Moxifloxacin (5 g), Penicillin-G (10 g), Norfloxacin (10 g), Gentamicin (10g), Ampicillin/Sulbactam (10/10g), Streptomycin (10g), Trimethoprim/Sulfamethoxazole (25 g), Netilmicin (30 g), Ceftazidime (30 g). The TGY agar samples with antibiotics were incubated at 20 °C for 24h. The diameters of the antibiotic inhibitory zones were measured after incubation.

**Biofilm formation**

For the observation of the biofilm development, *Pseudomonas mandelii* KGI_MA19 culture in 200 µl TGY medium ($OD_{600}$: 0.5) in a 96 well plate was maintained at six different temperatures (4 ℃, 10 ℃, 15 ℃, 20 ℃, 25 ℃, 30 ℃) for 48 h. After incubation, the medium in the 96 well plate was poured. The plate was washed twice

with $dH_2O$. After waiting for air dry at room temperature, 200 µl of 0.1% Crystal violet dye was added and incubated for 15 min at room temperature. The plate was then washed with $dH_2O$. After air drying, 200 µl of 95% ethanol was added, and the absorbance of the samples was measured at 570 nm. Triplicate repetitions were performed for the measurement of all temperatures.

### 2.2.3 Amplification of chitinase gene

#### 2.2.3.1 Genomic DNA isolation

An inoculum of -80 ℃ stock culture was inoculated into a 5 ml TGY medium and cultivated for 18 hours at 20 ℃ before being harvested. A total of 4 ml of culture was precipitated after incubation, and DNA was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, #K0722) in accordance with the additional procedure for gram-negative bacteria. The obtained DNA was checked on a 0.7 % agarose gel under 120V, 20 min conditions.

#### 2.2.3.2 Primer design

Oligo Analyzer Tool was used to construct gene-specific primers in order to determine the % GC content and Tm (°C) of the primers. When developing gene-specific primers, formation of heterodimer and homodimer, hairpin structures, and secondary structures should be avoided. These primers matched the chitinase gene from the *Pseudomonas mandelii* KGI_MA19 strain. Both the Forward gene specific primer and the Reverse gene specific primer have a restriction enzyme recognition site for *Sac*I and *Not*I, respectively (Table 2.1). The restriction sites were added to PCR primers to facilitate the insertion of amplified products into vector cloning sites. Primers were constructed to have a melting temperature that is close to that of one another, allowing them to be employed concurrently at different stages of the combined overlap PCR process.

**Table 2. 1** Forward and reverse primers designed specifically for the chitinase gene.

| Name | Sequence (5' to 3') | GC Content (%) | Tm (°C) | Length (bp) |
|---|---|---|---|---|
| **Forward Gene Specific Primer (*Sac*I)** | CGGAGCTCATGTCAAAATTCGACTTTACG | 34.38 | 61 | 32 |
| **Reverse Gene Specific Primer (*Not*I)** | AAGCGGCCGCCTGCTGGCTGGCGACGTCAGGTAT | 34.38 | 63.8 | 32 |

### 2.2.3.3 Conventional PCR

Three alternative annealing temperatures were attempted for the optimization of the polymerase chain reaction in order to get the chitinase enzyme gene using the primer set, and the gene area of the relevant enzyme was amplified using proper PCR conditions. The PCR conditions and components of the reaction are listed in (Table 2.2).

**Table 2. 2** Content(A) and conditions (B) of polymerase chain reaction of chitinase enzyme.

**A)**

| Content of Reaction | Volume |
|---|---|
| $dH_2O$ | 8.25 µl |
| DMSO | 0.75 µl |
| F Primer (10µM) | 1.25 µl |
| R Primer(10µM) | 1.25 µl |
| Template | 0.5 µl |
| Phusion® High-Fidelity HF PCR Master Mix (2X) | 12.5 µl |
| Phusion DNA Polymerase (5u/µl) | 0.25 µl |
| Total Volume | 25 µl |

**B)**

| PCR Processes | Time | Temperature | |
|---|---|---|---|
| Initial Denaturation | 30 sec | 98 °C | |
| Denaturation | 10 sec | 98 °C | 30cycle |
| Annealing | 30 sec | 60 °C- 65 °C- 70 °C | |
| Elongation | 33 sec | 72 °C | |
| Final Elongation | 10 min | 72 °C | |

The PCR products obtained at the end of the reaction were visualized on a 0.7% agarose gel at 120 V for 20 min and the appropriate PCR condition was determined.

#### 2.2.3.4 PCR product purification

Determining that the optimum PCR condition was at 60 ℃, a PCR reaction was set up at 60 ℃. Then the purification of the PCR products was carried out. with PCR Purification Kit (*EcoSpin*, EcoPP). Briefly, 375 µl of binding buffer was added onto 75 µl of PCR product and mixed, then 100 µl of isopropanol was added. The column included in the kit was placed in a 2 µl centrifuge tube and the mixture containing the PCR product was added to the column. Then it was centrifuged at room temperature, 30 sec, >12.000 *x*g. The flow through was discarded. 700 µl of wash buffer was added to the column and centrifugation was performed under the same conditions. In order to remove the wash buffer thoroughly, the column without adding any solution was centrifuged at room temperature, 1 min, at >12.000 *x*g. The column was transferred to a sterile 1.5 ml tube and 50 µl of elution buffer was added in the middle of the membrane inside the column. After waiting for 2 min at room temperature, centrifugation was performed at room temperature, 30 sec, at >12.000 *x*g. After the column was removed, the control of the purified DNA sample in the tube was maintained on a 0.7% agarose gel at 120V for 25 min.

#### 2.2.3.5 Digestion of PCR product and vector

The *Sac*I and *Not*I restriction endonucleases were used to digest the chitinase gene and expression vector pET28a (+). Two distinct responses were designed to account for the target gene and the expression vector. 20 µl of DNA, 3 µl of 10X Tango buffer, 6 µl of $dH_2O$, and 1 µl of *Sac*I (50 U/ µL) were mixed for chitinase gene digestion. The expression vector pET28a (+) was then combined with 15 µl of plasmid, 2 µl of 10X Tango buffer, 2 µl of $dH_2O$, and 1 µl of *Sac*I. The reactions were incubated at 37 ℃ for 1 hour. Following the incubation time, 3.75 µl of 10X Tango buffer, 64.25 µl of $dH_2O$, and 2 µl of *Not*I (50 U/ µL) enzyme were added to the previously prepared digestion reaction of chitinase, and then 2.5 µl of 10X Tango buffer, 75.5 µl of $dH_2O$, and 2 µl of *Not*I enzyme were added to the previously prepared digestion reaction of pET28a (+), followed by 1 h at 37 ℃ incubated. Following the incubation time, the PCR purification step was carried out, and the products were tested on a 0.7 % agarose

gel at 120 V for 25 min. The DNA quantity of four reactions was evaluated using a nanodrop spectrophotometer after the digestion process and the generated gel identity.

### 2.2.3.6 Ligation

The ligation reaction was performed out at a 1:5 ratio (vector:insert). Using the Ligation calculator online web tool (NEB), the reaction was set up with the appropriate amount of vector to insert ratio. The components and quantities of the ligation reaction are listed in Table2.3. For a 1:5 ratio, a ligation reaction volume of 20.5 µL was prepared using 12.5 µL insert DNA, 5 µL vector, 2 µL T4 DNA Ligase enzyme (Roche, #10 716 359 001), and 1.5 µL 10X Ligation Buffer (Roche, #11 243 292 001). For 16 hours, the reaction tubes were incubated at 15 °C. Following incubation, the tubes were incubated at 60 °C for 10 min to inactivate the T4 DNA ligase.

**Table 2. 3** Content of ligation reaction.

| **1:5 ratio (Vector:Insert)** | |
|---|---|
| **Components** | **Volume** |
| Insert DNA | 12.5 µL |
| pET-28a (+) Vector | 5 µL |
| T4 DNA ligase enzyme (5 U/µl) | 2 µL |
| 10X Ligation Buffer | 1.5 µL |
| Total | 20.5 µL |

### 2.2.3.7 Transformation and selection of colonies

After the ligation step, *E.coli* BL21 (DE3) competent cells prepared previously and stocked at -80 ℃ were used for transformation. *E.coli* BL21 cells from -80 ℃ were kept on ice for 15-20 min. 5 µl of ligation product was added onto 200 µl competent cells thawed on ice and incubated at 42 °C for 2 min. The cells were then incubated on ice for 5 min. At the end of the incubation, 1 ml of LB medium was added to the cells and incubated at 37 ℃ for 1 h with shaking at 200 rpm. At the end of the incubation, centrifugation was performed at 12.000 *x*g for 5 min at room temperature and the supernatant was removed. 200 µl of LB medium was added to the pellet and the pellet was thawed. By pipetting of 100 µl of the dissolved pellet, the cells were spread on LB agar containing 40 mg/ml kanamycin by taking 100 µl of the dissolved pellet. The plates were incubated at 37 ℃ for 16 h.

At the end of the incubation, 16 colonies were selected, colony PCR was set up from the selected colonies, and each selected colony was incubated in 3 ml of LB medium containing kanamycin (40 mg/ml) at 37 ℃ for 16 h with shaking at 200 rpm. Following incubation, 1 ml of culture was taken from each tube and the cells were centrifuged at 12000 *x*g for 5 min, and then glycerol stocks of each colony were prepared.

### 2.2.3.8 Colony PCR

For the performing of colony PCR selected 16 colonies were dissolved in 20 µl of sterile $dH_2O$. Dissolved colonies were used as template in the reaction. The components and conditions of colony PCR are as described in Table 2.4.

**Table 2. 4** Content(A) and conditions (B) of colony PCR.

**A)**

| Content of Reaction | |
|---|---|
| $MgCl_2$ | 0.75µl |
| Buffer | 2.5 µl |
| dNTP | 0.5 µl |
| Reverse Primer (10 µM) | 1.25 µl |
| Forward Primer (10 µM) | 1.25 µl |
| Selected colony (20 µl) | 1 µl |
| *Taq* Polymerase (5 U/µl) | 0.25 µl |
| $dH_2O$ | 18.25 µl |
| Total Volume | 25 µl |

**B)**

| PCR Processes | Time | Temperature | |
|---|---|---|---|
| Initial Denaturation | 94°C 4min | 98 °C | |
| Denaturation | 94°C 30 sec | 98 °C | 35cycle |
| Annealing | 60°C 45 sec | 60 °C | |
| Elongation | 72°C 93 sec | 72 °C | |
| Final Elongation | 72°C 8 min | 72 °C | |

The colony PCR products obtained at the end of the reaction were visualized on 0.7% agarose gel at 120 V for 20 min and the positive colonies were determined.

### 2.2.3.9 Plasmid isolation and control cleavage with restriction enzyme *EcoR*V

Each of the six positive colonies selected as a result of colony PCR were incubated in 3 ml of LB medium containing 40mg/ml kanamycin at 37℃ for 16 h with shaking at 200 rpm. At the end of the incubation, plasmid isolation (Ecospin, EcoPI) was performed. Briefly, 2 ml colony cultures were taken and centrifuged at 6000 *x*g for 2 min at room temperature and the supernatant was removed. The pellet was treated with 250 µl of resuspension buffer and then 20 µl of RNAse was added to the mixture. After

adding 250 µl of lysis buffer to the mixture, it was made upside down 6-7 times and incubated for 3 min at room temperature. At the end of the incubation, 350 µl of binding buffer was added to the mixture and the solution was mixed by making it upside down again. The mixture was centrifuged at >12000 *x*g for 5 min at room temperature. The supernatant obtained at the end of centrifugation was transferred to the column in the kit, then centrifuged at room temperature, 30 sec, >12000 *x*g, and flow-through was removed. 400 µl wash buffer 1 was added to the column and centrifuged at room temperature, 30 sec, >12000 *x*g. After the flow-through was removed, 500 µl wash buffer 2 was added to the column and centrifuged at room temperature, 30 sec, >12000 *x*g. The column was transferred to a new ependorf and 200 µl Wash Buffer 2 was added on it and centrifuged at room temperature, 2 min, >12000 *x*g. At the end of centrifugation, the column was transferred to a sterile 1.5ml tube and 40 µl of elution buffer was added to the column and incubated for 2 minutes at room temperature. At the end of the incubation, it was centrifuged at room temperature for 30 sec at >120000 *x*g and the column was removed. The plasmid samples obtained were controlled on a 0.7 % agarose gel under 120 V 20 min conditions and the samples were stored at -20 ℃.

For each plasmid obtained as a result of plasmid isolation, cutting was performed using Fast Digest *EcoR*V (Thermo Scientific, #FD0303) and 10X FastDigest Buffer (Thermo Scientific, #00101053). The reaction content is indicated in Table 2.5. After the reaction content was prepared, the reaction tube was incubated at 37 °C for 15 min. At the end of the incubation, the reaction was incubated at 60 °C for 20 min and enzyme inactivation was achieved. At the end of the incubation, the pET-28a (+) vector with a length of 5369 bp and a 1062 bp insert with a total length of 6441 bp were checked on a 0.7 % agarose gel at 120 V, 20 min. Plasmids with the desired bp length were selected for sequencing.

**Table 2. 5** The components of digestion reaction of plasmid DNA.

| Components | Volume |
|---|---|
| 10X Fast Digest Buffer | 2 µL |
| Plasmid DNA | 1 µL |
| Fast Digest *EcoR*V enzyme | 1 µL |
| $dH_2O$ | 6 µL |
| Total | 10 µL |

After checking plasmids with agarose gel, positive colonies were transformed into *E. coli* BL21 (DE3) competent cells as previously described, and -80 ℃ stocks containing 15 % glycerol were prepared.

**Sequencing**

Selected plasmids were sequenced using the universal T7 Promoter (5'-TAATACGACTCACTATAGGG-3') and T7 Terminator (5'-GCTAGTTATTGCTCAGCGG-3') primers. Sequencing using the Sanger sequencing method was performed by Genometry Biotechnology Limited Company (Izmir, Turkey). The results were checked through SnapGene Viewer program.

### 2.2.4 Expression of chitinase

The expression of the chitinase gene, which was correctly cloned as a result of Sanger sequencing, was controlled by the commercially available MagicMedia™ (Thermo Fisher Scientific) medium. Briefly, a positive colony containing the chitinase gene with a stock of -80 ℃ was seeded on LB agar medium containing kanamycin (40mg/ml) and incubated overnight at 37 ℃. After a single colony was selected and incubated at 200 rpm at 37 ℃ 18 h 200ml in LB medium containing kanamycin (40mg/ml) culture was prepared. 1 ml of preculture was added to Magic Media containing kanamycin (40mg/ml) and incubated at 37 ℃ for 17h at 200 rpm. At the end of the incubation, the cells were precipitated at 4 ℃ for 30 minutes at 9000 rpm. Precipitated cells were treated with 1ml of lysis buffer (0.1 M Tris-HCl pH.8, 0.3M NaCl). Ultrasonication was performed using MS73 probe under 5 min 40W 20 sec pulse on, 20 sec pulse off conditions and 100 µL lysate was separated. After, the cell debris was removed by centrifugation at 9000 rpm, 4 ℃ for 30 min. The supernatant was separated, and expression control was checked by 10 % SDS-PAGE.

#### 2.2.4.1 SDS-PAGE analysis

Firstly, the stacking gel concentration was set at 5 %, while the resolving gel concentration was set at 10 %. Appendix C lists the components of each buffer and gel used in this investigation.

Before loading the samples onto the gel wells, 20 µl of protein samples were mixed with 5 µl of sample application buffer and warmed for 15 min at 95 °C using a heater. 3 µl of PageRuler Plus Prestained Protein Ladder (Thermo Scientific PageRuler Plus,

#26616) was put into the first well without heat denaturation as a molecular weight marker. After the proteins had reached the resolving gel, a steady voltage of 120 V was applied until the front dye had reached the bottom of the resolving gel. Then, the gel was shaken overnight with Coomassie Brilliant Blue R-250 stain. After the incubation, destaining buffer was used for removing excess stain about three hours.

### 2.2.5 Purification of chitinase

#### 2.2.5.1 His-tag affinity chromatography

The positive chitinase colony, whose expression was controlled, was incubated with 100ml Magic Media containing kanamycin (40 mg/ml) at 37 ℃ for 17h at 200 rpm. At the end of the incubation, the cells were precipitated at 4 ℃ for 30 min at 9000 *x*g. Precipitated cells were treated with 6 ml of lysis buffer (0.1 M Tris-HCl pH.8, 0.3 M NaCl). Using MS73 probe, ultrasonication was performed under 5 min 40W 20 sec pulse on, 20 sec pulse off conditions and 200 µl lysate was separated. After the lysate was centrifuged at 9000 *x*g 4 ℃ for 30 min to remove the cell debris and the supernatant was separated. For the purification of chitinase protein, affinity chromatography was performed using HisTrap™ Fast Flow (Sigma Aldrich) column with 1 ml capacity. First, the elution (500mM imidazole) and lysis (5mM imidazole) buffers, the contents of which are included in the kit's manual, were prepared. Buffers containing 20mM, 40 mM, 60 mM, 80 mM, 100 mM, 150 mM, 200 mM, 250 mM imidazole were prepared for applying gradient elution affinity chromatography with the help of the prepared buffers. In order to prepare the column before chromatography, first 3 column volumes of dd$H_2O$ and then 5 column volumes of Binding Buffer were passed. 6ml sample called clear lysate was passed through the column and flow-through was collected. After the sample was passed, 4 column volumes of 20mM, 2 column volumes of 40 mM, 2 column volumes of 60mM, 2 column volumes of 80mM imidazole-containing buffers were passed, respectively, and the collected elutions were named as W1, W2, W3, W4. Then, 2 column volumes of buffers containing 100mM, 150 mM, 200mM, 250mM, 500mM imidazole were passed as 2 column volumes and the collected elutions were named as E1, E2, E3, E4, E5. Purification result was checked with 10 % SDS-PAGE at 90 V 120 min and Western Blot analysis.

### 2.2.5.2 Western blot analysis

First, SDS PAGE was used to analyse the samples. The PVDF membrane to be used for the transfer step was trimmed to fit the size of the gel and shaken for 10 min in 100 % methanol. A western blot transfer equipment was used to sandwich the membrane and gel (Mini Trans-Blot Electrophoretic Transfer Cell, 170-3930, 170-3935, Bio-Rad). For this procedure, 1X Transfer Buffer is applied to the sponge. In addition, filter paper is saturated in transfer buffer and put on a sponge. SDS-PAGE electrophoresis gel was delivered to it. The membrane was appropriately put on this gel, as were the filter paper and sponge. It was covered with a cylindrical substance to prevent the production of air bubbles. After appropriately positioning the transfer equipment in the tank, a cold 1X transfer buffer was added to the tank, and the transfer procedure was carried out at 90 V for 1 h. This procedure was carried out at 4 °C, and an ice pack was put in the tank. The blocking step was performed after the protein transfer. In this blocking procedure, the membrane was initially incubated for 1 h at room temperature in 1X TBST solution containing 5% skim milk. Following incubation, 1X TBST blocking buffer was withdrawn. 6x-His-tag monoclonal antibody (3D5), AP (Thermo Scientific, #R932-25) was mixed to 1X TBST buffer with 2.5 % skim milk, and membrane was treated overnight at 4 °C with this buffer. After the buffer was withdrawn, the membrane was stirred for 5 min with 1X TBST for 5 times. The antibody and colored bands were identified using 1-Step NBT/BCIP Substrate (Thermo Scientific, #34042) as directed by the manufacturer.

### 2.2.5.3 Ultrafiltration

Buffer exchange was performed with the help of a 30.000 MWCO (Merck Millipore) ultrafiltration membrane selected in accordance with the size of the E5 sample obtained as a result of the purification. In order for the membrane to be ready, 15 ml dd$H_2O$ was passed through the membrane at 4 ℃ for 10 min at 4000 *x*g Then, 15 ml phosphate buffer (pH:7) was passed at 4 ℃ for 10 min at 4000 *x*g. After the buffer was completely passed, 2 ml of E5 sample was added and a total of 30 ml of phosphate buffer was passed through and centrifugation was carried out at 4000 *x*g for 10 min. After passing 30 ml of buffer, approximately 1.5 µL of sample remaining was collected by pipetting and transferred to a new sterile tube.

### 2.2.6 Enzyme activity assay

#### 2.2.6.1 Biochemical characterization

**Bradford Assay**

The Bradford method was used to determine the concentration of the protein obtained after purification. Bovine Serum Albumin (BSA) concentrations ranging from 0.125 to 2 mg/ml were used as a standard. Briefly, 5 µl of sample was mixed with 195µl of Bradford's reagent to make up to 200 µl and the measurement was taken at 595 nm. Then, the concentration of the enzyme was calculated by comparing the absorbance values of the standards.

**3, 5 – Dinitrosalicilic acid (DNS) assay**

The chitinase enzyme breaks down the β-1-4 glycosidic bonds in the N-acetyl glucosamine polymers in chitin by hydrolyzing. N-acetyl glucose amine (NAG) is an amine sugar with reducing properties. Thanks to its reducing property, color change is observed by reacting with 3,5-Dinitrosalicylic acid (DNS). By using the color change, the amount of reducing sugar in the environment is determined. Thus, enzyme activity can be observed. The activity of the chitinase enzyme was measured using the DNS method. Briefly, at the end of the 50 µl reaction established with 1 mM purified enzyme and 1% colloidal chitin, 50 µl DNS was added to the reaction. The prepared mixture was incubated at 100 ℃ for 5 min and then allowed to cool. Measurements of the cooled samples were taken at $OD_{540}$. N-acetyl glucosamine (Sigma-Aldrich, #A8625) with a concentration between 0.125 and 2 mg/ml was used as a standard.

#### 2.2.6.2 Preparation of the colloidal chitin

100 mg of the chitin (Sigma-Aldrich, #C9752) was incubated with 1.2 ml of HCl at 4 ℃ overnight. After the incubation, 40 ml of ethanol was added and incubated at 4 ℃ overnight with mixing. Then, it was washed with $dH_2O$ with 6000 *x*g for 25 min at 4 ℃ until the pH reached 7. The sample with pH value 7 was dried at room temperature and stored at 4 ℃.

#### 2.2.6.3 Determination of optimum temperature and thermal stability

In order to determine the optimum temperature of enzyme activity, reactions containing 50 µL of 1 mM enzyme and 1% colloidal chitin were set up at 4 ℃, 10 ℃, 15 ℃, 20 ℃, 25 ℃, 30 ℃, 35 ℃, 40 ℃, 45 ℃, 50 ℃, 55 ℃, 60 ℃ for 90 min

incubations were performed. The activity of the samples was measured with the DNS method mentioned previously. All analysis was performed in triplicate.

To determine the thermal stability of the enzyme, the enzyme in 50mM potassium acetate buffer (pH: 6.00) at 4 ℃, 10 ℃, 15 ℃, 20 ℃, 25 ℃, 30 ℃, 35 ℃, 40 ℃, 45 ℃, 50 ℃, 55 ℃, 60 ℃ for 90 min kept on hold. The enzyme incubated at different temperatures was treated with 1% colloidal chitin for 90 min and then the enzyme activity was measured by DNS method. The experiment was done in triplicate repetitions.

#### 2.2.6.4 Determination of optimal pH and pH stability

To establish the optimal pH for enzyme activity, reactions containing 50 µL of 1mM enzyme and 1% colloidal chitin were set up in the pH 3-10 range (Sodium Citrate (pH:3), Sodium Acetate (pH 4-6), Tris-HCl (pH:7), Glycine NaOH (pH:8-9-10)) at 30℃ for 90 min. The activity of the samples was measured with the DNS method mentioned previously. All analysis was performed in triplicate.

In order to determine the pH stability, 1mM enzyme pH values between 3-10 (Sodium Citrate (pH:3), Sodium Acetate (pH 4-6), Tris-HCl (pH 7), Glycine NaOH (pH 8-10)) was left to stand for 90 min. After the enzyme incubated at different pH values was treated with 1% colloidal chitin for 90 minutes, the enzyme activity was measured by DNS method. The experiment was done in triplicate repetitions.

#### 2.2.6.5 Effects of metal ions on the chitinase enzyme

$Fe^{+2}$, $Zn^{+2}$, $Al^{+2}$, $Mg^{+2}$, $Mn^{+2}$, $Co^{+2}$, $Ba^{+2}$, $Ca^{+2}$ metal ions were used to determine the effects of metal ions on the chitinase enzyme. 1mM and 10mM amounts of each metal ion were treated with chitinase enzyme for 90 min at 30 ℃. At the end of the incubation, 1% colloidal chitin was added to the medium and the reaction was carried out at 30 degrees for 90 min. At the end of the reaction, enzyme activity was measured by DNS method. Each reaction was performed in triplicate.

#### 2.2.6.6 Effects of some organic solvents and detergents on chitinase enzyme

Urea (10 mM, 1 mM), EDTA (10 mM, 1 mM), Tween-80 (20 %, 5 %), DMSO (20 %, 5 %), SDS (5 %, 1 %), β-mercaptoethanol (20 %, 5 %) and methanol (20 %, 5 %) were used to determine their effects on the chitinase enzyme. The concentrations of the

prepared solvent and reagents were incubated with 1mM chitinase enzyme at 30 ℃ for 90 min. At the end of the incubation, 1% colloidal chitin was added to the medium and the reaction was carried out at 30 ℃ for 90 min. When the reaction was finished, the activity of the enzyme was measured by DNS method. Each reaction was performed in triplicate repetitions.

# 3. RESULTS

## 3.1 Identification of *Pseudomonas mandelii* KGI_MA19

### 3.1.1 Obtaining environmental samples

12 different sediment and soil samples were collected from eight different regions of the Antarctic continent as part of the 2nd National Antarctic Science Expedition (TAE-2) by Prof. Dr. Nurgül Çelik Balcı during the expedition to the continent are shown in Table 3.1. The locations of the coordinates on the map are shown in Figure 3.1.

**Table 3. 1** The numbered and coordinates of 12 samples obtained from eight distinct areas of King George Island.

| Region | Samples/Coordinates | Region | Samples/Coordinates |
|---|---|---|---|
| **1** | **VC (Sample 18)**<br>422947,66 / 3106001,65 | **5** | **P4 (Sample 24)**<br>62°9’36.27’’S<br>58°28’31.45’’W |
| | **EG2(Sample 19)**<br>423236,96 / 3106008,24 | | **PFC7 (Sample 25)**<br>62°9’’33.69’’S<br>58°28’27.52’’W |
| **2** | **TC (Sample 20)**<br>62°12’59.65’’S<br>58°27’25.21’’W | | **PFC2 (Sample 29)**<br>62°9’42.45’’S<br>58°28’40.04’’W |
| **3** | **S3 (Sample 21)**<br>424290,07 / 3100816,64 | **6** | **ULI (Sample 26)**<br>62°12’55.29’’S<br>58°27’18.98’’W |
| | **MCI (Sample 30)**<br>62°13’16.40’’S<br>58°27’9.05’’W | **7** | **PFCI (Sample 27)**<br>62°9’43.68’’S<br>58°28’39.06’’W |
| **4** | **S2 (Sample 22)**<br>422208 / 3105481,49 | **8** | **MLI (Sample 31)**<br>62°13’9.43’’S 58°27’20.21’’W |

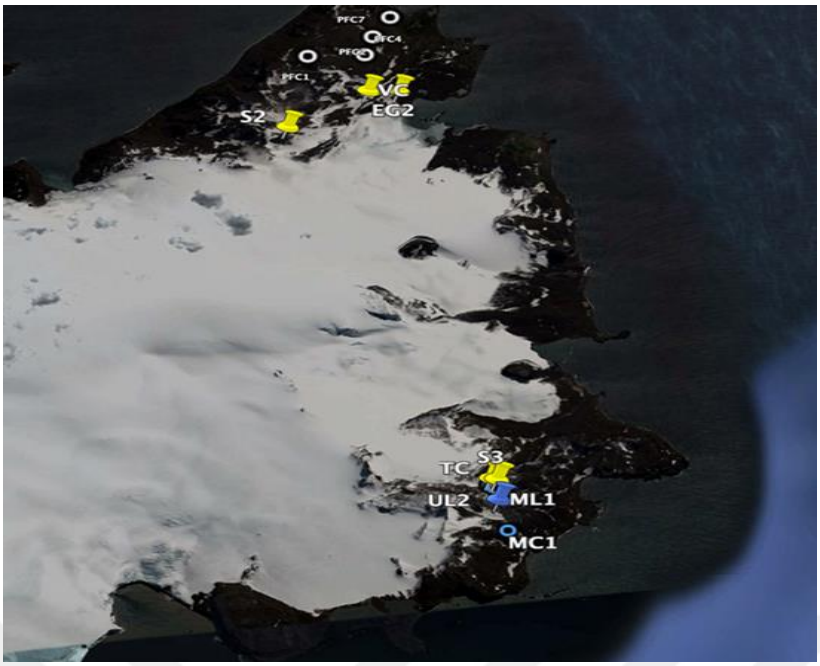


**Figure 3. 1** View of King George Island on the a map showing twelve samples.

### 3.1.2 Enrichment culture

Sediment samples, which are cultivated at 4 ℃, 96 h shaking with 200 rpm in TGY medium, were cultured in TGY agar media and incubated at 4 ℃ for 96 hours. Then, opaque, white-coloured and morphologically different colonies were observed in the medium of each sample (Figure 3.2).

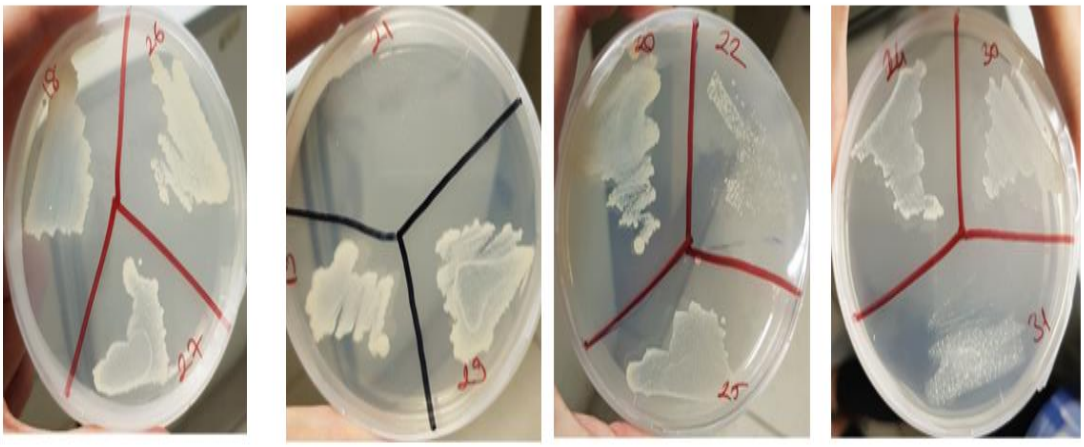

**Figure 3. 2** Colonies belonging to each sediment sample.

DNAs were isolated from the enriched culture of each sediment sample and checked on a 0.7% agarose gel (Figure 3.3).

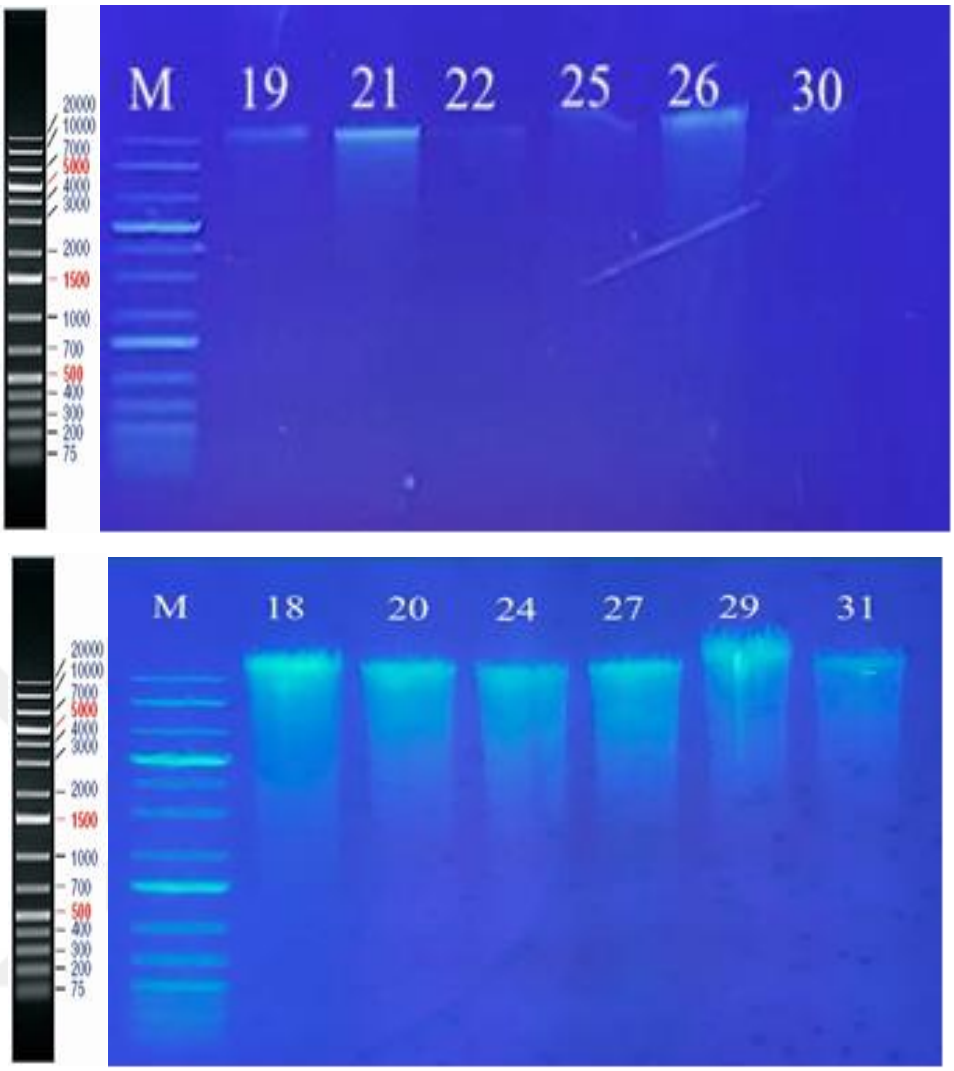


**Figure 3. 3** Isolation of complete genomic DNA from enrichment cultures. M: Marker, GeneRuler 1 kb, and DNA Ladder.

Eight environmental DNAs were submitted for 16S rRNA analysis to represent each location. According to the 16S rRNA analysis, *Pseudomonas* sp and *Janthinobacterium* sp species in sample location 18, *Pseudomonas* sp and *Paeniglutamicibacter* sp species in sample location 20, *Flavobacterium* sp species in sample location 21, *Pseudomonas* sp and *Pseudomonas frederiksbergensis* in sample location 24, *Pseudomonas* sp and *Paeniglutamicibacter* sp species in sample location 26, *Pseudomonas* sp and *Arthrobacter* sp species in sample location 27, and *Pseudomonas* sp and *Pseudomonas frederixbergensis* for Antarctica 30 sample were observed as dominant species in their regions (Figure 3.4).

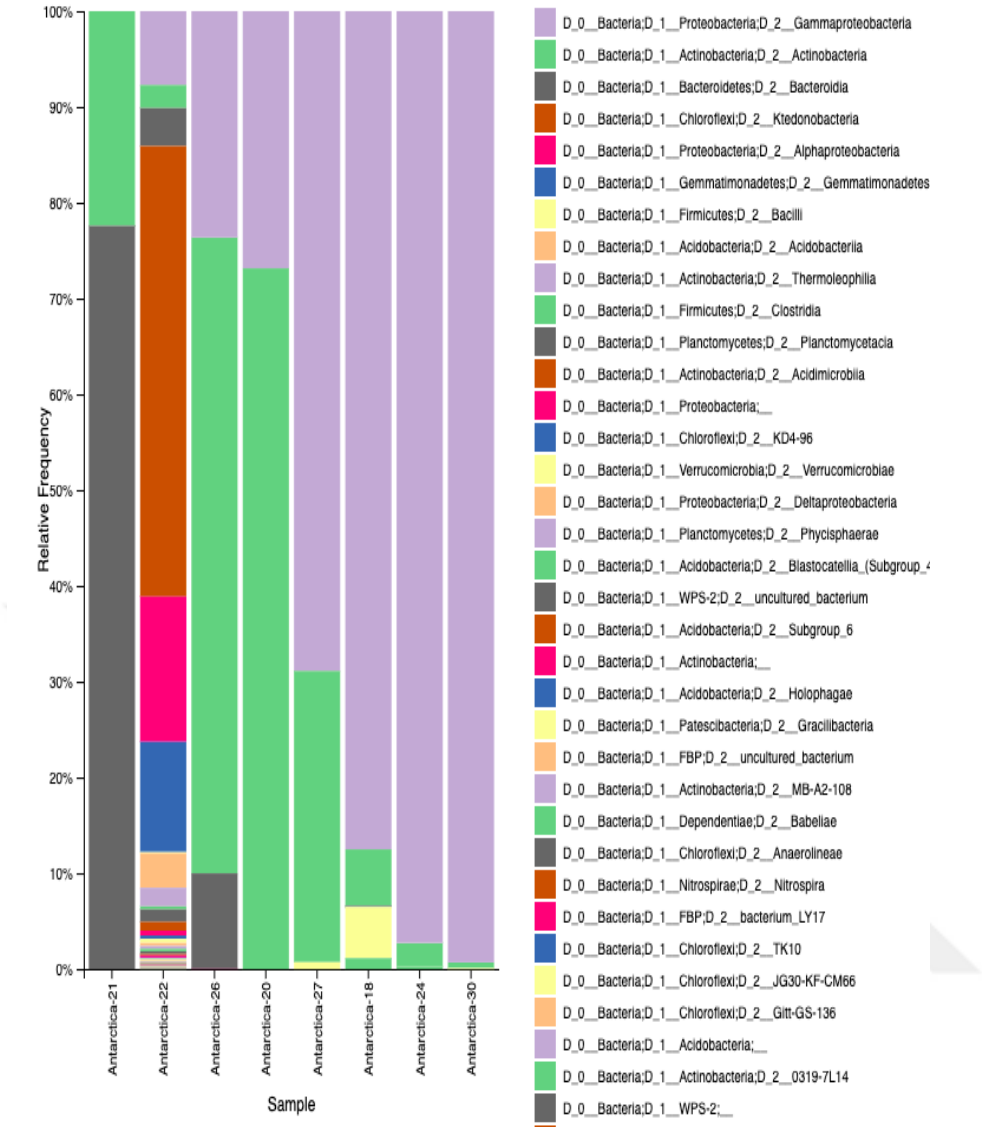


**Figure 3. 4** Biodiversity of eight different sample origins according to the 16S rRNA analysis. Column 1. Sample 21. Column 2. Sample 22. Column 3. Sample 26. Column 4. Sample 20. Column 5. Sample 27. Column 6. Sample 18. Column 7. Sample 24. Column 8. Sample 30.

### 3.1.3 Freeze-thaw stress

Only five of twelve samples were found to be capable of withstanding the provided stress conditions of -20 ℃/ +4 ℃ after freeze-thaw (Figure 3.5). From the petri dish samples, one big and one tiny colony deemed to be morphologically distinct were chosen. Then DNA of selected colonies was isolated after growing in a TGY medium with Thermo Scientific GeneJET Genomic DNA Purification Kit. The genomic DNAs were then observed on a 0.7% agarose gel as a control (Figure 3.6).

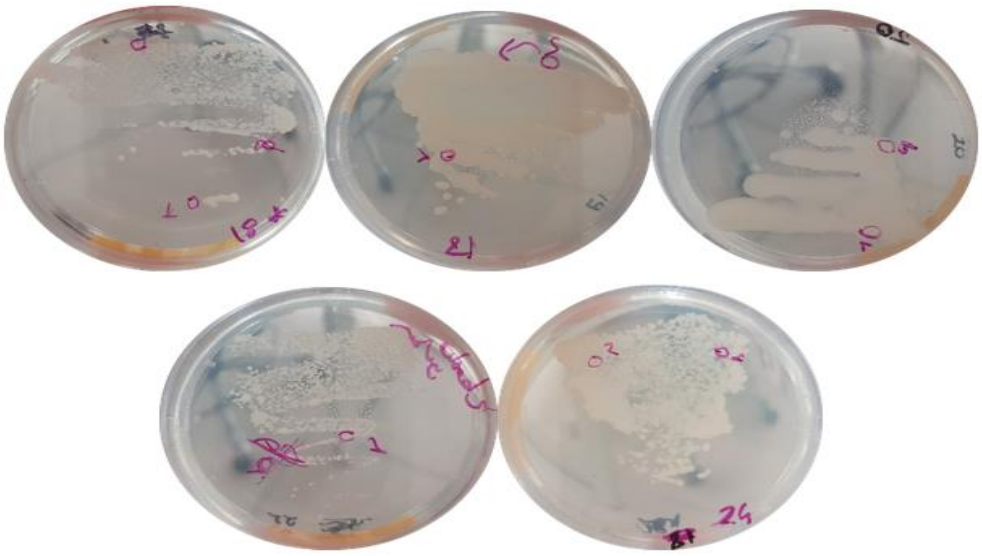

**Figure 3. 5** Stress-resistant colonies were obtained on TGY solid medium after freeze-thaw stress.

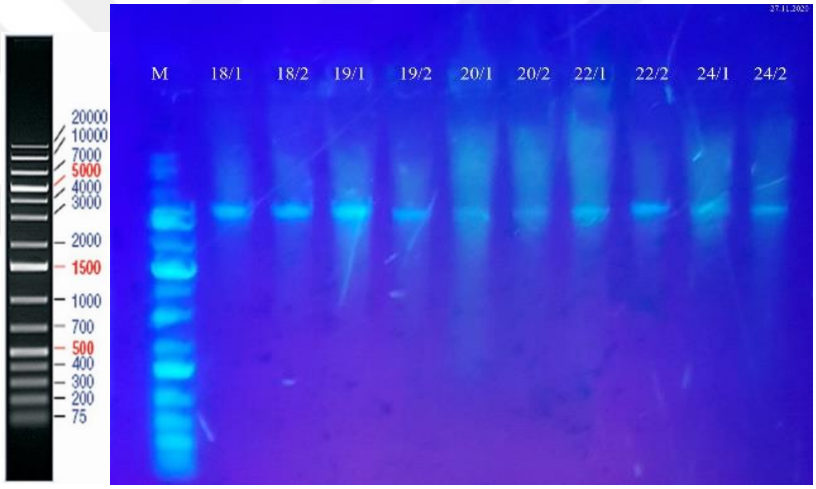


**Figure 3. 6** Genomic DNA isolations from freeze-thaw resistant colonies. M: Marker, GeneRuler 1 kb and DNA samples of single colonies.

The results of 16S rRNA analysis on colonies picked following freeze-thaw stress revealed that all of the bacteria were *Pseudomonas* (Table 3.2). This is an anticipated conclusion given that the dominant species of mixed cultures were identified as a consequence of microbial diversity. When the microorganisms were compared to the *Pseudomonas* species previously discovered in Antarctica, *Pseudomonas mandelii* was shown to be a common species. To conduct genetic and phenotypic research, it was decided to continue with sample 19-2.

**Table 3. 2** Similarity rate of single colonies according to the NCBI database.

| Sample | Organism Type | Similarity Rate |
|---|---|---|
| 18-1 | *Pseudomonas umsongensis* strain SeaQual_P_81/6 | 100 |
| 18-2 | *Pseudomonas sp.* strain Sl7 | 100 |
| 19-1 | *Pseudomonas* sp. st. PAMC 27357 | 99,83 |
| 19-2 | *Pseudomonas* sp. st. 2200 | 99.72 |
| 20-1 | *Pseudomonas sp.* strain HS001 | 100 |
| 20-2 | *Pseudomonas sp.* strain P2647 | 100 |
| 22-1 | *Pseudomonas sp.* strain GP_566 | 100 |
| 22-2 | *Pseudomonas* sp. ADAK2 | 99.82 |
| 24-1 | *Pseudomonas sp.* strain PAMC 27339 | 99.80 |
| 24-2 | *Pseudomonas sp.* strain BIS1137 | 100 |

### 3.1.4 Whole genome analysis

Following the freeze-thaw stress, the genetic material of the 19-2 colony was isolated via the Thermo Scientific GeneJET Genomic DNA Purification Kit, and the amount of DNA obtained was quantified in the nanodrop. As a consequence of the isolation, 120 ng/l DNA was recovered, with ratios of 260/280-230/260 ranging between 1.8 and 2. De-novo sequencing from the acquired gDNA was used to undertake a whole-genome sequencing analysis. The genomic DNA length was determined to be 6749770bp, and the genome had 19 rRNAs, 70 tRNAs, and 6074 protein-coding areas as a result of the sequencing (Figure 3.7).

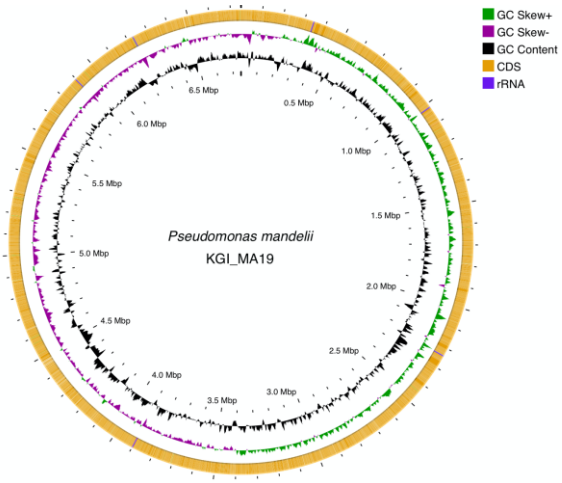


**Figure 3. 7** *Pseudomonas mandelii* KGI_MA19 genomic map in circular form.

When the full-length 16S rRNAs from the complete genome were compared to the NCBI database, 99.94% of the sequences were identical to those from *Pseudomonas mandelii* LMG 21607, *Pseudomonas mandelii* JR-1, and *Pseudomonas* sp SW-3. By comparing the ANI values of the acquired genomic DNA to those of these three bacteria, the greatest ANI value was discovered to be 98.86%. Because the acquired organism's complete genome similarity is less than 99%, it was designated as a novel strain of *Pseudomonas mandelii* and given the name *Pseudomonas mandelii* strain KGI_MA19. The novel strain's whole genome has been deposited in the NCBI GenBank database (Accession number: CP081178.1).

## 3.2 Characterization of *Pseudomonas mandelii* KGI_MA19

### 3.2.1 Phenotypic characterization

#### 3.2.1.1 Growth curve

Microorganisms obtained from Antarctica are classified as either psychrophilic or psychrotolerant. To characterize the newly found KGI MA19 strain, the growth curve versus temperature was plotted to establish its pattern of growth under different pH and NaCl conditions (Figure 3.8). The optimal growth temperature was determined to be 20 °C using the logarithmic phase and doubling time of the growth curve. *Pseudomonas mandelii* KGI_MA19 strain was determined as psychrotolerant due to its optimum temperature of 20 °C and no growth at 37 °C.

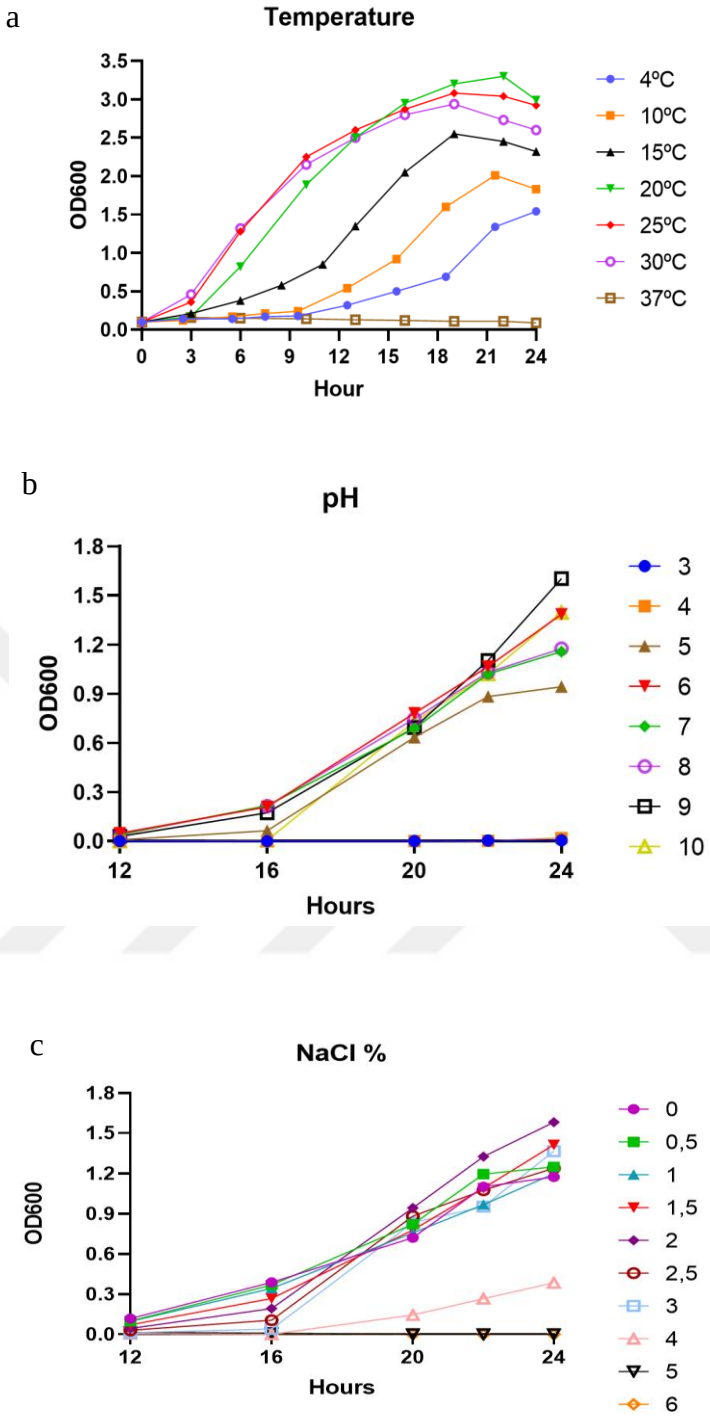


**Figure 3. 8** Growth curve of *Pseudomonas mandelii* KGI_MA19 (a) at different temperatures (b) at different pH (c) at different NaCl concentrations.

An experiment was conducted at 20 ℃ at different pH ranges to see at which pH values the KGI MA19 strain, whose optimal temperature was established, endures. The optimum pH value for the growth of the organism was observed as 6. Furthermore, no

growth was detected at pH levels 3 and 4. Organism, which has been found to grow between pH 5 and 10, could dwell in a wide pH range.

The effect of NaCl content on the development of the KGI_MA19 strain was also analysed in another experiment. In the experiment performed with ten different salt concentrations, no growth was observed at 5-6 % NaCl concentrations. The optimal NaCl concentration was determined to be 2 %, and its growth was observed between 0 and 4 % NaCl concentrations. As a result of this situation, strain KGI MA19 was identified as halotolerant bacteria.

### 3.3 Biochemical characterization

During the biochemical characterization, it was discovered which carbon sources were utilised and which enzymes were active in KGI_MA19. Biochemical characterisation investigations using *P. mandelii* were reviewed throughout the literature review, and it was discovered that the biochemical characterizations of the *P. mandelii* 6A1 strain and its mesophilic homologue, *P. mandelii*, were extensively explored. When the three strains were compared, it was discovered that the KGI MA19 strain differed from the other two in several ways (Table 3.3).

**Table 3. 3** Differences in phenotypic characteristics between *P. mandelii* KGI_MA19 and *Pseudomonas* sp. 6A1 from Antarctica and *P. mandelii.* ***N/D:** Not determined.

| **Test (active ingredients)** | **Detail** | ***P. mandelii* KGI_MA19** | ***Pseudomonas* sp.6A1** | ***P. mandelii* (1999)** |
|---|---|---|---|---|
| **Urease** | Activity | - | - | - |
| **Tryptophan deaminase** | Activity | - | N/D | N/D |
| **Acetoin production** | Activity | - | N/D | N/D |
| **Gelatinase** | Activity | + | - | - |
| **Catalase** | Activity | + | N/D | N/D |
| **Oxidase** | Activity | + | + | + |
| **β -galactosidase** | Activity | - | - | - |
| **Arginine dihydrolase** | Activity | + | - | - |
| **Ornithine decarboxylase** | Activity | - | - | - |
| **Nitrate reduction** | Activity | + | N/D | N/D |
| **Glucose** | Fermentation | + | N/D | N/D |
| **Mannose** | Fermentation | + | + | + |
| **Inositol** | Fermentation | + | + | + |

**Table 3.3 (continued)** Differences in phenotypic characteristics between *P. mandelii* KGI_MA19 and *Pseudomonas sp*. 6A1 from Antarctica and *P. mandelii*. ***N/D:** Not determined.

| **Test (active ingredients)** | **Detail** | ***P. mandelii* KGI_MA19** | ***Pseudomonas* sp.6A1** | ***P. mandelii* (1999)** |
|---|---|---|---|---|
| **Sorbitol** | Fermentation | W | - | + |
| **Rhamnose** | Fermentation | + | - | - |
| **Sucrose** | Fermentation | w | - | - |
| **Melibiose** | Fermentation | + | - | - |
| **Amygdalin** | Fermentation | - | N/D | N/D |
| **Arabinose** | Fermentation | + | + | + |
| **L-tyrosine** | Utilization | + | - | + |
| **L-tryptophan** | Utilization | - | + | - |
| **Glycine** | Utilization | - | + | + |
| **D-Glucose** | Utilization | + | + | + |
| **D-Galactose** | Utilization | + | + | + |
| **D-Fructose** | Utilization | + | + | + |
| **D-Sorbose** | Utilization | - | N/D | N/D |
| **Sucrose** | Utilization | + | - | - |
| **Maltose** | Utilization | + | - | - |
| **Lactose** | Utilization | + | - | - |
| **Malic acid** | Utilization | - | + | + |
| **Malonic Acid** | Utilization | - | + | + |
| **Citric acid** | Utilization | - | + | + |
| **Fumaric Acid** | Utilization | - | N/D | N/D |
| **Alpha keto glutaric acid** | Utilization | - | + | + |
| **2 oxo glutaric acid** | Utilization | - | N/D | N/D |
| **Oxalic acid 2 hydrate** | Utilization | - | N/D | N/D |
| **Mandelic acid** | Utilization | - | N/D | N/D |
| **Mannitol** | Utilization | + | + | + |
| **Glycolic acid** | Utilization | - | - | + |
| **Maleic Acid** | Utilization | - | N/D | N/D |
| **Sulfonilic acid** | Utilization | - | N/D | N/D |
| **Glycerol** | Utilization | - | + | + |
| **Citrate** | Utilization | + | + | + |

It was observed that gelatinase and arginine dihydrolase enzymes were able to perform catalysis in the KGI_MA 19 strain, while the other two strains did not have this feature. Unlike the others, KGI_MA19 was found to have the ability to use disaccharides such as rhamnose, sucrose, maltose, and lactose as a carbon source. In addition, while the other 6A1 and mesophilic strains could assimilate acids such as malic acid, malonic acid, citric acid, and alpha-ketoglutaric acid, it was observed that KGI_MA19 did not have such an ability.

### 3.3.1 Antibiotic resistance

To determine the antibiotics to which the KGI_MA19 strain is susceptible and resistant, the disc diffusion method was performed using eighteen different antibiotics.

After spreading the KGI_MA19 strain on TGY solid medium, discs were placed with a distance between each antibiotic-containing disc (Figure 3.9).


**Figure 3. 9** Antibiotic resistance of theKGI_MA19 strain was observed as a result of the disk diffusion method.

At the end of the incubation, it was observed that the strain was resistant to Cefaclor, Novobiocin, Vancomycin, Penicillin G, Trimethoprim/ Sulfamethoxazole, Ampicillin, Clindamycin, and Ceftazidime antibiotics. The severity of KGI_MA19's sensitivity to antibiotics increases or decreases according to the zone diameters formed. While it is very sensitive to tetracycline with the largest zone diameter, it is resistant to tetracycline against chloramphenicol with the smallest zone diameter. The measurements of the zone diameters are as follows; Tetracycline (5cm), Norfloxacin (4.6cm), Amikacin (3.5cm), Netilmicin (3cm), Gentamycin (2.7cm), Moxifloxacin (2.6cm), Streptomycin (2.3cm), Kanamycin (1.5cm) and Chloramphenicol (1.4cm). The antibiotic susceptibility of the KGI_MA19 strain is Tetracycline, Norfloxacin, Amikacin, Netilmicin, Gentamycin, Moxifloxacin, Streptomycin, Kanamycin, and Chloramphenicol, respectively, from highest to lowest (Table 3.4)

**Table 3. 4** Antibiotic resistance and susceptibility of *P. mandelii* KGI_MA19.

| **ANTIBIOTIC** | ***P. mandelii* KGI_MA19** |
|---|---|
| Cefaclor | R |
| Novobiocin | R |
| Vancomycin | R |
| Tetracycline | S |

**Table 3.4 (continued)** Antibiotic resistance and susceptibility of *P. mandelii* KGI_MA19.

| ANTIBIOTIC | ***P. mandelii* KGI_MA19** |
|---|---|
| Gentamycin | **S** |
| Penicillin G | R |
| Trimethoprim/ Sulfamethoxazole | R |
| Norfloxacin | S |
| Streptomycin | S |
| Ampicillin | R |
| Clindamycin | R |
| Ceftazidime | R |
| Netilmicin | S |
| Moxifloxacin | S |
| Kanamycin | S |
| Amikacin | S |
| Chloramphenicol | S |

S=Sensitive / R=Resistance

### 3.3.2 Biofilm formation

Microtiter plate assays with CV were used to examine biofilm development at various temperatures (4 °C, 10 °C, 15 °C, 20 °C, 25 °C and 30 °C). Based on the data collected throughout 48 h, it was determined that the biofilm growth at higher temperatures was robust (Figure 3.10). The biofilm developed at 25 °C was found to be three times as thick as the biofilm formed at 4 °C and half as thick as the biofilm formed at 20 °C (Figure 3.11).

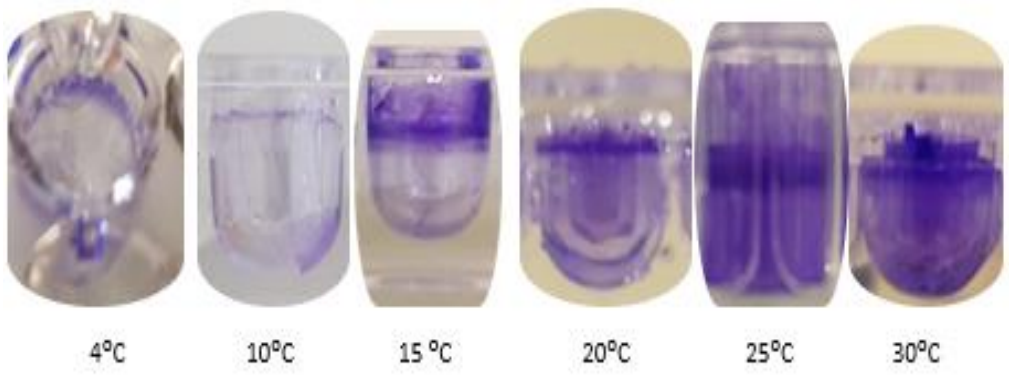


**Figure 3. 10** View of biofilm formation on the microplate at different temperatures.

Consequently, the production of biofilms by bacteria is recognized as a means of protecting themselves and surviving in difficult environments. It is known that psychrophilic and psychrotolerant microbes can form large biofilms at low

temperatures (Chrismas et al., 2016; Vásquez-Ponce et al., 2017; Ricciardelli et al., 2019). At 25 °C, *P. mandelii* KGI_MA19 produces more biofilm than the *P. mandelii* 6A1 strain (Vásquez-Ponce et al., 2017). In addition to low temperatures, environmental variables such as UV light and high salinity and pH variations are known to affect biofilm development (Rühs et al., 2013; Poulin and Kuperman, 2021). Due to the significant rise in the number of cells between 25 °C and 30°C, the carbon resources in the environment are projected to be quickly exhausted by the KGI_MA19 strain. Biofilm production at 25 °C and 30 °C is expected to rise as a result of nutritional deprivation, which is a new severe situation for the microorganism during growth.

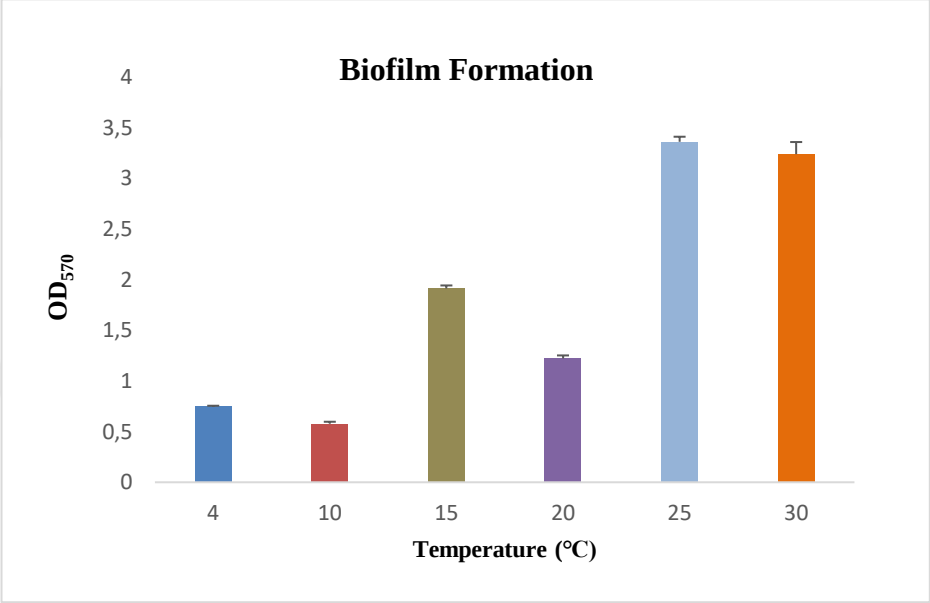


**Figure 3. 11** Temperature-dependent biofilm formation data.

### 3.4 Amplification of Chitinase Gene

#### 3.4.1 Genomic DNA isolation

To produce the chitinase protein of the *P. mandelii* KGI_MA19 strain recombinantly, the first step was to isolate the genomic DNA of the strain. The GeneJET Genomic DNA Purification Kit (Thermo Scientific) was used for genomic DNA with a length of 6749770 bp and two different *P.mandelii* DNAs were successfully isolated with the same kit. At the end of isolation, genomic DNAs were checked in a 0.7 % agarose gel under 120 V for 20 min (Figure 12).

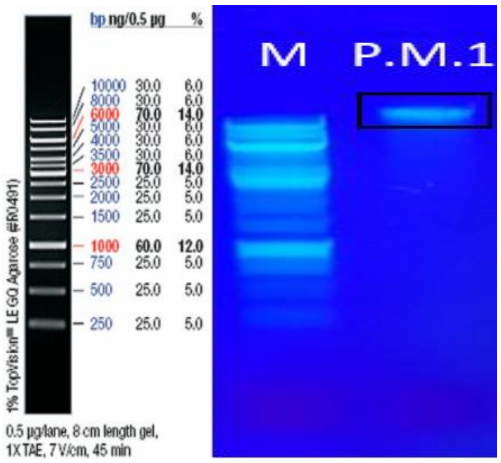


**Figure 3. 12** Genomic DNA isolation of P.mandelii KGI_MA19 M: Marker, GeneRuler 1 kb, and DNA Ladder, 1. DNA isolation of *Pseudomonas mandelii* KGI_MA19 (P.M.1)

DNA concentration values (260/280) were detected via nanodrop (Table 3.5).

**Table 3. 5** Nanodrop values of genomic DNA.

| **Absorbance of gDNA (260nm)** | |
|---|---|
| **P.M. 1** | 37.8 ng/ µl |

### 3.4.2 Conventional PCR

In the light of the data obtained at the end of the de novo whole genome analysis, it was observed that there was a single chitinase gene belonging to the *P. mandelii* KGI_MA19 strain with a length of 1059 base pairs, consisting of 353 amino acids, and the molecular weight of the protein was 38.68 kDa. According to the BLASTn database, the nucleotide sequence of the gene was 98.78 % identical to *Pseudomonas mandelii* LMG 21607. Furthermore, according to the Protein Data Bank database, it has an 84.7 % similarity to the chitinase protein of the *Pseudomonas* sp BG5 strain.

A gradient PCR reaction was performed with a primer pair containing the previously designed gene specific *Sac*I and *Not*I restriction enzyme sites. As a result of the applied PCR, it was observed on the agarose gel that the amplification was higher at 60 ℃ (Figure 3.13). In order to increase the amount of PCR product, the reaction was carried out at 60 ℃ with the same reaction content (Figure 3.14) PCR products were controlled at 0.7 % agarose gel.

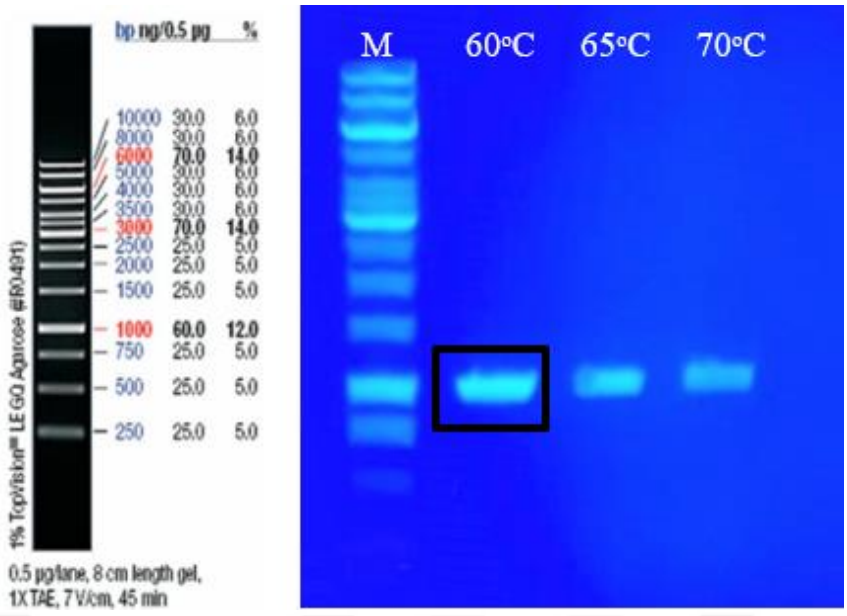


**Figure 3. 13** Image of a PCR result on an agarose gel electrophoresis. Three distinct temperatures were used for gradient PCR: 60 °C, 65 °C, and 70 °C were used for the first, second, and third lane. M: Marker, GeneRuler 1 kb, and DNA Ladder 1. 60 °C PCR product 2. 65 °C PCR product 3. 70 °C PCR product.

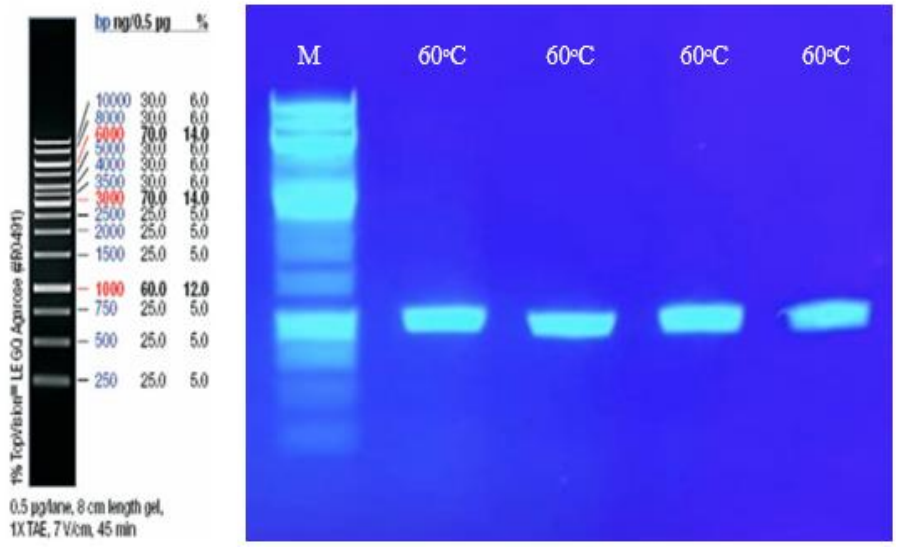


**Figure 3. 14** Image of a PCR result on an agarose gel electrophoresis. 60 °C were used for the first, second, third and fourth lane. M: Marker, GeneRuler 1 kb, and DNA Ladder, Lane 1,2,3,4. 1. 60 °C PCR product.

### 3.4.3 PCR product purification

For PCR purification, PCR purification kit was used, and the results were confirmed on a 0.7 % agarose gel (Figure 3.15). A purified PCR product with 1062 base pairs and the correct size was observed at the end of the process.

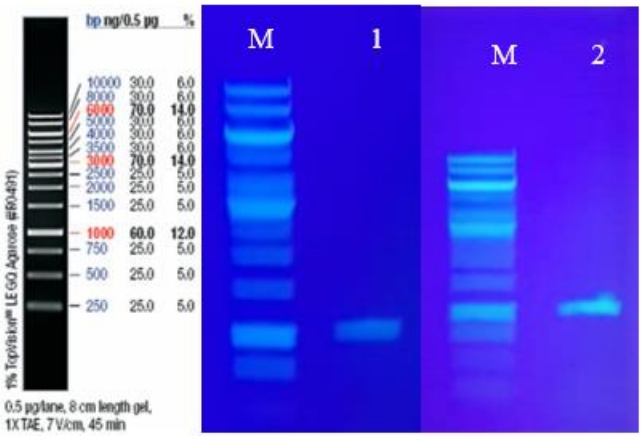


**Figure 3. 15** PCR purification products controlled with 0.7 % agarose gel electrophoresis. M: DNA ladder, 1 and 2: Product of PCR purification.

### 3.4.4 Digestion of PCR product and vector

The cut and uncut states of the PCR product and the expression vector pET28a (+) vector were visualized on a 0.7 % agarose gel (Figure 3.16). Then, their concentrations were measured in the nanodrop after enzyme digestion with *Not*I and *Sac*I enzymes for the PCR product and the expression vector pET28a (+). Then, passed into the ligation step.

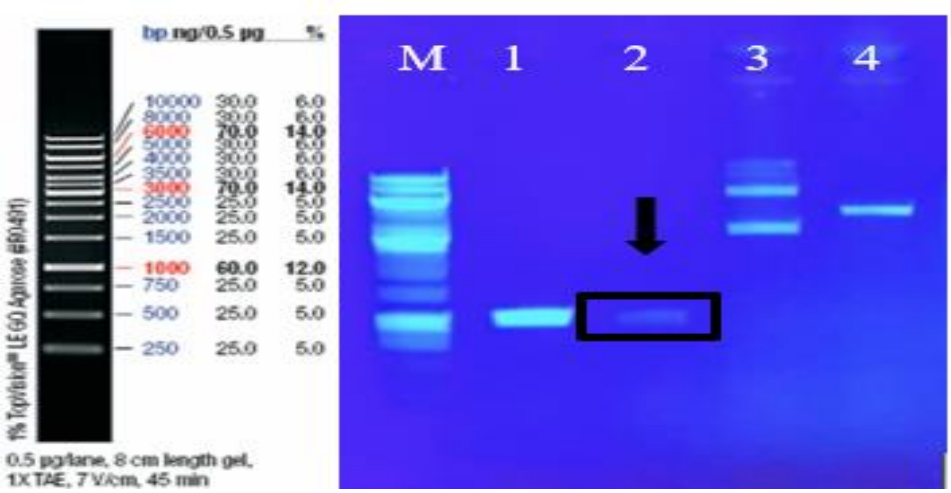


**Figure 3. 16** Control of the digestion PCR product and expression vector on 0.7% agarose gel. M: Marker 1. Uncut PCR product 2. Cut PCR product 3. Uncut pET28a (+) DNA 4. Cut pET28a (+).

**Table 3. 6** Nanodrop values of PCR product and plasmid before and after digestion.

| **Absorbance of Ligation Product (260nm)** | |
|---|---|
| 1 | 14 ng/ µl |
| 2 | 4 ng/ µl |
| 3 | 53.9 ng/ µl |
| 4 | 10 g/ µl |

### 3.4.5 Ligation

The chitinase gene was cloned into the pET28a (+) expression vector and converted into *E.coli* BL21 cells, which were then incubated overnight at 37 °C in LB media with kanamycin (40mg/ml). Following that, the transformation products were observed. Among the colonies that reproduced, 16 colonies in the center, particularly the little ones, were chosen (Figure 3.17).

**Figure 3. 17** Imaging of plates containing kanamycin at the end of the transformation. Colonies formed by BL21 competent cells of the ligation product formed at the end of the ligation step established at a ratio of 1:5 (vector:ligation).

### 3.4.6 Colony PCR

Among the colonies obtained as a result of ligation and transformation, 16 were selected. To determine whether the selected colonies contain gene products or not, the colony PCR reaction was established by using the sample taken from each colony and gene-specific primers. The PCR products obtained at the end of the reaction were checked on a 0.7% agarose gel (Figure 3.18).

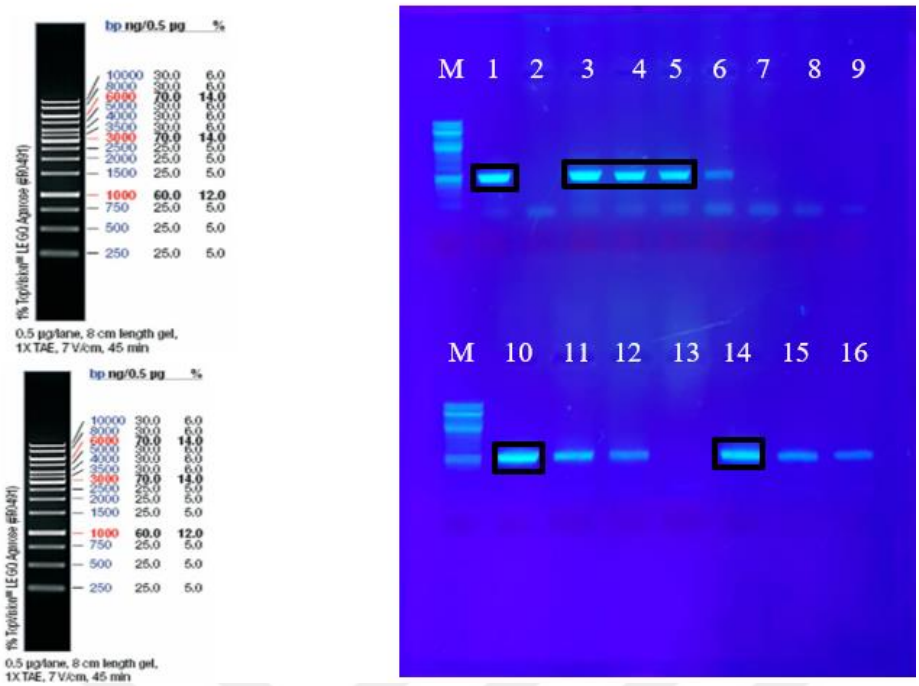


**Figure 3. 18** Control of colony PCR products on 0.7% agarose gel. M: Marker Lane 1-16. Colony 1-16.

According to the agarose gel results, 11 positive colonies were observed, which were thought to carry the gene product of 1062 bp length.

### 3.4.7 Plasmid isolation and control cleavage with restriction enzyme *EcoR*V

At the end of the colony PCR, the 1st and 3rd colonies, which were the brightest bands, were grown in LB medium, and plasmid isolations were performed as previously described. The plasmids obtained were cut with the fast digestion *EcoR*V enzyme, which cuts the plasmid from a single site, and then controlled with agarose gel (Figure 3.19).

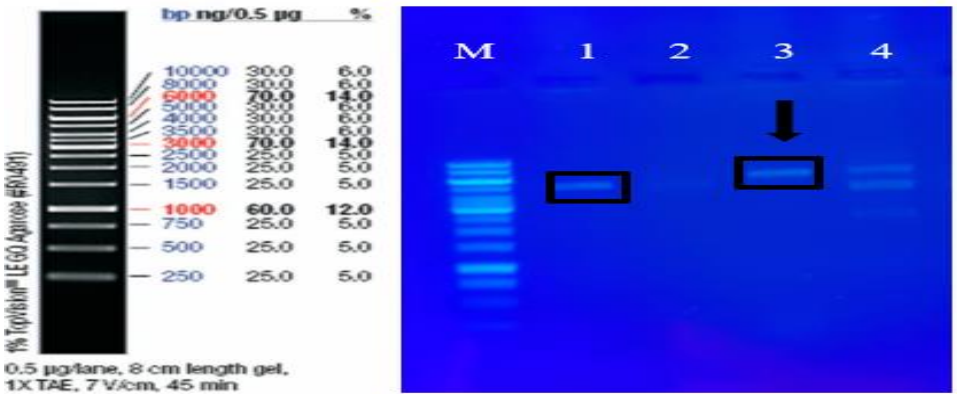


**Figure 3. 19** Control of the presence of the gene product in the pET28a (+) by EcoRV enzyme digestion on a 0.7% agarose gel. M. Marker 1. Cut pET28a (+) plasmid 2. Selected Colony 1 3. Selected Colony 3 4. Uncut pET28a (+).

Since the genomic size of the pET 28a (+) plasmid is 5369 bp and the size of the PCR product is 1062 bp, the length of the base pair to be obtained should be approximately 6500bp on the agarose gel. As a result of the agarose gel, 3rd colony was considered to have the gene product and therefore the sample was sent for Sanger sequencing analysis.

Sanger sequencing was done by Genometry Biotechnology Limited Company (Izmir, Turkey). Snapgene Viewer tool was used to validate the results. According to the data, the chitinase gene was appropriately and accurately introduced into the pET28 a (+) expression vector. The chitinase gene sequence established as a result of Sanger sequencing analysis with the Snapgene Viewer program:

5'-
CGGAGCTCATGTCAAAATTCGACTTTACGTTATTGAAATCACCACTCGCC
GACGCCGCTTCGTTGATGCCGAGCATTGCCGGCAAAAAGATCCTCATGGG
CTTCTGGCACAACTGGCCCGCCGGGCCGAGCGACGGTTATCAGCGTGGCC
AGTTCGCCAACATCGCGCTGCAGGATGTGCCCAAGGATTACAACGTGGT
GGCCGTGGCCTTCATGAAGGGCAACGGCATCCCGACCTTCAAGCCGTAC
AACTTGTCCGACGCCGAGTTCCGTCGCCAGGTCGGCGTGCTGAATAGTCA
GGGCAGGGCGGTGCTGATTTCCCTCGGTGGCGCCGACGCGCACATCGAG
TTGCACAAAGGTAACGAACAGCCGCTGGCCAACGAAATCATCCGTCTGG
TGGAAACCTATGGCTTCGATGGGCTGGACATCGATCTTGAACAGAGCGC
GATTGATTTCGCCGACAACAAGAGCGTCCTGCCTGCCGCGCTGAAGCTGG
TCAAGGACCATTACGCCGGTGAAGGCAAACACTTCATCATCAGCATGGC
GCCGGAGTTTCCGTACCTGACCACCGCCGGCAAATACGTCGGCTACCTCC
AGGCCCTGGAAGGCTATTACGACTTCATCGCCCCGCAGTATTACAACCAG
GGCGGCGACGGAATCTGGGTGCCGGAAGCCAATAACGGCAATGGCGCGT
GGATTGCGCAGAACAACGACGCGATGAAAGAGGACTTCCTGTTTTACCT
GACCGAAAGCCTGGTGAGCGGCACTCGCGGGTTCACGAAGATCCCGGCG
GACAAGTTCGTGATCGGCCTGCCTGCCAACGTCGACGCGGCGGCCACCG
GTTACGTGATCAATCCGGCGGCAGTGGTGAATGCCTTCAAACGTCTGGAT
GCCAAAGGACTTTCTATCAAGGGCCTGATGACCTGGTCGGCGAATTGGG
ACAACGGGGTCAATAGGGACCACGTGCCTTACAACTGGGAGTTCAGCCG
TCGTTACGGGCCGTTGATCAATGGCAAGCAGCTCTCTTCACAGGAAGACA
GCGCGATACCTGACGTCGCCAGCCAGCAGTAACGCCGGCGAA-3'

### 3.5 Expression of Chitinase

To enhance expression especially for *E. coli* cells, commercially available Magic Media$^{TM}$ medium was utilized for expression control (Figure 3.20).

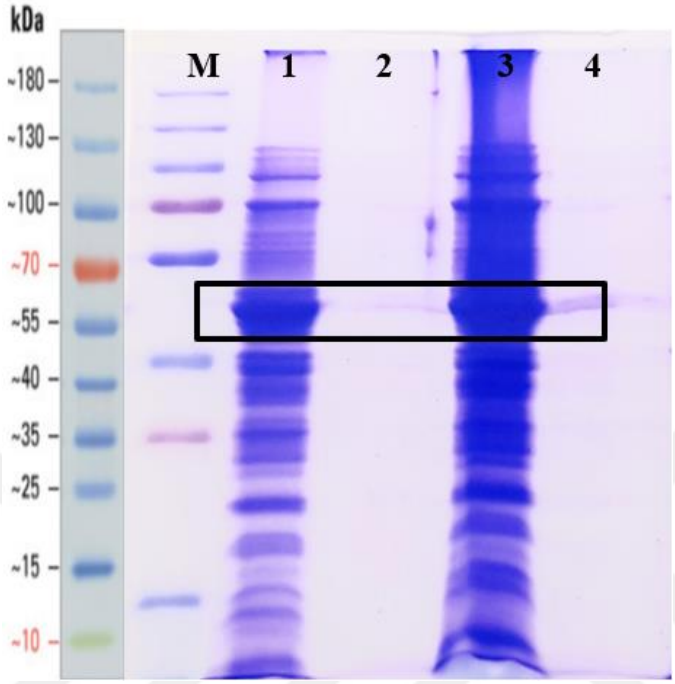


**Figure 3. 20** Control of expression of strain KGI_MA19 by SDS-PAGE. M: Molecular weight marker; Lane 1: Clear lysate (10µl) obtained after Magic Media expression, Lane 2: Supernant portion of cells precipitated at the end of expression, Lane 3: Clear lysate (20 µl) obtained after Magic Media expression, Lane 4: Supernant portion of cells precipitated at the end of expression.

According to the SDS-PAGE data, the expression of the required chitinase protein occurred successfully. The chitinase protein's molecular weight was determined as 38.68 kDa using the Protein Molecular Weight tool (https://www.bioinformatics.org/sms/prot mw.html). On SDS-PAGE, the detected bands of overexpression seem to be about 44 kDa in size. This is owing to the presence of twelve histidine tags in the N and C terminal regions. The protein's molecular weight was measured to be 43.96 kDa after six histidines were added to the N and C terminal regions. This demonstrated that the band that was overexpressed belonged to the chitinase protein.

### 3.6 Purification of Chitinase

After the expression control, the purification step of the chitinase protein was performed by gradient affinity chromatography using His-Trap columns (Figure 3.21).

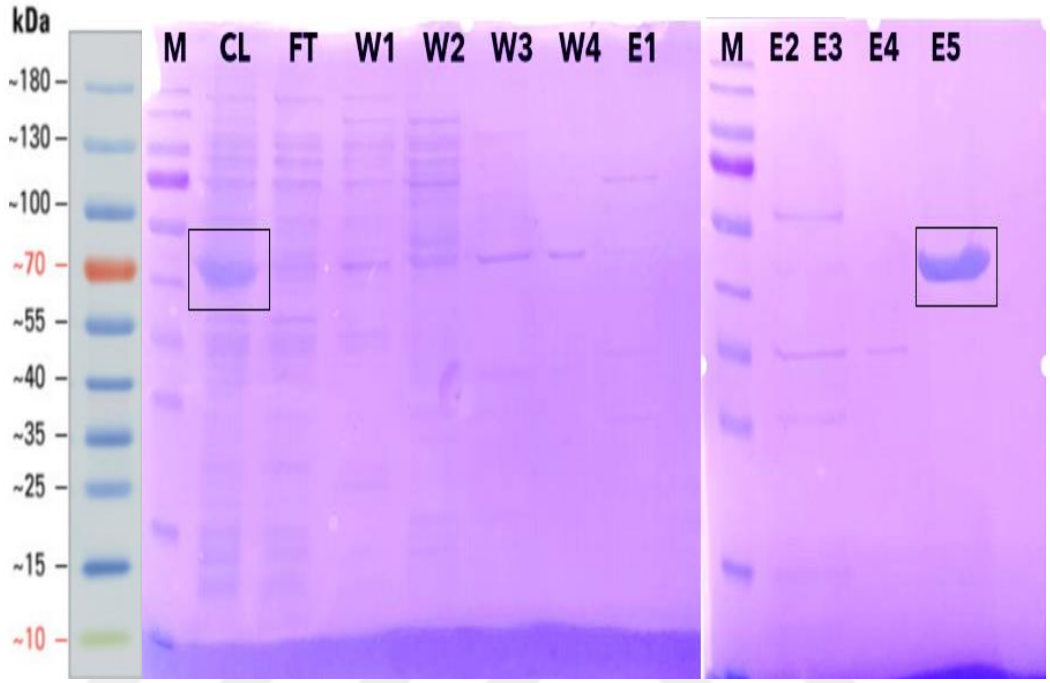


**Figure 3. 21** Control of purification of strain KGI_MA19 by SDS-PAGE. M: Molecular weight marker; Lane 1: Clear lysate obtained after Magic Media expression, Lane 2: Flow through obtained during purification, Lane 3: 20mM imidazole concentration(W1), Lane 4: 40mM imidazole concentration(W2), Lane 5: 60mM imidazole concentration (W3), Line 6: 80 mM imidazole concentration(W4), Lane 7: 100mM imidazole concentration (E1), Lane 8: 150 mM imidazole concentration(E2), Lane 9: 200 mM imidazole concentration(E3), Lane 10: 250 mM imidazole concentration (E4), Lane 11: 500 mM imidazole concentration(E5).

According to the SDS-PAGE result, it was observed that other proteins produced by the *E. coli* cells and causing impurities were separated from the chitinase protein at different imidazole concentrations. The chitinase enzyme was shown to be purely and concentratively purified in the E5 sample with 500 mM imidazole concentration.

During the western blot analysis, antibodies specific for the Histidine residue at the C terminus were used. The result of Western blot analysis for clear lysate obtained after expression with *E.coli* BL21 (DE3) competent cell and elutions obtained after His-Tag purification is shown in Figure 3.22.

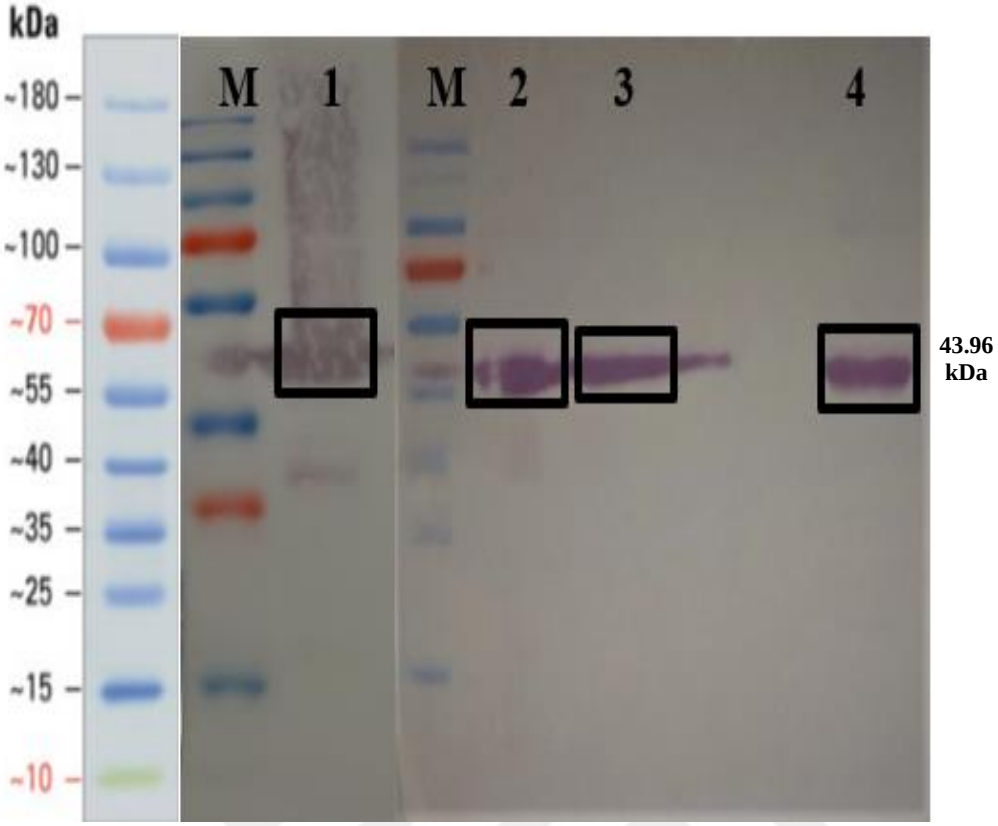


**Figure 3. 22** Western blot analysis of purified samples after His-Tag purification. M: Molecular weight marker, 1. Clear lysate obtained after Magic Media expression, 2,3,4. Purified chitinase by His-Tag chromatography.

According to the Western blot result obtained, the sample showing overexpression and purified by His-Tag affinity chromatography was shown to belong to the chitinase enzyme, which was successfully cloned.

### 3.7 Ultrafiltration

After purifying the chitinase protein purely by affinity chromatography, ultrafiltration was performed with a 30,000 MWCO ultrafiltration membrane to remove the imidazole content, concentrate the enzyme and replace the buffer. As a result of ultrafiltration, the control of the enzyme was achieved with 10% SDS-PAGE (Figure 3.23).

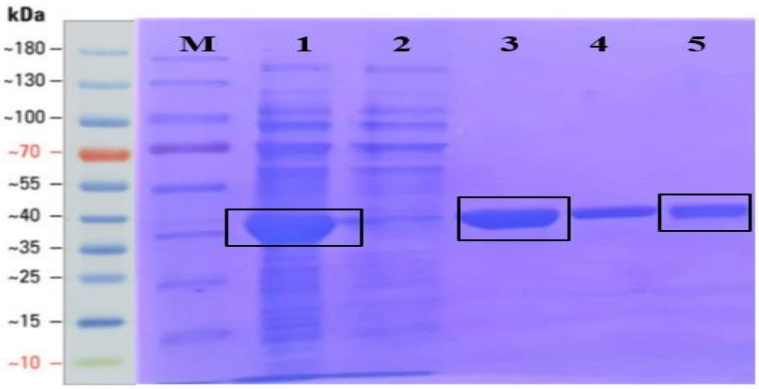


**Figure 3. 23** Ultrafiltration control of strain KGI_MA19 by SDS-PAGE.M: Molecular weight marker; Lane 1: Clear lysate obtained after Magic Media expression, Lane 2: Flow through obtained during purification, Lane 3: 500 mM imidazole concentration(E5) (20µl), Lane 4: 500 mM imidazole concentration(E5) (10 µl), Lane 5: Ultrafiltration product.

According to the results of SDS-PAGE, the enzyme clear lysate obtained at the end of ultrafiltration and its elution obtained after purification were compared. As a result, it was observed that the concentration of the enzyme increased at the end of ultrafiltration. In addition, the buffer (50 mM $KH_2PO_4$ pH:7) solution of the enzyme was changed.

### 3.8 Biochemical Characterization of Chitinase

#### 3.8.1 Determination of optimum temperature and temperature stability

As a result of the experiment, it was observed that the relative activity of chitinase enzyme reactions, in which 1 % colloidal chitin was used as a substrate, showed 100% of its relative activity at 30 ℃. Therefore, the optimum temperature of the cold adaptive chitinase enzyme was determined as 30 ℃ (Figure 3.25a). It has been observed that the activity of the enzyme is higher than 60 % at low temperatures such as 4 ℃ to 15 ℃ degrees. In addition, it was determined that the activity of the enzyme decreased up to 40% at elevated temperatures such as 50 ℃ and 60 ℃.

It was determined that the activity of the chitinase enzyme of *Exiguobacterium antarcticum*, another bacterial species isolated from Antarctica, was at an optimum temperature of 30 ℃ (Park et al., 2009). In addition, in studies with cold adaptive chitinases, it has been observed that the optimum temperatures may be variable between 15 ℃ and 50 ℃ (Ramli et al., 2011; Liua et al., 2019; Fu et al., 2020; Liua et al., 2020; He et al., 2021).

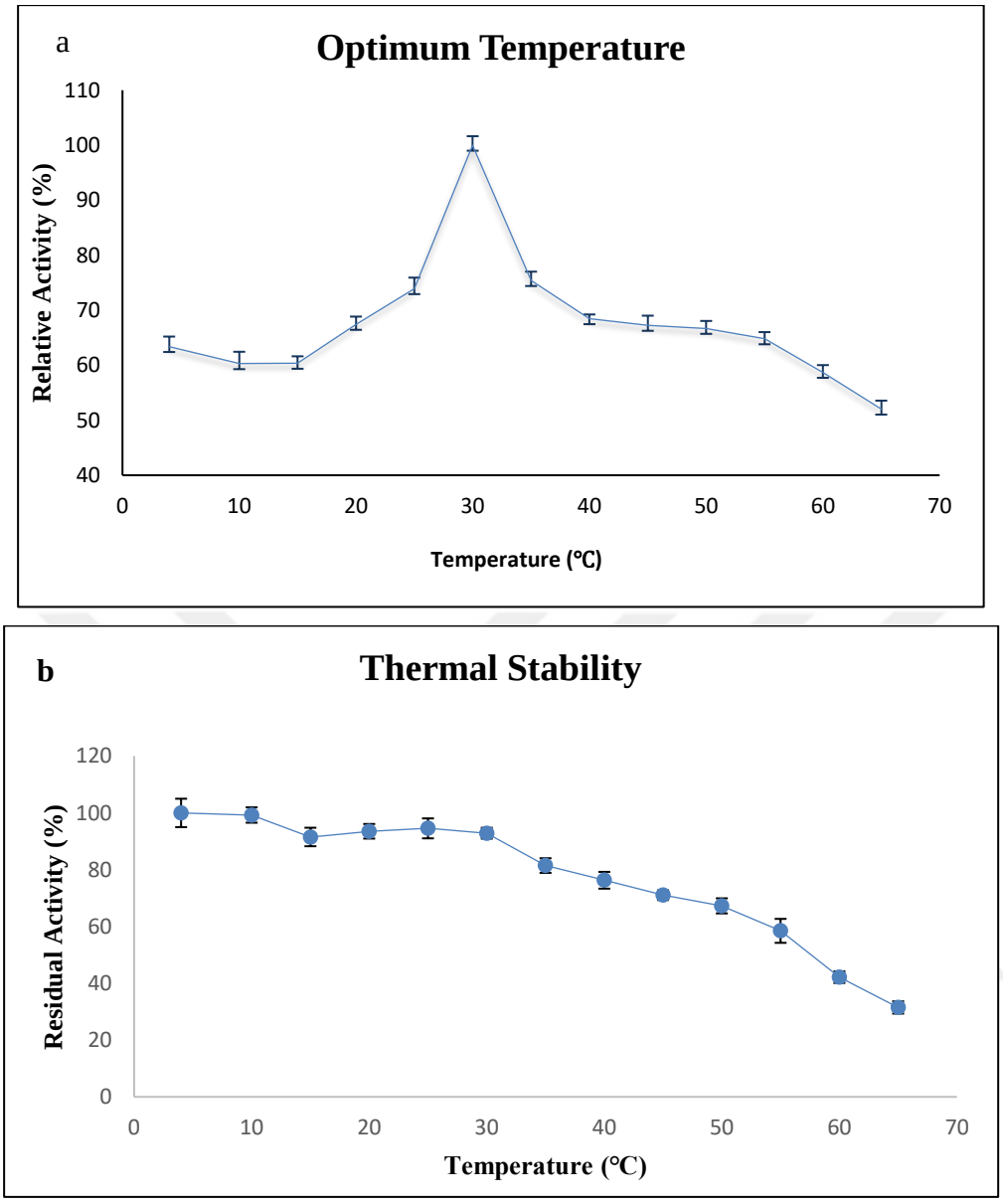


**Figure 3. 24** Optimum operating temperature and thermal stability of chitinase enzyme of *Pseudomonas mandelii* KGI_MA19 organism. **(a)** optimum operating temperature **(b)** thermal stability.

As a result of the experiment performed to determine its thermal stability, it was determined that the thermal stability of the chitinase enzyme belonging to the KGI_MA19 strain was between 4 ℃ and 35 ℃ (Figure 3.25b). Based on the information obtained for the thermal stability of the enzyme, its residual activity was observed above 90% between 4 ℃ and 30 ℃. It was observed that the thermal stability of the enzyme started to decrease at 35 ℃ and its residual activity decreased to 80%. In addition, it was determined that the thermal stability of the enzyme decreased up to 30% by increasing the temperature up to 65 ℃. In studies with *Sanguibacter*

*antarcticus* KOPRI 21702T chitinase, it has been observed that the basic stability of the enzyme can reach up to 40 ℃ (Park et al., 2009).

### 3.8.2 Determination of optimal pH and pH stability

After the enzyme activity assays performed at different pH levels, it was observed that the relative activity of the enzyme was 100% at pH 7. For this reason, the pH value at which the enzyme works optimally was determined as 7.00. In addition, while the residual activity of the enzyme is 50 % at low pH values, the enzyme activity decreases to 40 % at high pH values such as 9.00 and 10.00. (Figure 3.26a). In the optimum pH studies of the chitinase enzyme in the literature, the pH ranges of the chitinases of different organisms vary between 4 and 7.6 (Park et al., 2009; Ramli et al., 2011; Liua et al., 2019; Fu et al., 2020; Liua et al., 2020).

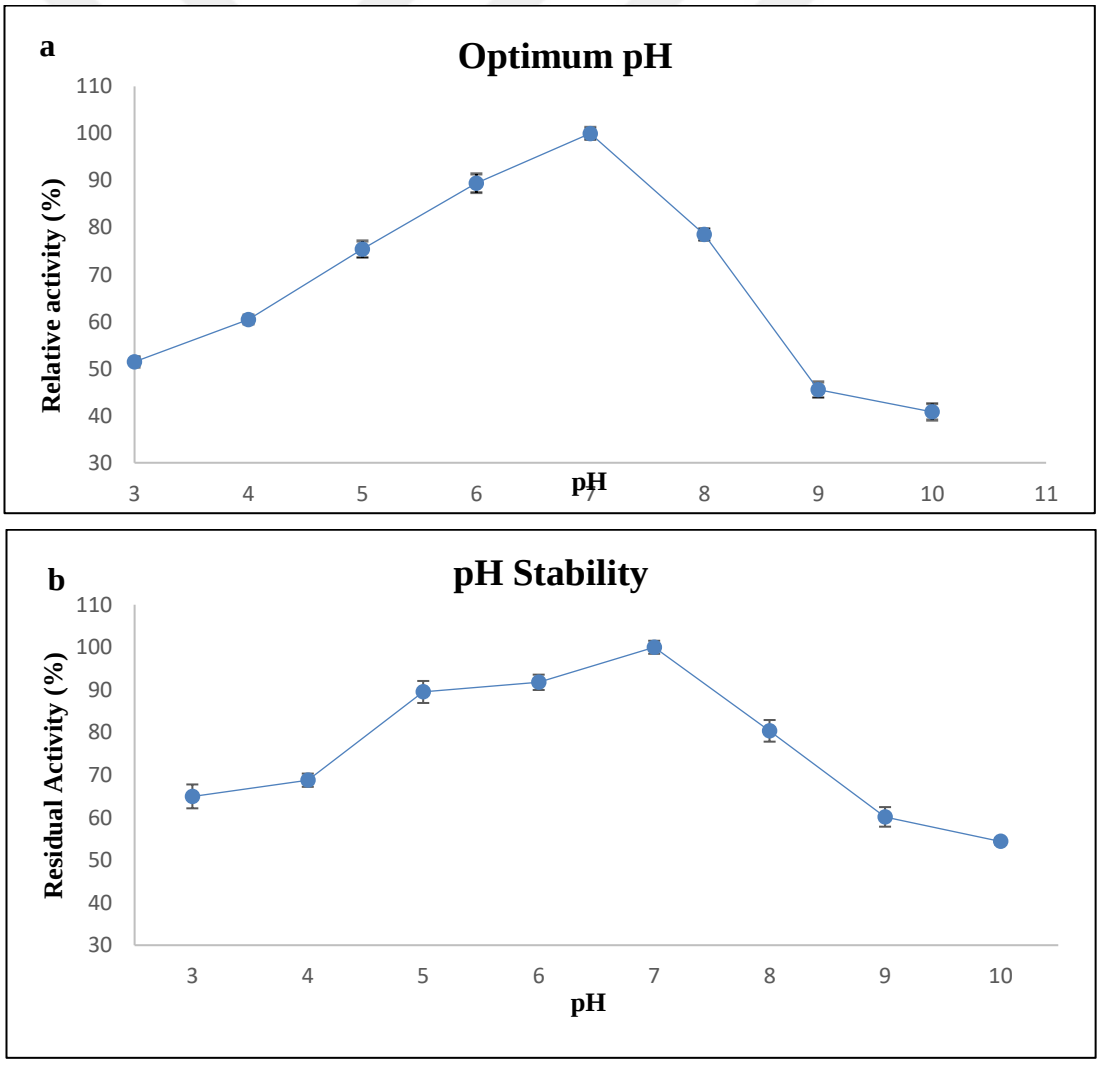


**Figure 3. 25** Optimum operating pH and pH stability of chitinase enzyme of Pseudomonas mandelii KGI_MA19 organism. (a) Optimum pH (b) pH Stability.

As a result of the experiment performed to determine its pH stability, it was determined that the thermal stability of the chitinase enzyme belonging to the KGI_MA19 strain was between 3.00 and 8.00 (Figure 3.26b). In determining the stability of the enzyme at different pH values, it was observed that the residual activity of the enzyme started from 60 % at low pH values and increased up to the optimum pH value. It was determined that the residual activity started to decrease at pH 8 and later and decreased up to 55 % at pH 10. In pH stability studies for cold adaptive chitinase enzymes in the literature, it has been known that enzymes belonging to different organisms remain stable between pH 4 and 11 (Park et al., 2009; Ramli et al., 2011; Fu et al., 2020; He et al., 2021).

### 3.8.3 Effects of metal ions, some organic solvents and reagents on chitinase enzyme

The result of the reaction established in order to observe the effects of metal ions, organic solvents and reagents on the chitinase enzyme is shown in Figure 3.27.

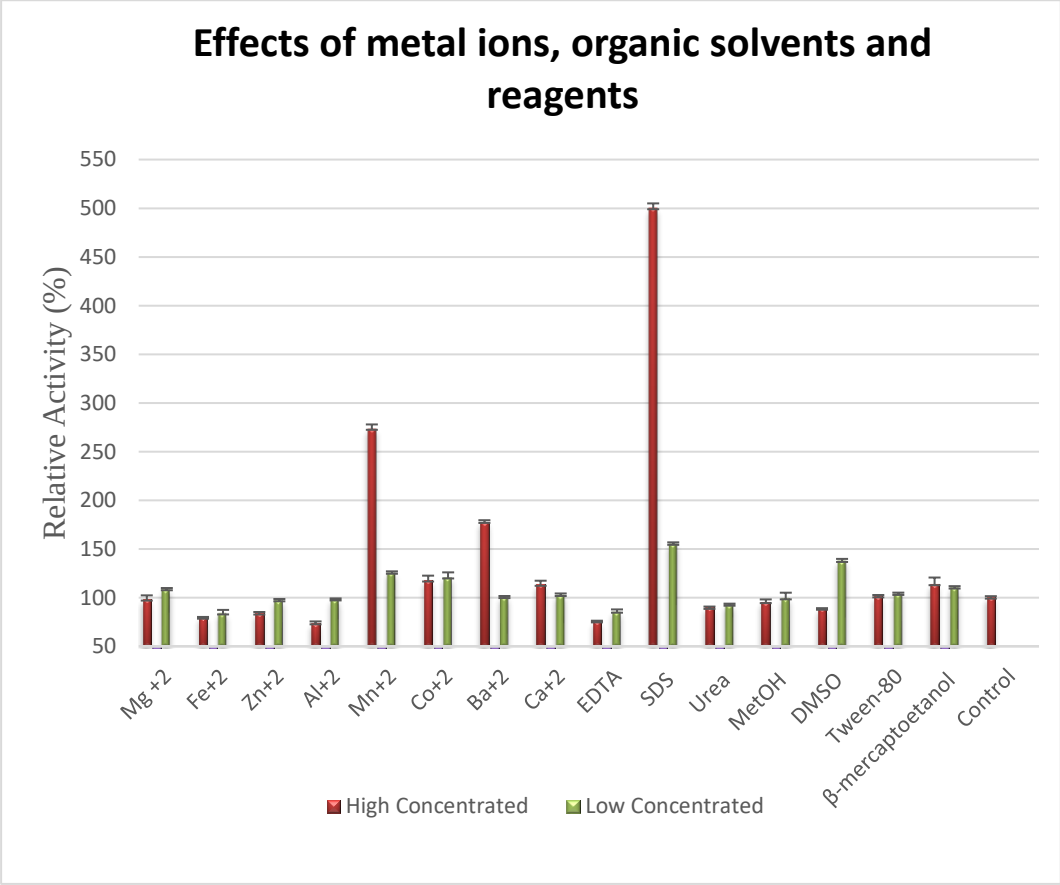


**Figure 3. 26** The effect of metal ions, organic solvent detergents on the chitinase enzyme of the organism *Pseudomonas mandelii* KGI_MA19. Red: Highly concentrated substances Green: Low concentrated substances.

Considering the effect of metal ions, it was observed that 10mM $Mn^{+2}$ increased the activity of the chitinase enzyme approximately 3 times, while 10mM concentration of $Ba^{+2}$ increased approximately 2 times. In addition, it was observed that 10 mM $Ca^{+2}$ and 10 mM $Co^{+2}$ had an activity-enhancing effect on the enzyme. Furthermore, it was observed that 10 mM $Al^{+2}$ reduced the enzyme's activity by approximately 1/4, and that both 1mM and 10mM concentrations of $Fe^{+2}$ ion had an inhibitory effect on the enzyme.

Among organic solvents and reagents, it was observed that 5% SDS concentration increased enzyme activity 5 times, and 10% and 20% β-mercaptoethanol increased enzyme activity. It was determined that 10% DMSO increased the enzyme activity as well as 20% DMSO had an inhibitory effect.

As a result, the activators of chitinase enzyme belonging to KGI_MA19 strain are $Mn^{+2}$ (10mM), $Ba^{+2}$ (10mM), SDS (1%, 5%), DMSO (10%), β mercaptoethanol (10%, 20%) while inhibitors, EDTA (1mM, 10mM), $Al^{+2}$ (10mM), $Fe^{+2}$ (1mM, 10mM), $Zn^{+2}$ (10mM), urea (1mM, 10mM) and DMSO (20%).

One of the inhibitors of EaChi39 obtained from *Exiguobacterium antarcticum*, which is a psychrotolerant organism with an optimum operating temperature of 30 degrees, has been determined in the literature as SDS. In addition, one of the inhibitors of the chitinase enzyme belonging to *Sanguibacter antarcticus* KOPRI 21702T, with an optimum operating temperature of 37 °C, is also reported in the literature as SDS. The effect of SDS on the chitinase enzyme of KGI_MA19 was determined to increase its activity 5-fold compared to the control.

According to the compare with literature, enzyme has the distinct advantage of gaining prominence in biotechnology because of its biochemical features. Enzyme industrial usage may save energy thanks to its optimal operating temperature of 30 ℃ which is near to room temperature. Furthermore, capacity of the enzyme to retain stability at acidic pH and activity at low pH values, enhances its additive value. Although there are common points with its homologs in terms of inhibitors and activators, the activator effect of SDS on the enzyme has not been defined in the literature as a result of comparisons.

To sum up, the enzyme chitinase was successfully produced recombinantly from an Antarctic-derived organism. It is thought that this enzyme will be one of the biocatalyst

candidates with industrial importance in recycling chitin, which is mostly produced by consuming seashells, without the requirement for high temperature.

## 4. CONCLUSION

*Pseudomonas mandelii* KGI MA19 was found to be a novel strain using a de-novo whole genome analysis after freeze-thaw stress and 16S rDNA analysis from soil samples collected from the Antarctic Peninsula. As a result of the studies carried out to characterise the strain, it was observed that the organism is gram-negative and rod-shaped. The optimum growth conditions of the organism include 20 ℃, NaCl 2 % and pH 6.00 and so, KGI_MA19 has psychrotolerant and halotolerant properties.

The biochemical characterisation of the organism has shown that it can break disaccharide molecules more effectively than other strains. Due to this feature, it is assumed that KGI_MA19 can be used in the industrial sector. In addition to being resistant to eight different antibiotics, the bacterium may also produce biofilms, according to its biochemical properties. Psychrophilic bacteria are known to form biofilms at lower temperatures, however, this study demonstrated that the biofilm development characteristic was strongest at the organism's optimal living temperature. It indicates that each strain is unique and that high temperatures produce a variety of stressful situations.

Chitin waste is growing with increasing demand for shellfish, especially in the outer shells of marine animals. The breakdown of chitin and the use of the products arising from its disintegration are of major significance in the industrial sector, particularly in the food, agricultural, and pharmaceutical industries. As a result of this study, 38.68 kDa chitinase enzyme belonging to *Pseudomonas mandelii* KGI MA19 bacterium was produced recombinantly and the characterization of the enzyme was accomplished. As a result of this study, the 38.68 kDa chitinase enzyme belonging to *Pseudomonas mandelii* KGI MA19 bacterium was produced recombinantly and the characterization of the enzyme was accomplished.

The characterization experiments represented that the optimum enzyme temperature and pH values were 30 °C and 7.00, respectively. Studies to evaluate its thermal and pH stability have shown that the enzyme is thermal stable between 4℃ and 35 °C and

has a wide pH range between 3.00 and 8.00. Additionally, it has been shown that $Mn^{+2}$, $Ba^{+2}$, SDS, DMSO, and β-mercaptoethanol are the enzyme activators. However, 20% of EDTA, $Al^{+2}$, $Fe^{+2}$, $Zn^{+2}$, urea and DMSO concentrations inhibit the enzyme activity. The phenotypic enzyme characterization will allow for taking a place of the chitinase enzyme, which comes to the forefront in terms of its properties, has a single domain and endochitinolytic activity, in the industrial area.

Biomolecules that have been discovered in the last several decades have an increasing demand in the industrial sector. New species and biomolecules discovered under harsh circumstances are being studied to fill in the gaps in the industry. Phenotypic and biochemical characterization of the organism reveals that the organism and biomolecules of *Pseudomonas mandelii* KGI MA19 display numerous distinct properties. Using these features in the industrial sector can reduce energy consumption and improve the speed and reliability of operations. In conclusion, *Pseudomonas mandelii* KGI_MA19 and its biomolecules are promising for the industry.

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## APPENDICES

**APPENDIX A:** Laboratory Equipment

**APPENDIX B:** Culture Media Composition

**APPENDIX C:** Buffers and Solutions

**APPENDIX D:** Chemicals, Enzymes and Kits

## APPENDIX A: Laboratory equipment

**Analytical Balances:** Precisa BT 610C

**Autoclave:** TOMY SX-700E High Pressure Steam Sterilizer

**Centrifuges:** Microfuge 18, Beckman Coulter; AvantiJ30I, Beckman Coulter.

**Deep freezers and refrigerators:** Ultra low freezer MDF-U4086S, Sanyo; Ultra low freezer MDF-U333, Sanyo; 1061 M refrigerator, Arcelik.

**Electrophoresis equipment:** Horizon 11.14, Whatman, Biometra Gel Casting System; Horizon 20-25, Whatman, Biometra Gel Casting System; Biorad Mini-PROTEAN® Gel Casting System; Mini Trans-Blot Electrophoretic Transfer Cell, 170-3930, 170-3935, Bio-Rad.

**Gel Documentation System:** UVIpro GAS7000; Biometra UVStar Transilluminator

**Heat Block:** BioSan Bio TDB-100

**Ice machine:** AF 10, Scotsman.

**Laminar flow cabinet:** Faster Laminar Flow BH-EN 2003.

**Magnetic stirrer:** Heidolph Standard

**Microplate reader:** Benchmark Microplate Reader, Bio-Rad; CLARIOstar Multimode Microplate Reader, BMG LABTECH

**Nanodrop:** Scientific

**Orbital shaker:** Forma orbital shaker, Thermo Electron Corporation.

**pH meter:** Inolab pH level 1, WTW GmbH & Co KG

**Pipettes:** epResearch Pipettes, Eppendorf

**Sonicator:** Sonoplus, Bandelin

**Thermal Cycler:** MiniAmp Plus

**Thermomixer:** Thermoshaker Ts1, Biometra.

**UV-Visible light spectrophotometer:** UV Star Biometra

**Vortex:** Scientific Industries

**Water Bath:** Memert

## APPENDIX B: Culture media composition

### Luria Bertani (LB) Medium (1000ml)

5 g yeast extracts, 10 g tryptone and 5 g NaCl were dissolved in distilled water up to 1 L. Autoclave was applied for 15 minutes at 121 °C to sterilize the LB medium.

### Luria Bertani (LB) Agar Medium (1000ml)

5 g yeast extracts, 10 g tryptone and 5 g NaCl were dissolved in distilled water up to 1 L. After that 15 g of agar was added in order to prepare solid media. Autoclave was applied for 15 minutes at 121 °C to sterilize the LB Agar medium.

### Tripton Yeast Glucose (TGY) Medium (1000ml)

5 gr tryptone,1 gr glucose and 3 gr yeast extract were dissolved in distilled water up to 1000ml. Sterilized by autoclaving at pressure 121°C for 15 minutes.

### Tripton Yeast Glucose (TGY) Agar Medium (1000ml)

5 gr tryptone,1 gr glucose and 3 gr yeast extract were dissolved in distilled water up to 1000ml. After that 15 g of agar was added in order to prepare solid medium. Sterilized by autoclaving at pressure 121°C for 15 minutes.

## APPENDIX C: Buffers and solution

**Agarose Gel (1 %):** 0,40 g agarose was melted in 40 mL TAE Buffer (1X), following to cooling down and gel was poured into the tray.

**Chloramphenicol stock solution:** 12.5 mg of chloramphenicol was dissolved in 1 mL of $dH_2O$, filter sterilized and stored at -20 °C.

**Citrate Buffer (50 mM, pH 3.0):** 1.285 g of Sodium Citrate and 121.1 mg of Citric Acid were dissolved in 45 ml of $dH_2O$. The pH was adjusted to 3.0 with 1 N HCl and the final volume was made up to 50 mL with $dH_2O$.

**Glycine-NaOH Buffer (50 mM, pH 9.0):** 0.187 g of glycine was dissolved in 45 ml of dH2O. The pH was adjusted to 9.0 with 1 N NaOH and the final volume was made up to 50 mL with $dH_2O$.

**Glycine-NaOH Buffer (50 mM, pH 10.0):** 0.187 g of glycine was dissolved in 45 ml of $dH_2O$. The pH was adjusted to 10.0 with 1 N NaOH and the final volume was made up to 50 mL with $dH_2O$.

**His-tag purification lysis buffer:** 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 8

**His-Trap binding buffer:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, pH 7.4

**HisTrap Elution Buffer 1:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 100 mM imidazole, pH 7.4

**HisTrap Elution Buffer 2**: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 200 mM imidazole, pH 7.4

**HisTrap Elution Buffer 3:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 250 mM imidazole, pH 7.4

**HisTrap Elution Buffer 4**: 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 500 mM imidazole, pH 7.4

**HisTrap Wash Buffer 1:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, pH 7.4

**HisTrap Wash Buffer 2:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 40 mM imidazole, pH 7.4

**HisTrap Wash Buffer 3:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 60 mM imidazole, pH 7.4

**HisTrap Wash Buffer 4:** 20 mM sodium phosphate buffer (pH 7.4), 0.5 M NaCl, 80 mM imidazole, pH 7.4

**Kanamycin stock solution:** 40 mg of kanamycin was dissolved in 1 mL of $dH_2O$, filter sterilized and stored at -20 °C.

**SDS-PAGE Destaining Solution:** 40 mL of methanol, 10 mL of acetic acid, 50 mL of $dH_2O$.

**SDS-PAGE Resolving gel (10 %):** 2.3 mL of $dH_2O$, 1.275 mL of 40 % Acrylamid/Bisacrylamid (37.5:1), 1.3 mL of Tris-HCl pH 8.8, 50 µL of 10 % SDS, 50 µL of 10 % APS, 5 µL of TEMED.

**SDS-PAGE Running buffer (10X):** 30.3 g Tris, 144 g glycine and 10 g SDS were dissolved in 1L with $dH_2O$. 10X Running buffer was diluting to 1X Running buffer with $dH_2O$.

**SDS-PAGE Sample loading buffer (5X):** 90 mM TRIS-HCI, pH 6.8, 20 % Glycerol, 2 % SDS, 0.02 % Bromophenol blue, 0.1 M DTT

**SDS-PAGE Stacking gel (5 %):** 1.4 mL of dH2O, 250 µL of 40 % Acrylamid/Bisacrylamid (37.5:1), 250 µL of Tris-HCl pH 6.8, 20 µL of 10 % SDS, 20 µL of 10 % APS, 5 µL of TEMED.

**SDS-PAGE Staining Solution:** 0.1 g of Coomassie Brilliant Blue R-250, 40 mL of methanol, 10 mL of acetic acid, 50 mL of dH2O.

**Sodium Acetat (50mM, pH:4.0):** In 45 ml of distilled water, 0.205 g of sodium acetate was dissolved. The pH was adjusted to 4.0 with 1N acetic acid and the final volume was completed to 50 ml with $dH_2O$.

**Sodium Acetat (50mM, pH:5.0):** In 45 ml of distilled water, 0.205 g of sodium acetate was dissolved. The pH was adjusted to 4.0 with 1N acetic acid and the final volume was completed to 50 ml with $dH_2O$.

**Sodium Acetat (50mM, pH:6.0):** In 45 ml of distilled water, 0.205 g of sodium acetate was dissolved. The pH was adjusted to 6.0 with 1N acetic acid and the final volume was completed to 50 ml with $dH_2O$.

**TAE Buffer (50X):** 242 g Tris base (MW=121.14 g/mol), 14.6 g EDTA and 57.1 mL glacial acetic acid were dissolved in distilled water up to 1 L. Solution was stored at room temperature.

**TBS Buffer (10X):** 24 g Tris base (MW=121.14 g/mol) and 88 g NaCl (MW=58.4) were dissolved in 900 mL distilled water, pH is adjusted to 7.6 using HCl, filled up to 1000 mL with distilled water.

**TBS-T Buffer (1X):** 100 mL of 10X TBS and 1 mL of Tween-20 detergent were mixed and filled up to 1000 mL with distilled water.

**Transfer Buffer (10X):** 144 g . glycine (MW= 75,07 g/mol) and 30.2 g Tris base (MW=121.14 g/mol) were dissolved in distilled water up to 1 L.

**Transfer Buffer (1X):** 100 mL of 10X Transfer buffer and 100 mL of methanol were mixed and filled up to 1000 mL with distilled water.

**Tris HCl (50mM, pH:7.0):** 0.30 g of Tris was dissolved in 45 ml of dH2O. The pH was adjusted to 7.0 with 1 N HCl and the final volume was made up to 50 mL with $dH_2O$.

## APPENDIX D: Chemicals, enzymes and kits

### Chemicals

**40 % Acrylamide Solution:** Bio-Rad

**Acetic acid:** Sigma-Aldrich

**Agarose:** Multicell

**Bromophenol Blue:** Sigma-Aldrich

**Calcium Chloride:** ZAG Kimya

**Chitin:** Sigma-Aldrich

**Chloramphenicol:** Biochemica

**Coomassie Brilliant Blue R-250:** Sigma-Aldrich

**dNTP:** Thermo Fisher Scientific

**EDTA:** Sigma-Aldrich

**Ethanol:** Sigma-Aldrich

**Glucose:** Sigma-Aldrich

**Glycerol:** Merck

**Glycine:** Sigma-Aldrich

**Hydrochloric Acid:** Sigma-Aldrich

**Imidazole:** Sigma-Aldrich

**Isopropanol:** Merck

**Kanamycin:** Merck

**Magnesium Chloride:** Sigma-Aldrich

**Methanol:** Sigma-Aldrich

**N-acetyl glucosamine:** Sigma-Aldrich

**His Trap Column:** Sigma-Aldrich

**Page Ruler Prestained Protein Ladder:** Thermo Scientific

**TEMED:** Sigma-Aldrich

**Skim Milk Powder:** Sigma-Aldrich

**Sodium Chloride:** Merck

**Sodium Dodecyl Sulphate:** BDH Laboratory

**Sodium Phosphate:** Sigma-Aldrich

**Tris Base:** Sigma-Aldrich

**Tryptone:** Sigma-Aldrich

**Yeast Extract:** Sigma-Aldrich

**β-mercaptoethanol:** Sigma

**1 kb DNA Ladder:** Bio-Rad

**1-Step NBT/BCIP Substrate™:** Thermo Scientific

**2X HF Buffer:** New England Biolabs

**6X DNA loading dye:** EZ-Vision

**6x-His Tag Monoclonal Antibody (3D5):** Thermo Fisher Scientific

**10X Tango Buffer:** Thermo Fisher Scientific

**10X Ligation Buffer:** Roche

**Enzymes**

**Fast Digest *EcoR*V:** Thermo Fisher Scientific

***Not*I :** Thermo Fisher Scientific

**Phusion DNA Polymerase:** New England Biolabs

***Sac*I:** Thermo Fisher Scientific

**Taq Polymerase:** Roche

**T4 DNA Ligase Enzyme:** Roche

**Kits**

**Bradford Assay Kits:** Bio-Rad

**High Pure Plasmid Isolation Kit:** EcoSpin

**High Pure PCR Product Purification Kit:** EcoSpin

**CURRICULUM VITAE**

**Name Surname** **:** Emine Tuğçe SARAÇ CEBECİ

**EDUCATION:**

- **B.Sc.** **:** 2012, Halic University, Faculty of Science and Letters, Department of Molecular Biology and Genetics

**PUBLICATIONS/PRESENTATION ON THE THESIS**

- **Saraç Cebeci E.T., Gül Karagüler N. 2022.** Recombinant Production and Characterization of Chitinase from *Pseudomonas mandelii* KGI_MA19. *IGRS'22 - Internatıonal Graduate Research Symposıum*, June 1-3,2022 Istanbul, Turkiye.