**KARADENİZ TECHNICAL UNIVERSITY**
**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

**DEPARTMENT OF MOLECULAR BIOTECHNOLOGY**

# EXTRACELLULAR EXPRESSION OF *IDEONELLA SAKAIENSIS* PETase MUTANT IN PHOTOSYNTHETIC MICROALGA *Chlorella vulgaris*

**MASTER of THESIS**

**Mehmet EL**

**FEBRUARY 2021**
**TRABZON**

**KARADENİZ TECHNICAL UNIVERSITY**
**GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES**

**DEPARTMENT OF MOLECULAR BIOTECHNOLOGY**

# EXTRACELLULAR EXPRESSION OF IDEONELLA SAKAIENSIS PETase MUTANT IN PHOTOSYNTHETIC MICROALGA *Chlorella vulgarıs*

**Mehmet EL**

**This thesis is accepted to give the degree of**
**"MASTER OF SCIENCE"**
**By**
**The Graduate School of Natural and Applied Sciences at**
**Karadeniz Technical University**

**The Date of Submission : 04.01.2021**
**The Date of Examination : 01.02.2021**

**Supervisor : Uğur UZUNER**

**Trabzon 2021**

## PREFACE

This study named "Extracellular Expression of *Ideonella sakaiensis* PETase Mutant in Photosynthetic Microalgae *Chlorella vulgaris*" was prepared as a "Master's thesis" in Karadeniz Technical University, Institute of Science, Department of Molecular Biotechnology.

I would like to show my greatest appreciation to my thesis advisor Assistant Professor Uğur Uzuner, who provided to support me to develop and complete this thesis. Having accepted my thesis defense jury membership and listened to me, Prof. Dr. Sabriye Çanakçı and Assoc. Prof. Utku Avcı.

At the same time, I would like to thank all my professors working in the Department of Molecular Biotechnology of Karadeniz Technical University.

Prof. Dr. Ali Osman Beldüz and Asst. Prof. Selcen Çelik Uzuner, who did not refrain from helping to study. I would like to thank Lect. PhD Elif Ayazoğlu Demir, Sinan Tetikoğlu, Ramazan Çakmak and Huriye Patan separately.

Also, I would like to thank all my friends in our department.

Finally, I would like to express my endless thanks to my dear family members Ömer, Rezzan and Mustafa El, who have been with me with all their financial and moral support since the first day I started my education.

I attribute this thesis to my mother, Nimet EL, who is my first teacher, my mentor, who always carried me forward with her great support.

Mehmet EL
Trabzon, 2021

## THESIS ETHICS STATEMENT

I declare that I have completed this study titled "Extracellular expression of *Ideonella sakaiensis* PETase enzyme in photosynthetic microalga *Chlorella vulgaris*", which I submitted as a master's thesis, under the responsibility of my advisor Assistant Professor Uğur UZUNER from the beginning to the end, collected the data/samples myself, conducted the experiments/analyzes in the relevant laboratories, revealed the information I received from other sources in the text and references, and acted in accordance with scientific research and ethical rules during the study and accept any legal consequences in case the opposite occurs 01/02/2021

Mehmet EL

**TABLE OF CONTENTS**

**Page Number**

Yüksek Lisans Tezi

# ÖZET

## *Ideonella sakaiensis* PETase MUTANTININ FOTOSENTETİK MİKROALG Chlorella vulgaris'de HÜCRE DIŞI EKSPRESYONU

Mehmet EL

Karadeniz Teknik Üniversitesi
Fen Bilimleri Enstitüsü
Moleküler Biyoteknoloji Anabilim Dalı
Danışman: Dr. Öğr. Üye. Uğur UZUNER
2021, 78 Sayfa

PETaz enzimi polietilen terefitalat (PET) türevi plastiklerin hidrolizinden sorumlu temel enzim olarak 2017 yılında ilk kez tanımlanmıştır. İki farklı çalışmada belirlenmiş ve yüksek oranda aktivite artışı sağlamış en yüksek aktiviteli 3 farklı mutasyon belirlenmiş ve bunların kümülatif aktivite artışı durumları *in silico* modelleme ve moleküler kenetleme analizleri üzerinden dğrulandıktan sonra tek bir PETaz sekansında birleştirilmiştir. Oluşturulan PETaz nükleotit dizisi, sentetik olarak ticari yollardan sentezlendi ve PETaz3M olarak adlandırıldı. PCR yoluyla çoğaltılarak, pJET1.2 klonlama vektörüne klonlandı.

PETaz3M'i kodlayan nükleotit bölgesi, mikroalg ifade vektörü pOPT_cCa_mRuby2_Paromy'e aktarıldı. PETaz3M kodlayıcı DNA fragmanı, HSP70/RBCS2 füzyon promotor'un arkasına yerleştirildi. pOPT_cCa_mRuby2_Paromy vektöründe bulunan Kırmızı Floresan Proteinini (RFP) kodlayan bölge, *Bgl*II ve *Eco*RI enzimleriyle kesilerek çıkarıldı ve PETaz3M kodlayıcı DNA fragmanının bu bölgeye ligasyonu sağlandı. Böylece, PETaz3M kodlayan bölge, HSP70/RBCS2 promotoru ve 3' translasyona uğramayan bölge (3'UTR) arasına entegre edilerek, mikroalg konaklarına transformasyon için hazırlandı. pOpt_cCA_mRuby2_Paro_PETaz3M vektörünün *C. vulgaris* hücrelerine transformasyonu elektroporasyonla gerçekleştirildi. PETaz3M pozitif transformantlar, 50 µg/ml paromomisin içeren TAP besiyerinde üç ardışık nesil boyunca seçime tabi tutuldu. *C. vulgaris* transformantları'nın hücre dışı ekspresyonu, SDS-PAGE ile doğrulandı. PETase3M enziminin fonksiyonelliği, saflaştırılmış PETase3M ile muamele edilmiş PET filmlerinin ışık mikroskobu altında yüzey analizleri yapılarak doğrulandı.

Sonuç olarak, bu çalışma ile bir PETaz mutantının, ekstrem şartlara toleranslı *C. vulgaris* konaklarında hücre dışı üretimi ilk kez gerçekleştirildi.

**Anahtar Kelimeler:** *C. vulgaris* 211/11J, elektroporasyon, hücre dışı ekspresyon, mutant PETaz, transformasyon

Master of Thesis

SUMMARY

# EXTRACELLULAR EXPRESSION OF *IDEONELLA SAKAIENSIS* PETase MUTANT IN PHOTOSYNTHETIC MICROALGA *Chlorella vulgaris*

Mehmet EL

Karadeniz Technical University
The Institute of Science
Molecular Biotechnology Graduate Program
Supervisor: Assoc. Prof. Uğur UZUNER
2021, 78 Pages

The first PET degrading enzyme was first identified in 2017. Subsequently, various enzyme engineering studies were carried out on PETase enzyme. Three different mutations were determined from two different studies with high activity improvement, and combined within one PETase aa sequence. After being evaluated through in silico modeling and molecular docking analysis, their integration into the encoding nucleotide sequence of PETase enzyme was performed. Subsequently, the newly created PETase DNA fragment was commercially synthesized as a synthetic gene and called as PETase3M. Upon PCR amplification, PETase3M was cloned into the pJET1.2 cloning vector for long term maintenance.

The corresponding DNa fragment of PETase3M enzyme was transferred into the expression vector pOPT_cCA_mRuby2_Paromy for expression in microalgae hosts. In this vector, PETase3M DNA fragment was positioned behind the HSP70/RBCS2 fusion promoter system. For this task, native DNA region encoding Red Fluorescent Protein (RFP) in pOPT_cCA_mRuby2_Paromy vector was removed using *Bgl*II and *Eco*RI restriction enzymes, and the DNA fragment of PETaz3M was ligated into this place. In this way, the DNA fragment encoding PETase3M was integrated between HSP70/RBCS2 promoter and the 3' untranslated region (3'UTR), making it ready for transformation into eukaryotic microalgae. Transformation of *C. vulgaris* microalgae cells with recombinant pOPT_cCA_mRuby2_Paromy_PETase3M vector was performed by a modified electroporation process. PETase3M positive transformants of *C. vulgaris* 211/11J cells were selected for three consecutive generations on TAP agar containing 50 µg/ml paromomycin. The presence of PETase3M insert within microalgae nuclear genome was confirmed through the amplification of PETase3M gene by PCR. Upon His tag based prufiication, the successful and extracellular expression of recombinant PETase3M in *C. vulgaris* transformants was confirmed by SDS-PAGE analysis of microalgae culture medium. PET films treated with purified PETase3M and degraded to a certain degree under light microscopy.

In conclusion, extracellular and functional production of a PETase3M mutant was carried out in *C. vulgaris* with high tolerance to extreme conditions for the first time. Thus, the devloped microalgae strain could find extensive utilizations in large-scale bioremediation and recycling applications of environmental PET wastes photosynthetically.

.

**Key Words:** *C. vulgaris* 211 / 11J, electroporation, extracellular expression, mutant PETase, transformation

## LIST OF FIGURES

**Page Number**

## LIST OF TABLES

**Page Number**

## ABBREVIATIONS

| | |
|---|---|
| BHET | : Bis Hydroxyethyl Terephthalate |
| bp | : Base pair |
| $CaCl_2$ | : Calcium Chloride |
| CTAB | : Cetyltrimethylammonium Bromide |
| *C.vulgaris* | : *Chlorella vulgaris* |
| $ddH_2O$ | : Dedistilled water |
| $dH_2O$ | : Distilled water |
| DMSO | : Dimethyl Sulfoxide |
| DMT | : Dimethyl Terephthalate |
| DNA | : Deoxyribonucleic acid |
| dNTP | : Deoxynucleotide triphosphate |
| EG | : Ethylene Glycol |
| FsC | : *Fusarium solani* cutinase |
| *gDNA* | *:* Genomic DNA |
| HCl | : Hydrogen Chloride |
| His | : Histidine |
| *I. sakaiensis* | : *Ideonella sakaiensis* |
| KCI | : Potassium Chloride |
| LB | : Luria Bertani |
| LCC | : Leaf-branch compost cutinases |
| M | : Molar |
| mA | : Milliamperes |
| mg | : Miligram |
| $MgCl_2$ | : Magnesium Chloride |
| MHET | : Mono (2-hydroxyethyl) terephthalic acid |
| MHETase | : MHET hydrolase |
| ml | : Milliliter |
| mM | : Millimolar |
| $^oC$ | : Degree Celcius |
| OD | : Optical Density |
| PAGE | : Polyacrylamide gel electrophoresis |
| PCR | : Polimerase Chain Reaction |

PET : Polyethylene Terephthalate
PETase : PET Hydrolase
PP : Polypropylene
PS : Polystyrene
PVC : Polyvinyl Chloride
rpm : Route per Minute
SDS : Sodium Dodecyl Sulfate
TAP : Tris-Acetate-Phosphate
TfH : *Thermobifida fusca* Hydrolase
Thc Cut-1 : *Thermobifida cellulosilytica* cutinase-1
Thc Cut-2 : *Thermobifida cellulosilytica* cutinase-2
TPA : Terephthalic Acid
µg : Microgram
µl : Microliter
µm : Micrometer

# 1. GENERAL INFORMATION

## 1.1. Introduction

Microalgae are essential photosynthetic microorganisms with enormous environmental and atmospheric benefits and industrial potentials. Microalgae remains dating back 3.4 billion years were found during an expedition in western Australia. In all of the researches, no changes were reported in their structure. On the other hand, other sources say that microalgae evolved 2.7 billion years ago and have survived until today. Thus, one group of researchers thinks that microalgae are the ancestors of all other plants. Thus, algae first formed marine plants and then, stepped on the lands just like animals and formed the plant community here. However, there are many issues that researchers need to prove with more scientific evidence (danger of drying, feed, reproduction, and protection from oxygen) and all are questions waiting to be answered. *Chlorella vulgaris* (*C. vulgaris*) and its products, which are still consumed as food supplements today, are also used for different purposes such as paint, medicine, animal feed, aquaculture and cosmetics. In addition, they are rich in protein, carbohydrates, vitamins, minerals and fatty acids. Moreover, microalgae use carbon dioxide as their main food source and accordingly produce almost half of the oxygen on our planet. Microalgae can grow in almost any environmental condition from the northernsites to the southernsites of the world. Discovered by Martinus Willem Beijerinck in 1890, the single-celled microalgae was named "Chlorella". They also play an important role in water treatment, as nitrogen, phosphorus, carbon dioxide; heavy metals are the leading nutrients that are vital for the growth of microalgae. Therefore, *C. vulgaris* makes a great contribution to complete elimination of ammonium and partial recycling of phosphorus from versatile wastewater reservoirs (Safi et al., 2014).

Polyethylene terephthalate, one of the fastest growing plastic types, is much stronger, harder and brighter than other thermoplastics. Polyethylene terephthalate, which provides superiority with its high impact experience, is also resistant to many solvents. Polyethylene terephthalate, which can be used in many areas such as food, beverage, beverage and synthetic fiber, has an important place in many areas with its advantages. Polyethylene terephthalate, synthesized by the polymerization method of ethylene glycol and terephthalic acid, is produced as a melted and sticky mass that can be processed plastic (URL-1).

PETase as the main biocatalyst for PET degradation is obviously secreted by *I. sakaiensis*. In addtion to *I sakaiensis,* several well known bacterial expression hosts including *Escherichia* and *Bacillus* have been established so far to develop synthetic PETase-hydrolyzing cell factories towards versatile and environmentally friendly biological recycling of PET wastes. Although some drawbacks such as the excessive amount of toxic PET monomers accumulated over time within the processing facilities or the imperative needs of costly carbon resources for large-scale processing applications stay as some of the considerable concerns to be addressed (Tanasupawat et al., 2016).

Interest in the use of different algae for biological degradation processes has increased in recent years. Our goal therefore was to developed a photosynthetic microalgae strain capable to degrade various environmental PET wastes to their monomers, facilitates the environmentally friendly recycling of PET chemicals. In this perspective, we planned to integrate highly active recombinant PETase gene into *Chlorella vulgaris* nuclear genome for the extracellular production of PETase enzyme.

### 1.2. Polyethylene Terephthalate (PET)

Plastics are synthetic or semi-synthetic chemical materials as high molecular weight polymeric substances whose principal supply is primarily petroleum. Plastics can find versatile utilizations in almost every day of our lives, have thus attracted excessive attentions of scientists since 1970s as they're durable, safe, light weighted, relatively cheap, and can easily be processed into many different forms such as highly flexible and extremely rigid plastics (Smith, 2013; Joo et al., 2018).

Linear plastic polymers incorporate into single linear monomer chains; branched polymers are linear with side chains and are therefore called as thermoplastic. Therefore, they are softening when heated. Cross-connected polymers are polymers with intercalated bonds among polymer chains are thermosetting, that is why they harden when heated. Due to those properties, plastics are extensively utilized in various industrial fields (Nkwachukwu et al., 2013).

Since plastics are inexpensive and can easily be produced into consumer products by fast automated machinery, they are widely used as packaging materials for farm, forest, dairy and other consumer products (Sinha et al., 2010). Plastic materials can be synthetic or semi-synthetic. Plastics as organic polymers have a high molecular mass but often contain other

substances. Synthetic plastics are most commonly derived from petrochemicals, thereby they ae partly natural (Dodbiba and Fujita, 2004).

Plastics are generally divided into six main classes: These are polyethylene terephthalate (PET), high density polyethylene, polyvinyl chloride (PVC), low density polyethylene, polypropylene (PP) and polystyrene (PS) (Dodbiba and Fujita, 2004; Jankauskaite, 2016).

Figure 1 shows the proportion of plastics demanded depending on the polymer type. According to this polyolefins, namely high-density polyethylene, low-density polyethylene and PP are the most widely used plastics and account for about half of the total production.

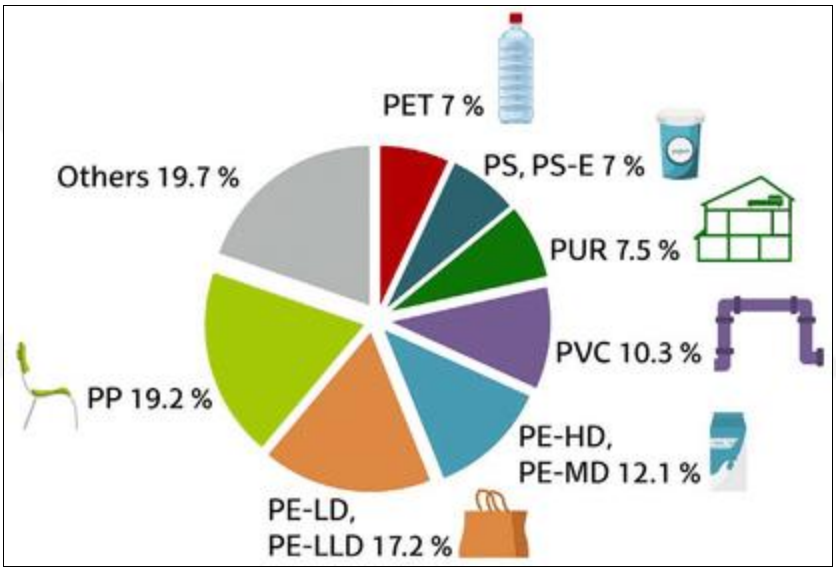



Figure 1. Current plastic distribution demand in Europe (Lopez et al., 2017).

PET was first polymerized John Rex Whinfield and his colleagues in 1941. E. I. DuPont de Nemours was the first person who used the trademark *Mylar* in June 1951 in Delaware, United States, and he received registration of it in 1952 (Barot et al., 2019). In 1973 Nathaniel Wyeth from Du Pont, patented the first PET bottle (Wyeth, 1988).

Stability, durability and transparency properties of PET made it the best known and widely used polyester worldwide and increased its usability especially in bottles, containers and films (Liu et al., 2019). Figure 2 reveals the molecular structure of the PET polymer.

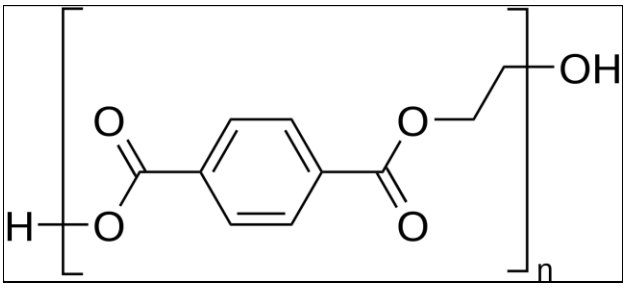



Figure 2. Molecular structure of a PET monomer (Fischer-Colbrie et al., 2004).

### 1.2.1. Production of PET

PET is a polymer resin that belongs to the thermoplastic group of polyester family (Poulikakos et al., 2017). It starts with the introduction of chemicals such as acid and alcohol into the environment in the production of polyester plastics and triggers the beginning of the formation of the products with the next step, polymerization. The first is a transesterification reaction; the second is an esterification reaction. PET can be synthesized from ethylene glycol (EG), dimethyl terephthalate (DMT) or terephthalic acid (TPA).

The first two chemical reactions result in the formation of bis (hydroxyethyl) terephthalate (BHET) as an intermediate and then, BHET is converted to PET through transesterification (Figure 3) (Cooper, 2013; Farzi et al., 2019; Sinha et al., 2010). On the other hand, during the DMT based production of PET, DMT and excess ethylene glycol are mixed and reacted with a basic catalyst at 150-200 °C. Methanol is distilled off due to the presence of excess ethylene glycol to further the reaction. Excess ethylene glycol is distilled at a higher temperature by using vacuum pump. In the second transesterification stage, the reaction is processed at 270-280 °C by continuously distilling ethylene glycol (Awaja and Pavel, 2005). The reactions are as follows

First step

$$C_6H_4(CO_2CH_3)_2 + 2\ HOCH_2CH_2OH \rightarrow C_6H_4(CO_2CH_2CH_2OH)_2 + 2\ CH_3OH \quad (1)$$

Seconder step

$$n\ C_6H_4(CO_2CH_2CH_2OH)_2 \rightarrow [(CO)C_6H_4(CO_2CH_2CH_2O)]_n + n\ HOCH_2CH_2OH \quad (2)$$

The second stage is the terephthalic acid process. In this step, the direct esterification of ethylene glycol and terephthalic acid is carried out at medium pressure (2.7-5.5 bars) and at higher temperature (220-260 °C). In this reaction, formed water is eliminated and further removed continuously by distillation (Awaja and Pavel, 2005).

$$(n\ C_6H_4(CO_2H)_2 + n\ HOCH_2CH_2OH \rightarrow [(CO)C_6H_4(CO_2CH_2CH_2O)]_n + 2n\ H_2O \quad (3)$$

It is a laborious task to obtain pure after polymer formation. Therefore, the purity of the intermediate products used is extremely important. By vacuum distillation, ethylene glycol must be obtained in pure form. Catalysts are used as diluents to get high reactions and reduce costs. The most common is antimony trioxide, but there are also many alternatives such as titanium or germanium (Goje and Mishra, 2003).

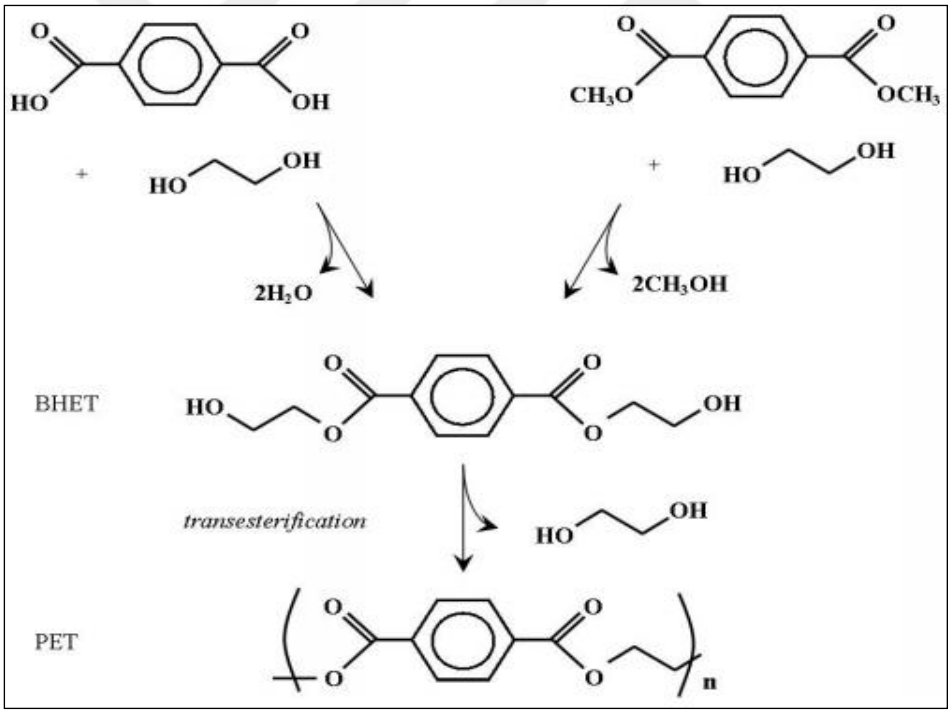



Figure 3. Chemical reaction of PET production (Webb et al., 2012).

### 1.2.2. Physical and ve Chemical Properties of PET

PET is colorless, semi-crystalline depending on the way it is processed, it can be semi-hard to hard and very light. Most PET products could be strong and durable, chemically and

thermally stable and reveals low gas permeability. PET can turn white color when exposed to some other chemicals such as chloroform as well as toluene. Oxygen, water vapor, odor and oil proof properties are favorable. Under 20 °C and 85% relative humidity conditions; the water vapor permeability of 100 µm thick PET material is 2 $g/m^2$ day, oxygen permeability is approximately 9.6 $cm^3/m^2$ bar per day (Bal et al., 2017).

PET has low moisture absorption, allowing the related material to maintain excellent structural stability at extreme temperatures and high humidity. Because of its good electrical insulation properties, PET has many uses in electrical industries.

When PET is heated above 72 °C, it transforms into a rubbery-like elastic form. PET polymers contains repeating units TPA and EG and these units possesses a molecular weight of about 200 Da (Farah et al., 2015).Some other physical, chemical and mechanical properties of PET are also summarized in Table 1. (Awaja and Pavel, 2005; Zein et al., 2010).

Table 1. Properties of PET

| **Physical Properties** | **Value** | **Chemical Properties** | **State** |
|---|---|---|---|
| Density ( g cm-3 ) | 1.3-1.4 | Acids resistance | Good for most mineral acids |
| Flammability | Self Extinguishing | Alcohols, Ketones, Halogens, Greases | Good |
| Refractive index | 1.58-1.64 | Alkalis resistance | Poor at high temperature |
| Water absorption | <0.7% | hydrocarbons resistance | Fair |
| **Mechanical Properties** | **Value** | **Thermal Properties** | **Value** |
| Coefficient of friction | 0.2-0.4 | Lower working temp(°C) | -40 to -60 |
| Hardness – Rockwell | M94-101 | Specific heat(J.K1 .kg-1 ) | 1200 – 1350 |
| Izod impact strength | 13-35 ( J.m-1 ) | Thermal conductivity | 0.15-0.4 @ 23 |
| Poisson’s ratio | 0.37-0.44 (oriented) | Upper working temperature | 115-170 |

### 1.2.3. Applications of PET

For more than 100 years, developments in plastic products have created a significant revolution in our lives. Polyethylene terephthalate or PET is a notable example in this regard. Around 56 million tones of PET were produced worldwide in 2013 alone; 15.4 million tons of these are used in food and liquid containers and 3.2 million tons in the packaging industry. This figure reached 41.56 million metric tons in 2014, and it is claimed that this production will reach approximately 73.39 million metric by 2020 (Yoshida et al., 2016).

Polyethylene terephthalate is a raw material, which is utilized for beverage, food and other liquid containers, thermoform applications, microwave food trays and food packaging. The finding such comprehensive utilization of PET in such different areas is partly due to its natural properties, which are very suitable for light, large capacity and break-resistant containers (Figure 4). Since it provides an excellent barrier against oxygen and carbon dioxide, PET fibers are widely used in the carbonated beverage industry, as well as in the clothing and carpet industry (Archna et al., 2015; Farzi et al., 2019; Raziyafathima et al., 2016; Sinha et al., 2010).


Figure 4. Applications of PET (Archna et al., 2015).

### 1.2.4. Effect on Environmental of PET

The increase in the use of plastic products in our daily life, especially the widespread use of disposable products produced as packaging materials, has been tremendously effective in the augmentation of plastic waste problems and solid waste management (Güçlü

& Acar, 2015). Today, only 14% of the plastic waste used in the world can be collected for recycling and almost the same amount of plastic is burned for energy. It is not arguable that the remaining 72% PET materials can cause particularly serious environmental pollution (Wei and Zimmerman, 2017). For this reason, tens of thousands of tons of plastic pollutants are released every day. Plastic pollution continues to be an ecological problem, since the durability of synthetically produced materials generally delays degradation (Geyer et al., 2017). Although the consequences of plastic pollution for ecosystems around the world are unpredictable until now, it is becoming increasingly apparent that plastic accumulated in nature is harmful to life. Monomers such as propylene, such as ethylene, obtained from fossil hydrocarbons are never biodegradable. One of the biggest problems of today's world is the formation and dispersion of tiny microplastics that are already in ocean waters (Palm et al., 2019).

Microplastics have been reported to pollute the seas and oceans due to their complex chemical structure and contain many compounds (Moog et al., 2019; Chae and Kim, 2018).

Micro-sized plastic waste can be detected in all major ocean basins in every region of the world, and it is estimated that in 2010, nearly 10 million tons of plastic were found in this environment. Like all synthetic wastes, plastics pollute fresh water resources, which are indispensable for human survival (Geyer et al., 2017).

Plastics constitute the biggest risk group due to the highly polluting chemical structures they contain. Thanks to these waste plastics, micromolecules that enter the bodies of many living things can reach us by being transferred according to the food chain. In addition, chemicals with high toxicity and PET and its derivatives that we use to make plastic affect living life extremely negatively. Therefore, degradation and/or eradication of micro and nanoplastics in ecosystems are of great importance (Archa et al, 2015; Chae and An., 2018).

### 1.3. PET Recyling

It is vital that the type of plastic known as PET can be recycled. In addition, it is necessary to reduce the accumulated plastic waste mass and even produce more valuable products at low cost. However, world countries are still insufficient for recycling PET and its derivatives (Jankauskaıtė at al., 2008).

Recycling of PET plastics can be done in different ways. These include mechanical recycling, chemical recycling and biocatalysis methods.

### 1.3.1. Mechanical Recycling

Mechanical recycling is also known as physical recycling. The basic structure of the waste plastic material is mainly not changed; instead, it is recycled into valuable resources. With this method, 1) raw plastic is directly recycled (i.e. high value end products) and yields quality products; or 2) down-loop i.e. low value end product as low quality plastic is obtained (Archna et al., 2015). In this recycling, bottles can be recycled into raw materials after going through a series of processes. How these waste bottles are used is very important in terms of their recyclability capacity (Koo et al., 2013).

### 1.3.2. Chemical Recycling

It is possible to convert plastic waste containing PET into chemicals that can be polymerized again. PET can be transformed by using many toxic substances and their derivatives. The most important methods towards chemical recycling are methanolysis, hydrolysis and glycolysis (Figure 5) (Abbasid et al., 2006). For monomers such as methanolysis, depolymerization is both more expensive and not useful than glycolysis. For this reason, glycolysis is more advantageous and partially more useful for PET (Wang et al., 2011). Chemical recycling process allocates any contaminants bound to the polymer chain to be removed by purification of monomers and/or oligomers. Although mechanical recycling process is only able to separate inorganic contaminants, during chemical recycling, the organic contaminants are remelted and are through extrusion process (Koo et al., 2013).

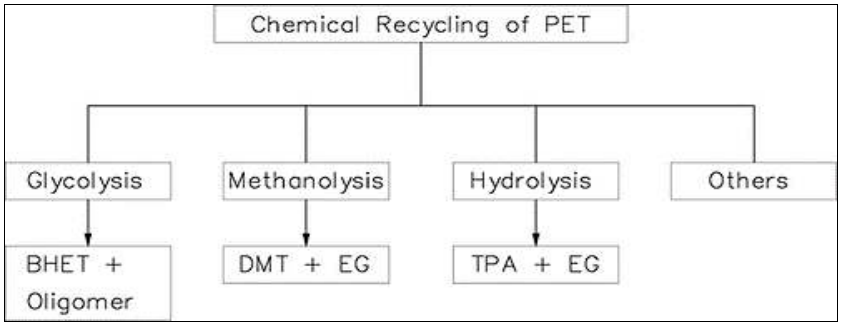



Figure 5. Current chemical recycling techniques of PET (Pudack et al., 2020).

### 1.3.3. Biological Recycling and Bioremediation of PET

Biologically, the breakdown of waste is the process of breaking down microbial life down to the smallest molecule they make in order to maintain their own lives (Zheng et al., 2005). The utilization of microbial and enzymatic approach towards the biodegradation of PET in nature is an economically viable and environmentally friendly recycling method (Nakkabi et al., 2015). In the presence of versatile technical impediments such as including energy consumption, availability of resources, capital investment and time, most of which are mainly experienced during the chemical degradation processes, chemical recycling of PET wastes seems to be an expensive and environmentally unfavorable approach. In addition, the necessity of processes such as the use of inorganic acids in chemical recycling, distillation, filtration, crystallization, and protection of certain high temperature and pressure conditions increase the need for a better solution (Koshti et al., 2018).

All microbes are constantly at war against biological pollution by converting almost all organic and inorganic wastes. Microorganisms use their enzymatic structures to meet their future energy needs by breaking down and storing polymers. Plastic waste has an important role for these microscopic creatures. To create one of the necessary steps to store and generate energy in order to use it as a carbon source (Amobonye et al., 2020).

Many microorganisms that carry out enzymatic activity also offer a low-cost solution to PET degradation. However, working in this area also increases all the maintenance costs of living things (Figure 6). Microbial degradation is generally an enzyme process that facilitates the separation of many polymer bonds by breaking them quickly. By using carbon as energy, they increase the degreda process. Visualization of degradation after this procedure is important for the health of the procedure (Bhardwaj et al., 2012 ; Moog et al., 2019; Wei and Zimmermann, 2017 ; Koshti, 2018).


Figure 6. PET biodegradation process by microorganisms (Amobonye et al., 2020).

### 1.3.3.1. Affecting PET Biodegradation

The biodegradation of PET includes many differences. These are classified according to the type of microorganism or polymer structural properties. In this way, it affects the biodegradation of PET (Artham and Doble, 2008). Various characteristics essential PET biodegradation were summarized in Figure 7. The higher the molecular weight of the polymer, the lower its structural degradation. Because it is difficult for microbes to use this type of polymer as a substrate (Mergaert and Swings, 1999). Physical forces such as drying,

thawing, freezing, heating or cooling are also important to overcome the physical barriers of biodegradation (Tokiwa and Calabia, 2004).

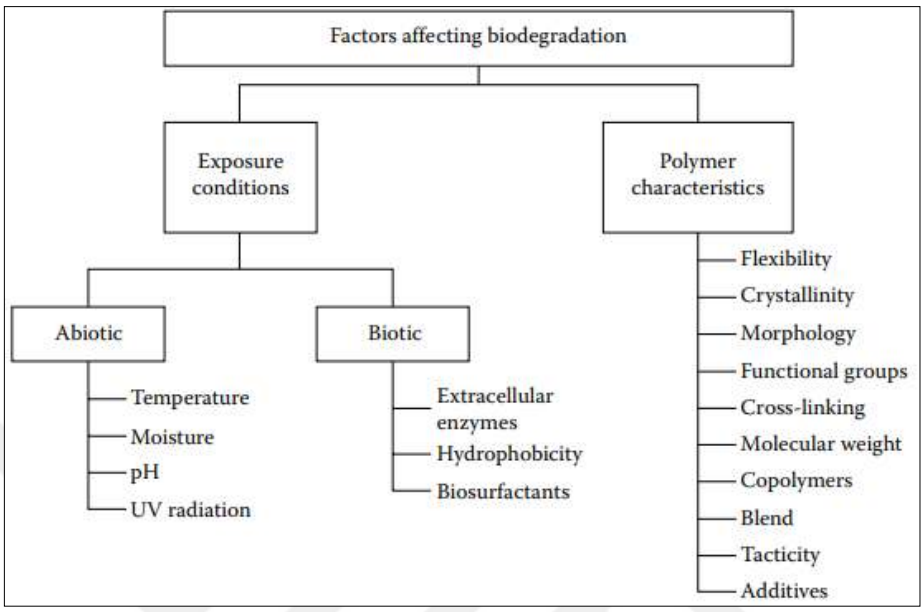



Figure 7. Factors affecting biodegradation (Kijchavengkul and Auras, 2008).

### 1.3.3.2. Role of Enzymes on PET Biodegradation

The use of microbes for PET bioremediation is the best choice even though several rate-limiting factors lessen its applicability. Growing a sufficient amount of microorganisms for the biodegradable can be a long-term and financially rewarding job. Chemical and physical decomposition methods may have adverse effects on many contaminants and chemical microorganisms released during decomposition. Plastic-degrading enzymes reveal high similarity to other enzymes thosse evolved in the biodegradation of polymers, have thus been classified into wide categories including extracellular and intracellular enzymes (Figure 8) (Gu, 2003).

Since extracellular enzymes come into contact with the monomers on the plastic, they are thought to contribute to all the reactions that occur (Chinaglia et al., 2018). Although the enzyme secreted by many organisms extracellularly is effective at the beginning of the degradation, it has been observed that organisms that secrete enzymes both intracellularly and extracellularly are more beneficial in terms of the sustainability of this work. In this way, the hydrophilic structure of the polymer is further developed. It is the environment in which

it is found that generally determines whether the degradation will be anaerobic or aerobic (Gug et al., 2014 ; Bhardwaj et al., 2012; Tokiwa and Calabia 2004; Koshti, 2018). Until now, enzymes such as esterases, lipases, cutinases and hydrolases have been stated that they give effective results in PET degradation (Amobonye et al., 2020).

Table 2. List of biochemically characterized PET hydrolytic enzymes (Adapted from Taniguchi et al., 2019).

| **Enzyme** | **Accession Number** | **Organism** |
|---|---|---|
| PET hydrolase (PETase) | GAP38371.1 | *I. sakiensis* 201 F6 |
| *Thermobifida fusca* hydrolase (TfH) | WP_011291330.1 | *T. fusca* DSM43793 |
| Leaf-branch compost cutinases (LCC) | AEV21261.1 | Uncultured bacterium |
| *Fusarium solani* cutinase (FsC) | 1CEX | *Fusarium solani pisi* |
| *Thermobifida cellulosilytica* cutinase-1(Thc Cut 1) | ADV92526.1 | *Thermobifida cellulosilytica* |
| *Thermobifida cellulosilytica* cutinase-2(Thc Cut 2) | ADV92527.1 | *Thermobifida cellulosilytica* |
| PET 2 | C3RYL0 | Uncultured bacterium |
| PET 5 | R4YKL9 | *Oleisira antarctica* |

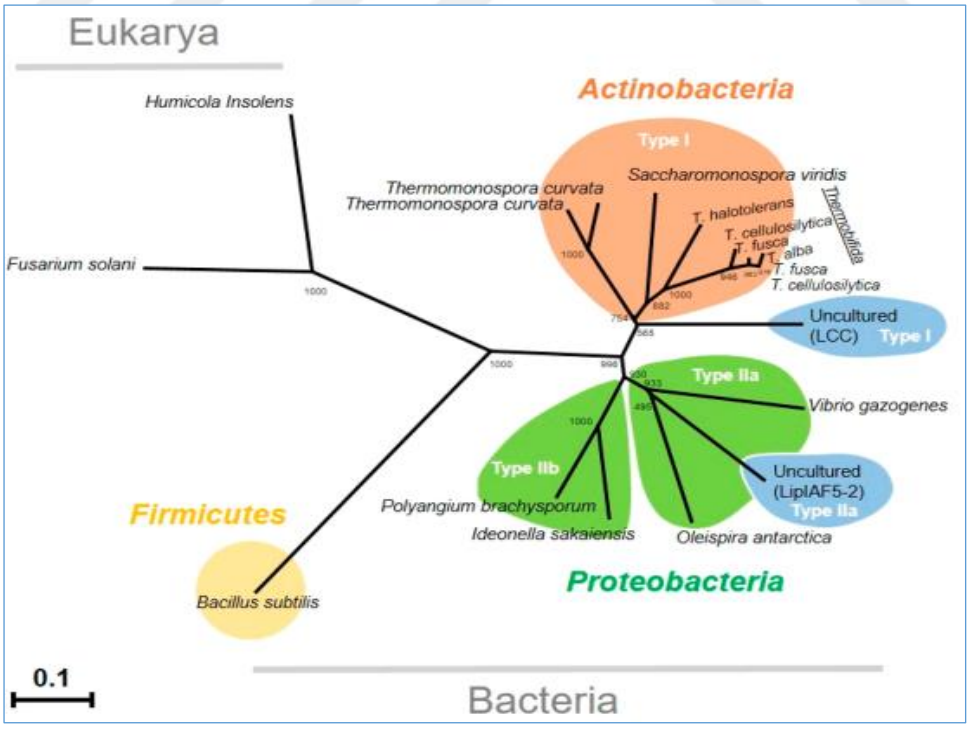



Figure 8. Phylogenetic tree created using the protein sequences from known PET hydrolytic enzymes (Taniguchi et al; 2019).

When looking at microorganisms and their PET degrading enzymes, there are only few illustrative and outstanding studies. For instance, in 2005, Müller and his colleagues first time revealed that PET could be degraded with a polyester hydrolase which known as TfH found in the *Thermobifida* species. The study using a PET sample melt pressed from a beverage bottle with 10% crystallinity resulted in a weight loss of about 10% (Müller et al., 2005). Ronkvist et al., (2009) reported that the cutinase described from *Thermomyces insolens* caused almost complete degradation of PET after 96 hours at 70 °C (Ronkvist et al., 2009).

The results obtained by Sharon and Sharon (2012) support that PET film was subjected to microbial degradation with the help of an esterase found in *Nocardia* (Sharon and Sharon, 2012). Wei and his colleagues (2014) showed that two polyester hydrolase found in *Thermomonospora curvata* can degrade PET (Wei et al., 2014). Kawai and his colleagues (2019) cloned a cutinase-like polyesterase from *Saccharomonospora viridis*, which can degrade PET film with up to 27% weight loss at 63 °C in 3 days (Kawai et al., 2019).

PETase is an enzyme of the esterase group that catalyzes the transform of PET to mono (2-hydroxyethyl) terephthalic acid (MHET) (Figure 9). In particular, it has been shown that PETase can degrade PET more significantly at 30 °C than the enzymes LCC, TfH and THc-Cut1 previously reported. Therefore, PETase is considered to be a potential tool for plastic degradation (Yoshida et al., 2016). PETases are also able to degrade PEF (polyethylene-2,5-furandicarboxylate) plastic, which is a biological derivative of PET substitute. However, PETase cannot hydrolyze polymers such as polybutylene succinate and polylactic acid (Austin et al., 2018).

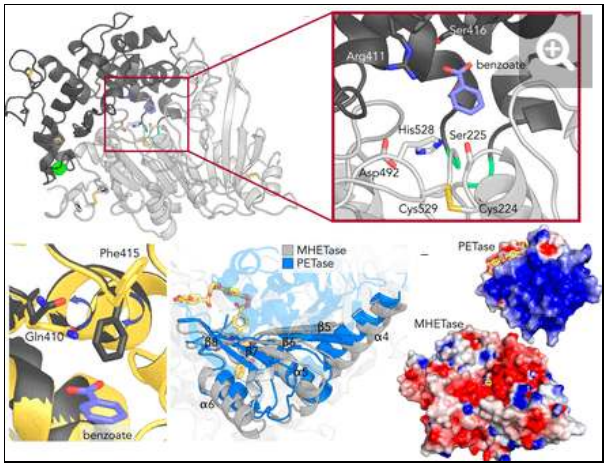



Figure 9. PETase and MHETase (Knott et al., 2020).

MHETase enzyme is responsible for the hydrolysis of mono-2-hydroxyethyl terephthalate substrate produced because of the catalytic activity of PETase. It is the second step enzyme towards PET hydrolysis, which is also crucial for PET degradation. The tannase-origin MHET hydrolase (or MHETase) uses MHET as a substrate to transform it to ethylene glycol and terephthalate (Figure 10). MHETase has a classic α / β-hydrolase domain and a cover domain that provides substrate specification (Yoshida et al., 2016).

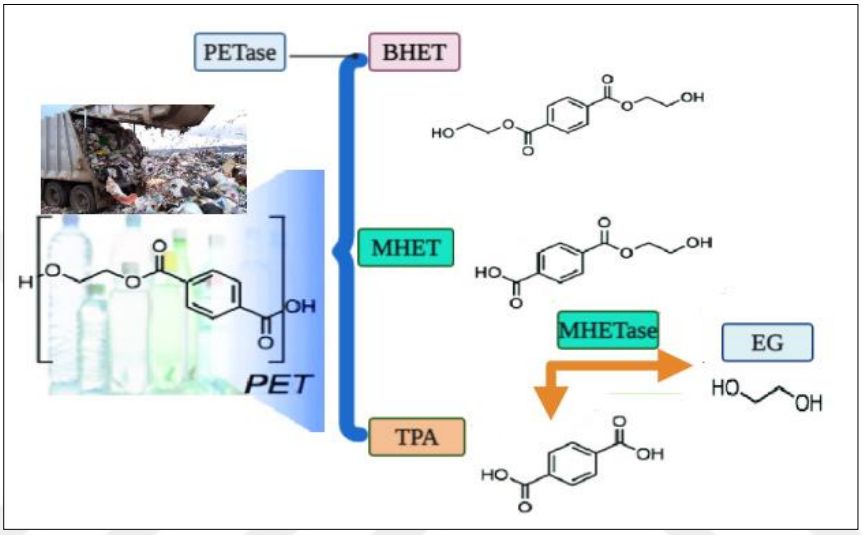



Figure 10. Degradation mechanism of PET (Austin et al., 2018).

The PETase enzyme is found and reported in *I. sakaiensis* and is a natural solvent that degrades PET, in addition to this, nowadays its activity is tried to increase by trying too much mustation. However, there are some disadvantages of using bacterial systems. As examples of such disadvantages, the compulsory needs to the presence of PET substrate or costly carbon resources for growth in cultures/bioreactors might be given as drastic pitfalls to be overcome (Tanasupawat et al., 2016).

### 1.4. Structure of Wild Type PETase

*I. sakaiensis* PETase 3D structure reveals a typical α/β hydrolase superfamily22–24, and contains single layer central twisted β-sheet structure, which is formed by nine mixed β-strands (β1–β9). The related β-sheet structure is enclosed by seven α-helices (α1–α7) (Figure 11). The active site of *I. sakaiensis* PETase consists of a serine hydrolase Gly–x1–Ser–x2–

Gly motif (Gly158–Trp159–Ser160–Met161–Gly162), which is also highly conserved within other α/β hydrolase superfamily proteins such as lipases and esterases.

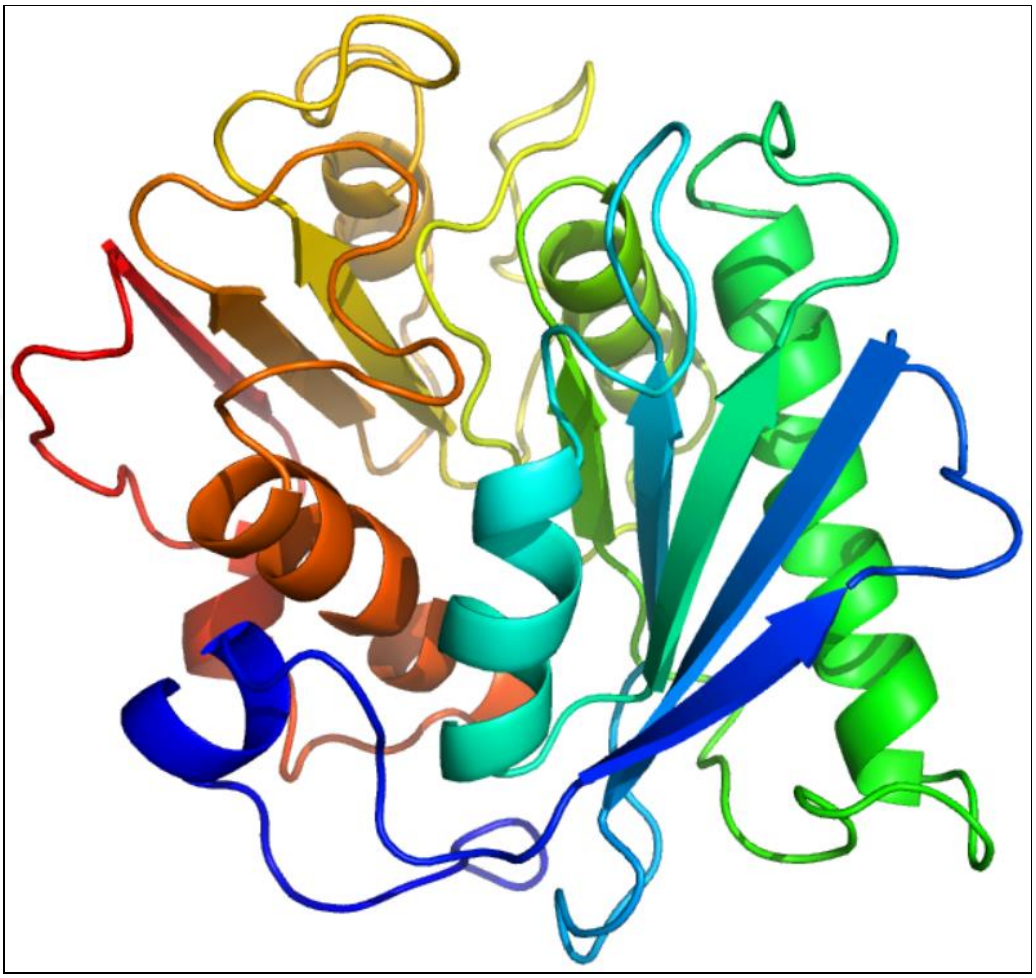


Figure 11. 3D structure of wild type PETase enzyme in cartoon form from *I. sakaiensis*

### 1.5. Microalgae

Microalgae are diverse group of photosynthetic unicellular or multicellular microorganisms, which can be either autotrophic, heterotrophic, or mixotrophic. Unicellular structure of microalgae enables them to easily reach nutrient supplies and transform solar energy into chemical energy through $CO_2$ fixation and $O_2$ production. In recent years, upward of 40000 new species of eukaryotic microalgae were documented as a different sources for augmentation, nutraceuticals and pharmaceuticals (Chu, 2012; Maruyama et al., 1997). The diversity of microalgae is vast and this is reflected in their metabolism and

biochemical properties i.e. pigment content, cell walls and bioactive compounds including fatty acids and lipid content, fats, sterols and secondary metabolites (Sathasivam et al., 2019) (Figure 12). It has been suggested that microalgae are a promising source for biodiesel production as global fossil fuel resources are depleted day by day (Mata et al., 2010; Safi et al., 2013). The rapid growth rate of microalgae, their adaptation abilities to environmental stress, and biochemically active natural compounds in their content allow them to be highly demanded in various industrial applications. Here, we highlight such freshwater microalga *C. vulgaris* with enormous capacities toward various industrial applications.

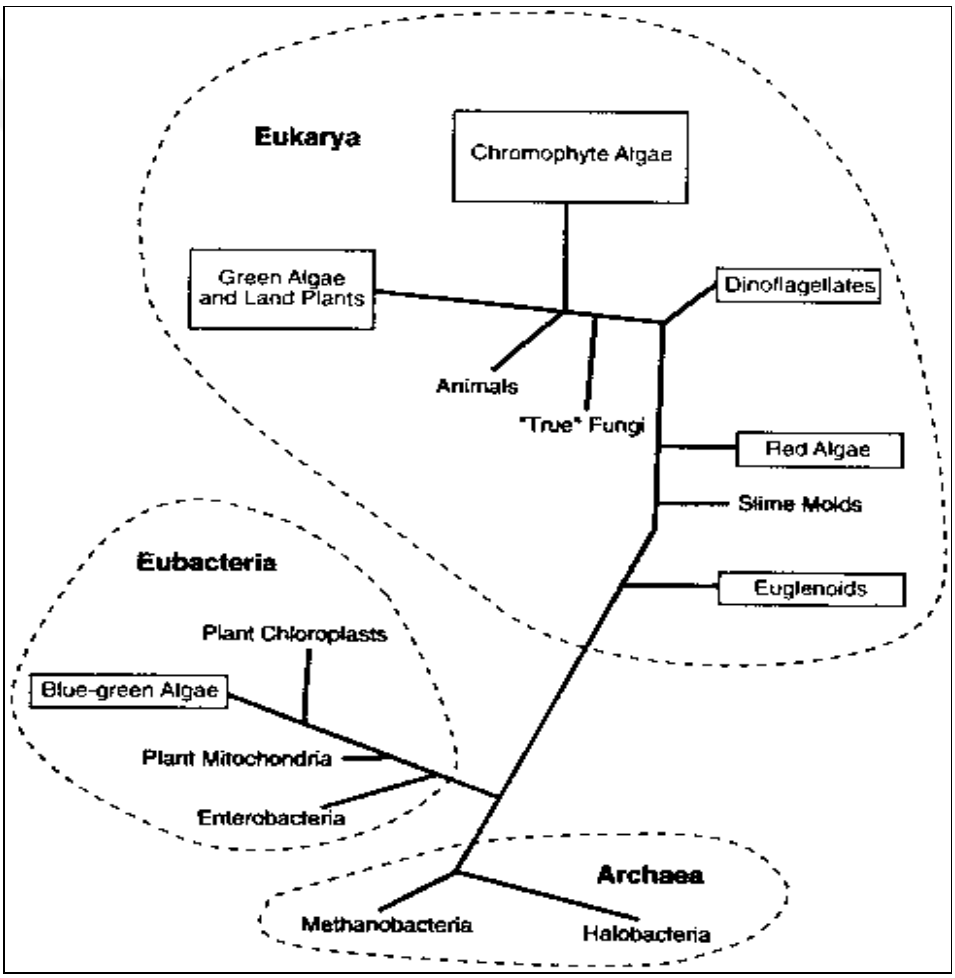



Figure 12. Depiction of the phylogenetic relatedness of some groups of microalgae. Lengths of line segments are proportional to evolutionary distance based on analysis of ribosomal RNA gene sequences (Radmer & Parker, 1994).

### 1.5.1. Industrial Bioremediation-Based Potentials of *C. vulgaris*

*C. vulgaris* is a green algae discovered by Martinus Willem Beijerinck in 1890. It is commonly found in sea, terrestrial environment and fresh waters. It has high photosynthetic activity and function (Tomaselli, 2004). Thanks to these features, microalgae have become remarkable for use in breeding and business production (Borowitzka, 2018). Microorganisms such as *Chlorella* species have existed in the world for many years (Krienitz et al., 2015; Safi et al., 2013). When looking at the general characteristics of *C. vulgaris* cells, it is seen that there are no whip protrusions in the form of spherical, ellipsoid, about 2 to 10 µm in diameter (Champenois et al., 2014; Garcia, 2012). They are single cell microalgae and may contain indistinguishable cells up to sixty four in their single colonies. *C. vulgaris* has a single chloroplast *C. vulgaris* reproduces by forming asexual otospores (Safi et al., 2013). When these otospores grow, the main cell wall is disrupted and a process called autosporulation occurs. In this case, it becomes food for daughter cells (Figure 13) (Yamamoto et al., 2004).

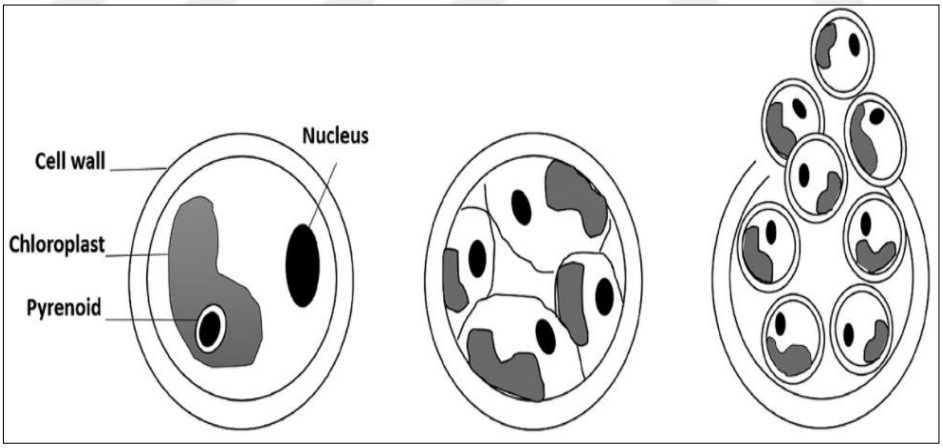



Figure 13. Auto sporulation manner of *C. vulgaris* (Tiong et al., 2020).

### 1.5.2. *C. vulgaris* Metabolites

Like many microorganisms, microalgae contain biomolecules in their structures. These biomolecules are structures such as carbohydrates, lipids, proteins and minerals (Maruyama et al., 1997; Safi et al., 2013; Spolaore et al., 2006). These biomolecules found in algae can be divided into primary and secondary. Primary metabolites are essential for

photosynthesis and respiration, while secondary metabolites have activities for environmental adaptations and food competitions (Guedes et al., 2011; Sigamani and Natarajan, 2016). Their chemical composition varies with microalgae species and way of life environments, salinity, temperature, and medium nutrients, pH (Sharma, 2012; Vitova et al., 2015).

Proteins are the principal ingredients of microalgae (Yaakob et al, 2014) Proteins found in algae are essential for cellular growth, healing and protection of cells. *C. vulgaris* cells have a protein content of 43-58% of their dry weight (Safi et al., 2013). Protein content is distributed in 20% cell wall, 50% in cells and 30% between inner and outer membrane of the cell membrane (Berliner, 1986). At the same time, there are essential and non-essential amino acids, which are protein building blocks in large amounts in their cytoplasm (Becker, 2007; Maruyama et al., 1997; Morris Quevedo et al., 1999; Safi et al., 2013).

Lipids are also vital ingredients for all microalgae cells, which are also crucial for cellular growth, metabolic activity and establish resistance against harsh stress conditions (Kottuparambil et al., 2019; Vitova et al., 2015). They can be either neutral or polar. Neutral lipids are found in motile organelles, while polar lipids produced by chloroplast are utilized in cellular membranes (Garcia et al., 2013). Microalgae might accumulate high amount of saturated and unsaturated forms of lipid derivatives, ranging from 2% to 77%, depending on the species and the environment in which they live. Although this ratio varies as microalgae species-specific it changes between 5 and 58% of the dry weight for *C. vulgaris* cells (Safi et al., 2013).

Carbohydrates, which are energy and carbon sources in microalgae cells, are used in forms of starch, glycogen, glucose. carbohydrates are found mostly in the form of amylopectin and amylose in *C. vulgaris cells*, as well as starch and cellulose. The carbohydrate content of *C. vulgaris* can be up to 55% of its dry weight (Choix et al., 2012).

Vitamins (A, B, C, D, E and K) commonly found in microalgae cells are organic molecules required for nutrition and growth. They are categorized as water and fat soluble. Since vitamins cannot be synthesized enough in the human body, they are taken exogenously in the diet. Vitamins available in *C. vulgaris* are vitamins A, B, C and E (Maruyama et al., 1997; Safi et al., 2013; Yeh et al., 2010). Minerals are inorganic materials that are needed and taken mostly by human body as externally. *C. vulgaris* cells are quite rich in terms of calcium, potassium, magnesium and zinc minerals (Mohd Yusof et al., 2011). Natural and

low-cost production of enormous amount of nutrients and minerals by *C. vulgaris* has made it as a high-quality food supply of micronutrients crucial to human body.

### 1.5.3. Applications of *C. vulgaris*

The large capacity and high productivity of freshwater microalga *C. vulgaris* diversifies its current industrial applications. Recent trends in microalgae biotechnology reveals that *Chlorella* strains can be vast production hosts for variety of industrially value-added products such as pharmaceuticals, vaccines, recombinant proteins, pigments, antioxidants, hormones, industrial enzymes, metabolites nutraceuticals… etc. Freshwater *Chlorella* stains as beneficial microorganisms can also be integrated into different environmental applications including agriculture, aquaculture, bioremediation, wastewater treatment and production of biofuels (Figure 14).

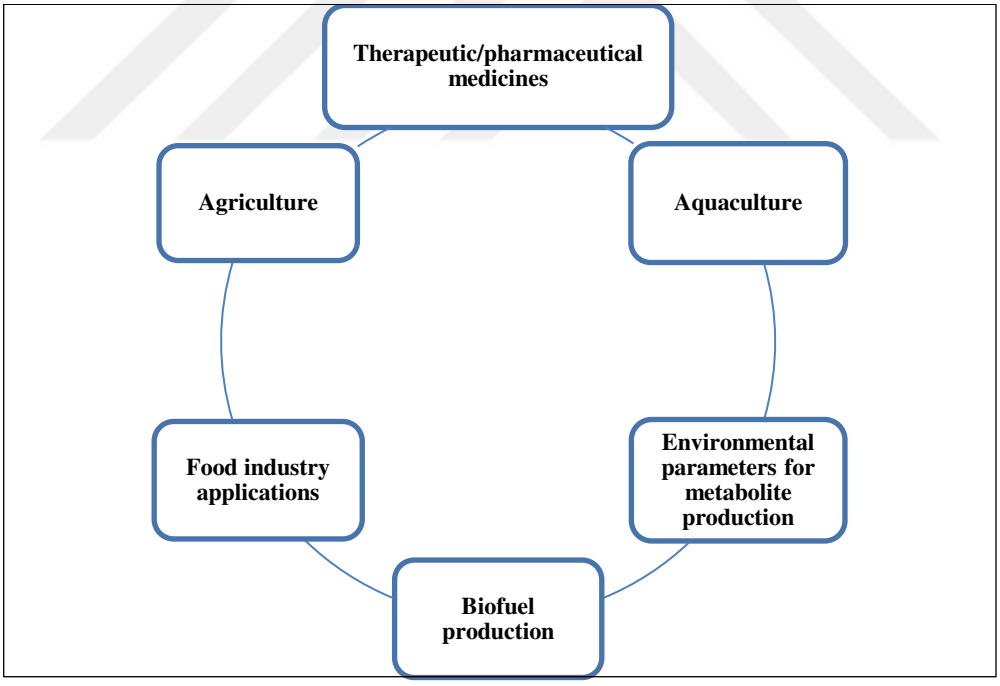



Figure 14. Applications of *C. vulgaris* (Tiong et al., 2020).

It has been reported that the bacteriocidal chlorellin found in *C. vulgaris* has a protective effect on infections caused by bacteria (Mostafa, 2012; Pratt et al., 1944). It is stated that the linolenic acid found in *C. vulgaris* cells has an antibacterial effect against S.

aureus infections and Salmonella typhi, and therefore *C.vulgaris* is used as a natural antibiotic source in the pharmaceutical field (Ahmad et al., 2020; Kumar P, 2017).

The nutritional supplements of *C. vulgaris* are used as raw materials in aquaculture due to their rich nutrients and high digestive content (Brennan & Owende, 2010). Pellets fortified with *C. vulgaris* cells and/or extracts ensure the survival rate of aquatic organisms by increasing resistance against pathogens and infections (Maliwat et al., 2017). The addition of *C. vulgaris* to fish diets was essential to develop skin pigmentation in ornamental fish and muscle color in bait fish (Gouveia et al., 2007). Saturated and unsaturated fatty acids produced and accumulated in microalgae stand as excellent resources for the manufactured of biodiesel (Frumento et al., 2016; Maruyama et al., 1997; Yeh et al., 2010). With high growth rate and elevated lipid accumulation potentials, biodiesel from microalgae can be the best alternative over diesel fuels from petroleum. In addition, as microalgae develop rapidly, require very little arable land and generate potential to reduce greenhouse gas effects, it sustainably stands as a promising biodiesel production host. *C. vulgaris* strains are able to perform the fixation of the atmospheric $CO_2$ at 10–50 times greater yield than terrestrial plants (Li et al., 2008). Although the production of biodiesel from *C. vulgaris* hosts is still expensive, combinatory approaches towards both wastewater treatment and following fuel production processes enabled notable and promising improvements in productivity.

The high protein content of *C. vulgaris* has also provided its use as an emulsifier in applications in the food industry (Gouveia et al., 2005; Raymundo et al., 2005). While some polysaccharides found in *C. vulgaris* are used for thickening and gelation Brennan and Owende, 2010). It has been found that there is an improvement in the stability of texture and color parameters through natural staining from *C. vulgaris* cells (Gouveia et al., 2007). The high amount of amino acids found in *C. vulgaris* cells act as phytosiderophs, facilitating the penetration and absorption of micronutrients (Shaaban, 2001a). *C. vulgaris* is also eligible to increase the growth of agricultural crops (nutrient content), by secreting plant growth promoting hormones and providing nutrients to plants and enhancing soil residences (Faheed and Fattah, 2008; Shaaban, 2001b). These findings thus endorse that the utilization of *C. vulgaris* is eligible to improve agricultural yields of crops and tends to reduce the environmental concerns related to excessive utilization of toxic chemical fertilizers.

*C. vulgaris* is a quite useful resource and production host for numerous value-added mmetabolites and recombinant proteins. In addition, distinct environmental parameters and related approaches were even reported as enhancing mecahnisms toimprove the metabolite

production titers. Nitrogen stand as a crucial nutrient since it is highly essential for cell growth and lipid productivity (Kong et al., 2011) thus consisting 1–10% of overall dry matter in microalgae (Wijffels et al., 2010). Nitrogen forms such as aminium, nitrate and urea play an important role in the growth of microalgae cells (Chen and Chen, 2006). If both ammonium and nitrate are present in the growth vicinity of it, *C. vulgaris* first tend to prefer ammonium. In a study, it was stated that *C. vulgaris* consumed urea before than ammonium and nitrate in mixotrophic (Kong et al., 2011). Considering this situation, it shows that *C.vulgaris* consumes different nitrogen sources in different culture media.

Zheng and his colleagues stated that the biomass efficiency of *C. vulgaris* enhanced when sodium bicarbonate was added, and reached the peak biomass capacity when 1.2 g / L NaHCO3 was added (Zheng et al., 2011). A similar study performed by Daliry and his colleagues revealed that *C. vulgaris* reaches the highest biomass productivity when there is 8% $CO_2$ available in the environment, (Daliry et al., 2017) while Yusof et al., (2011) showed that *C. vulgaris* was able to reach its highest growth rate when cultured under 10% $CO_2$ and 24 hours light (Mohd Yusof, 2011). It is affected by the carbon dioxide content of the pH value of the culture medium. Therefore, optimum conditions should be provided in order not to affect the biomass yield of microalgae cells. Kumar et al., 2010).

### 1.5.4. Purpose of the Study

The use of different algae for the biodegradation processes has been the subject of research and development for several decades. Here we hypothesize that photosynthetic microalgae strains could address the PET wastes dependent global environmental pollutions. Our long-term goal therefore is to develop a photosynthetic microalgae strain capable to degrade various environmental PET wastes to their monomers, facilitates the environmentally friendly recycling of PET chemicals. In this perspective, we plan to integrate highly active recombinant PETase gene into *C. vulgaris* nuclear genome for the extracellular production of PETase enzyme. By doing so, we further aim to establish a low-cost and consistent PET degradation process through the recombination of microalgae strains. We further would like to revolutionize the establishment of an additional PET recycling process within current industrial microalgae processing systems such as processing biofuels productions, waste water treatments, electricity production etc. We further propose the utilization of microalgae culture liquid effluents towards to the efficient degradation of

PET wastes through integrated processes and develop a novel and low-cost processing systems for PET waste recycling systems for nations.

## 2. MATERIAL AND METHODS

### 2.1. Chemicals, Enzymes and Kits

Tris base (Multicell Wisent Inc # 600-127-CG), Yeast extract (Merck), NaCl (Merck), Tripton (Merck), Taq DNA Polymerase (Promega), dNTP (Promega), EDTA (Merck), $CaCl_2$ (Aktar Kimya), Kanamycin (Applichem), Ethyl Alchol, Ampicillin (Applichem), *EcoRI* (Promega), *BamH*I (Neb), T4 DNA Ligase (Neb), *Xba*I (Neb), *Bgl*II (Neb), Gel Extracting Kit (Thermoschintific), $Na_2HPO_4$ (Merck) $NaH_2PO_4$ (Merck), β-mercaptoethanol (Merck), Methanol (Isolab), Acetic acid (Isolab), Glyserol (Merck), Amonium Sulfate (Merck), TEMED (Janssen Chimica), KCl (Merck), $MgCl_2$ (Sigma), LiCl (Sigma), $CuCl_2$ (Sigma), Commassie Brillant Blue G-250, Commassie Brillant Blue R-250 (Merck), Phosphoric Acid (Merck), BSA (NEB), Sodium Acetate (Merck), *Trisma* Base (Sigma), Acrylamide (Sigma), Bis-Acrylamide (Promega) Fetal Bovine Serum (Thermo # 10270106), Penicillin-Streptomycin (Multicell Wisent # 450101100), Tripsin-EDTA (Multicell Wisent # 3255542100), DMEM (Multicell Wisent # 319-005CL), RPMI 1640 (Multicell Wisent # 350-000-CL), PBS (Capricorn # PBS-1A), Opti-Protein XL Marker (ABMGOOD # G266).

### 2.2. Strain Medium and Culture Conditions

*C.vulgaris (*SAG: 211/11J) strain used in this study was purchased from Department Experimental Phycology and Culture Collection of Algae, (University of Göttingen, Germany). The collection synonyms of this species is also descripted as follows: CCAP 211/11J, UTEX265, Sequences: GenBank AY591497.1. *C.vulgaris* 211/11J strain was grown in Tris-Acetate-Phosphate (TAP) medium. It was also cultured in TAP agar plates for long-term storage. *C. vulgaris* inoculum taken from some amount of liquid culture was cultivated in fresh TAP medium until reaching to the desired cellular density**.**

### 2.3. Synthesis of Chimeric PETase Gene

Chimeric PETase enzyme sequence was originated from *I. sakaiensis* strain 201-F6 and retrieved from UniProt database (*https://www.uniprot.org/*). Primers of wild and mutant PETases were designed using *OligoAnalyzer* server from IDTDNA technologies and synthesized by ordering from *Macrogen Inc*.

Rational design of new PETase enzyme was performed through *in silico* analysis. Two major rational engineering studies recently preformed on wild type PETase enzyme was used as reference to build up a novel chimeric enzyme PETase3M. Austin et al (2017) identified that the active site of PETase from *I. sakaiensis* strain 201-F6 was three times wider than that of the active site of cutinase enzyme from *Thermobifida fusca*, revealing a high 3D structure similarity to PETase (PDB ID: 6EQE) (Austin et al., 2018). In the related study, researchers were performed mutations on the active site of PETase to narrow its catalytic cleft to make it more similar to the related cutinase to see whether the specific activity improvement of the enzyme is possible or not. They introduced several mutations onto the active site of enzyme and identified that narrowing the binding cleft of PETase via the susbstitutions of amino acids in cutinases, researchers observed an improved PET degradation by developed mutants. Thus, we selected the two best substitutions towards improved PETase activity (Trp159His and Ser238Phe) from the related study. Yet in our study, the corresponding residues were numbered with different residue numbers since those researchers started counting from the signal peptide of the enzyme. Therefore, we defined the two amino acid substitutions in wild type PETase as first (Trp132His and Ser211Phe). As similar, in another study by Ma et al (2018) revealed that further rational engineering studies performed on near regions of substrate binding cleft could also improve the enzymatic activity of PETase (Ma et al., 2018). The researchers were focused on six key residues around the substrate-binding cleft to develop novel high-efficiency PETase mutants through enzyme engineering and 8 corresponding amino acid substitution at the active site of PETase. They identified that increasing the hydrophobicity range of the enzyme active site with more hydrophobic amino acids improved the catalytic efficiency of PETase. Although most of their mutants were able to improve specific enzyme activity, we selected Iso179Phe substitution to create a new PETase mutant with enhanced catalytic activity. Because the Km of the I179F mutant was identified as 3.8 times lower than wild type PETase and its Kcat/Km value was identified as 15.6 times higher than that of wild type PETase.

Overall, we picked three of the most promising mutations (Trp159His, Iso179Phe and Ser238Phe) out of those substitutions in the studies reported above and analyzed their coordination towards a more enhanced PETase activity through a homology modeling and molecular docking studies. After obtaining quite promising theoretical data about the presence of those three mutations on PETase enzyme together, we combined those mutations as a new triple mutant of PETase enzyme, and placed those amino acid substitutions into the encoding DNA sequence of wild type PETase before the synthetic synthesis of gene fragment. After this, we called the new PETase mutant as PETase3M.

### 2.4. Homology Modeling of Chimeric PETase

Three consecutive mutations as amino acid substitutions in the precise orientations were placed into the amino acid sequence of *I. sakaiensis* strain 201-F6 PETase (in 6EQE amino acid sequence without its signal peptide sequence, the corresponding substitution regions were identified as Trp132His, Iso181Phe and Ser211Phe). The related amino acid sequence was then utilized for 3D structure prediction and following structure validation and energy minimization analyses.

Upon obtaining true folded protein structure of PETase3M, the related 3D structure was utilized for comparative molecular docking studies. A comparative docking study between wild type PETase (PDB ID: 6EQE) and PETase3M modeled structure was operated by using 2 PET molecule as substrate. The related comparative analysis was performed by using AutoDock Vina program.

These new PETase primers were redesigned using the *SnapGene* program and synthetically synthesized by Macrogen.

Table 3. Primer Sequences

| Primer name; | Primer sequences |
|---|---|
| PETase_F | 5’ – GCG GAT CCC AGA CCA ACC CTT AC-3’ |
| PETase_R | 5’ - GCG ATT TCT TGG TGG TGG TGG TGG T – 3’ |

### 2.5. Amplification of *PETase3M* gene by PCR

Table 4. PCR amplification of *PETase3M* gene

| Reaction components | Amounts | PCR Cycle |
|---|---|---|
| 10x Buffer | 5 µl | 98 ºC-105 second (sec) |
| dNTP | 1 µl | 98 ºC- 20 sec ⎫ |
| DMSO | 0,2 µl | 68 ºC-35 sec ⎬ 37 cycle |
| $MgCl_2$ | 0,5 µl | 72 ºC-40 sec ⎭ |
| Forward Primer | 1 µl | 72 ºC-7 minute (min) |
| Reverse Primer | 1 µl | 4 ºC-∞ |
| Template DNA | 0,7 µl | |
| O5Taq DNA polymerase | 0,25 µl | |
| $dH_2O$ | 15,35 µl | |
| Final | 25 µl | |

Sequences edited for GenScript order
PETase3M: codon optimized sequence with 3 mutations and + 6XHis

### 2.6. Cloning of PETase DNA Fragment into pJET1.2/blunt Vector

The PETase gene obtained as the PCR product was cloned into the pJET1.2/blunt cloning vector using the commercial kit (Thermo Fischer CloneJET PCR Cloning Kit; Catalog number: K1231). The ligation product was transformed into *E. coli* JM101 competently prepared by Calcium Chloride ($CaCl_2$) method. After taking 200 µl of the freshly prepared competent cell, 10 µl of ligation product was added. It was then kept in a basin of ice for 30 minutes. Thus, the transformation process was realized in a healthy way. After incubation, the samples were shocked in 42 ºC heater block for 2 minutes to allow the plasmid to enter the cell. At the end of a two-hour incubation at 37 ° C, it was inoculated on Luria-Bertani (LB) agar containing 50 µg / µl of ampicillin by the spreading method and incubated for 16 hours at 37 ° C. Transformants thought to be positive among the colonies that grew the next day were selected and isolated according to the alkaline lysis method. The isolated plasmids were cut with EcoRI and Ndel enzymes and checked in agarose gel to verify the positivity of the clones.

## 2.7. Excision of Chimeric DNA Fragment From pJET1.2 Vector to Clone Into pOpt_cCA_mRuby2_Paro Expression Vector

In order to express PETase3M protein in *C. vulgaris*, a recently developed expression vector pOpt_cCA_mRuby2_Paro was purchased from *Chlamydomonas Resource Center* (*https://www.chlamycollection.org/*) and utilized during microalgae transformation experiments (Figure 15). Using PETase specific primer pair, *Bam*H1 and *Eco*RI restriction endonuclease recognition regions were added to both 5' and 3' ends of PETase3M gene by PCR. Therefore, the recombinant PJET1.2 vector and POpt_cCA_mRuby2_Paro expression vector were digested with *Bam*H1and *Eco*R1 by following the reaction conditions suggested by the providing company (New England Biolabs Inc).

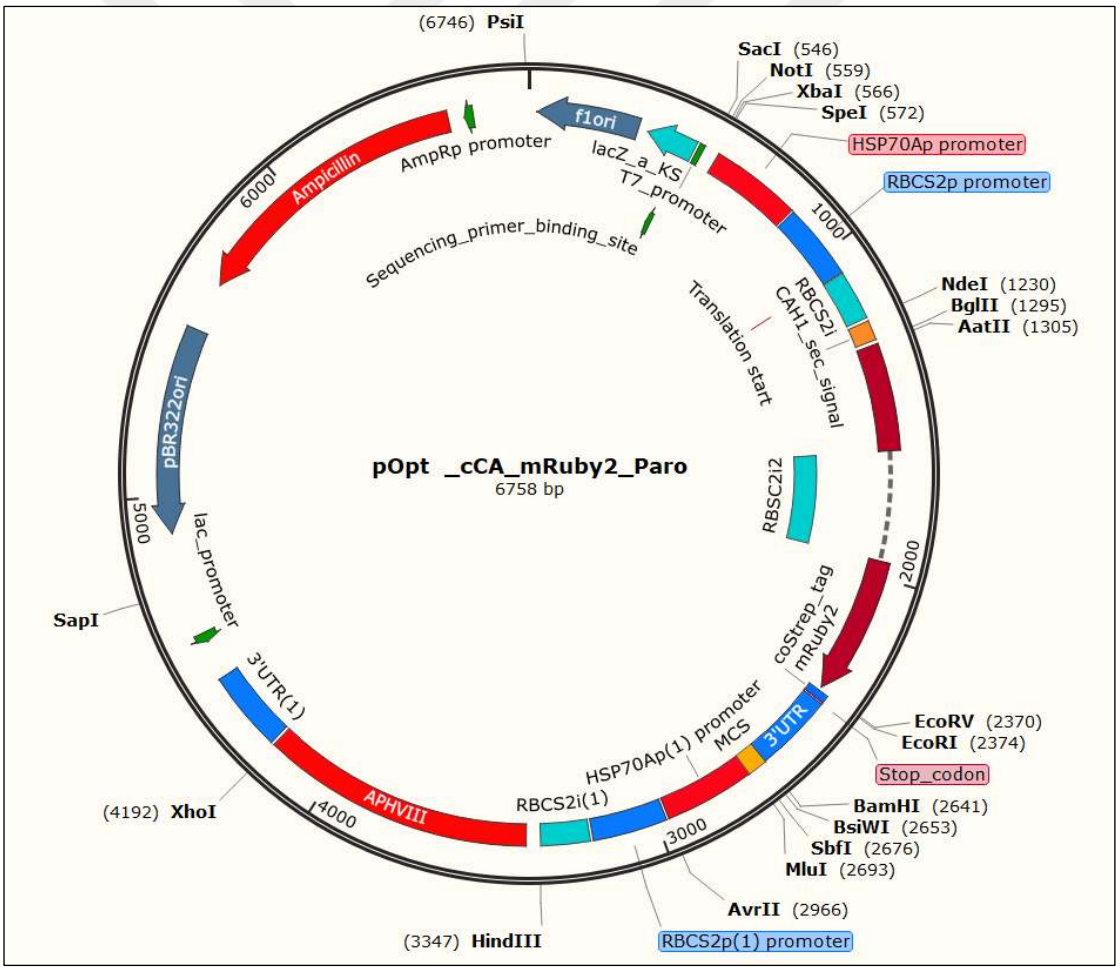



Figure 15. Schematic representation of pOpt_cCA_mRuby2_Paro *Chlamydomonas* expression vector. mRuby2 coding region was excised and related vector was initially linearized before the insertion of PETase3M coding DNA fragmenti through ligation.

### 2.8. Linearization of pOpt_cCA_mRuby2_Paro Vector

Likewise, the pOpt_cCA_mRuby2_Paro expression vector was prepared by cutting with *Bam*HI and *Eco*RI endonucleases enzymes.

### 2.9. Ligation of PETase Towards pOpt_cCA_mRuby2_Paro Vector Cloning and Transformation

The PETase gene and the pJET 1.2 vector were cut with restriction enzymes and cloned together with the kit. JM101 competent cells prepared by the CaCl2 method were used. When transforming, 10 µl of ligation product and 200 µl of our freshly prepared competent cells were used. Cells that were incubated in ice for 30 minutes were taken into a thermo shaker to be subjected to heat shock and were subjected to shock at 42 ° C for 2 minutes. Cells kept at 37 ° C for 2 hours without waiting were spread in LB agar containing 50 µg / µl paromomycin (Paro) and incubated overnight at 37 ° C.

### 2.10. Plasmid Isolation and Identification of PETase3M_ pOpt_cCA_mRuby2_ Paro Positive Clones

Colony selection was made on following day. Plasmid DNAs were isolated quickly and safely using one of the traditional methods, alkaline lysis method. Isolates digested with *EcoRI* and *Nde1* restriction enzymes were checked by agarose gel electrophoresis and recombinant clones were identified.

### 2.11. Enzymatic Treatment of *C. vulgaris* 211/11J cells for Protoplasting

In order to perform the electroporation-based transformation of *C. vulgaris* 211/11J host cells which harbor quite thick cell wall, we have treated the related microalgae cells from a logarithmic growth phase with a cell wall degrading enzyme cocktail (Figure 22). A time-dependent enzyme cocktail treatment was applied onto related microalgae cells to optimize protoplasting process for further experiments (Figure 23 and Figure 24). By doing so, we aimed to obtain microalgae protoplasts with thinner cell walls facilitating the DNA

transition from the cellular membrane at their outside, such application was a remarkably crucial treatment to prepare such persistent cells to nuclear gene transfer.

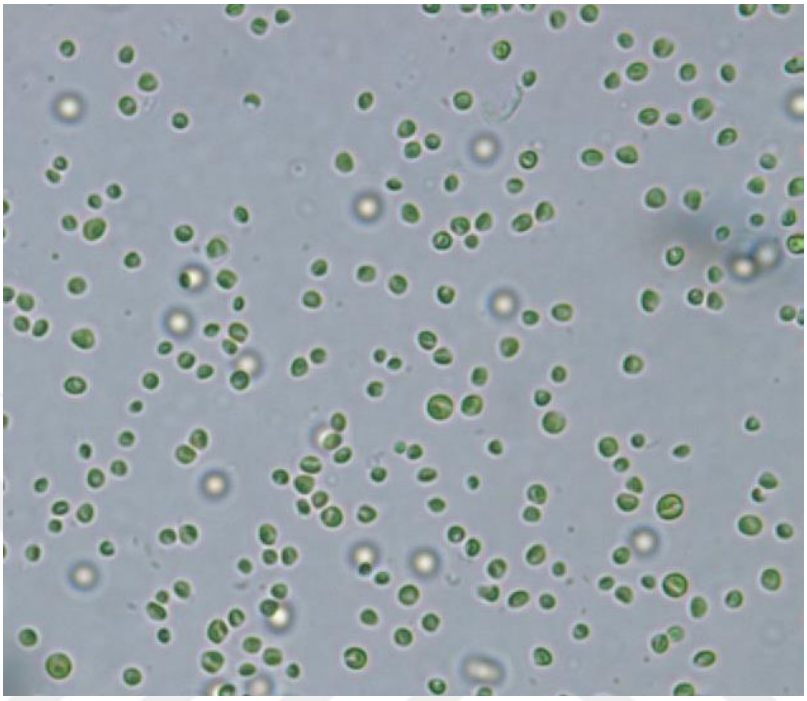


Figure 16. Visualization of untransformed *C. vulgaris* 211/11J cells before enzyme treatment

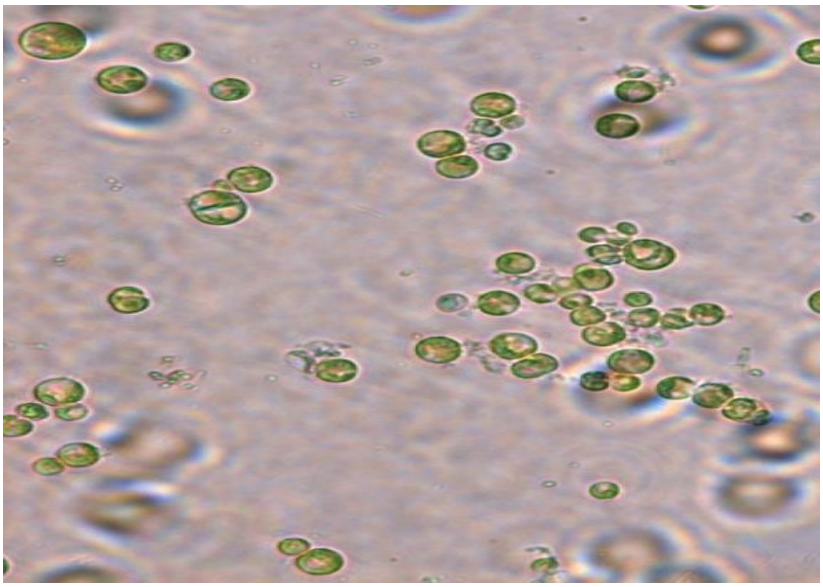


Figure 17. Microscopic visualization *C. vulgaris* 211/11J cells after treating with enzyme mixture for 1 hour

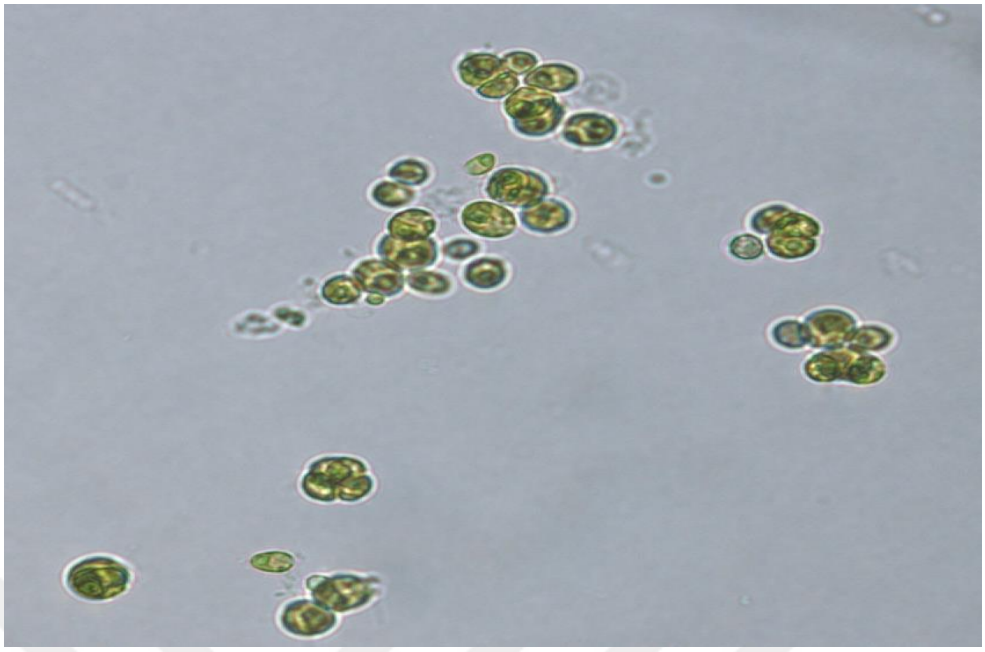


Figure 18. Visualization of *C. vulgaris* 211/11J cells after 2 hours treatment with enzyme cocktail

### 2.12. Transformation *C. vulgaris* 211/11J by Electroporation

Since *C. vulgaris* 211/11J has a thick wall and cell wall, enzyme treatment was applied to weaken the cell wall before electroporation.

Protoplasting enzyme mixture: Enzyme mixture containing 50 mM $CaCl_2.2H_2O$, 1.0 mg/ml lysozyme, 0.1% MES, 0.6 M sorbitol, 0.25 mg/ml chitinase and 1.0 mg / mL sulfatase was prepared in 10 mL sterile water. The mixture was dissolved at 4 ° C and filter sterilized using a 0.22 µm nylon filter. In the early growth phase, a total of $4 \times 10^7$ cells ml (Optic Density 680 = 2.0) were used to prepare protoplasts in 10 ml of the mixture solution. Cells were incubated at room temperature in the dark for up to 15 hours with gentle shaking in the shaker at 50 rpm.

The cells were harvested by centrifugation at 1350Xg for 10 minutes. The most intensive cell collection process was performed by increasing or decreasing the centrifuge speed according to the durability of your cells.

Electroporation process was performed by modifying the method determined by Bai et al., 2013; Run et al., *C vulgaris* 211/11J *p*rotoplast cell ($1 \times 10^6$) were used for transformation.

55 µl of cooled osmotic buffer (0.2 M mannitol and 0.2 M sorbitol) were added to the algae cells in Eppendorf tubes. Then, Eppendorfs were incubated for 20 min in ice. Eppendorf's taken from ice were kept at room temperature for a few minutes. It was again incubated in ice for another 20 min.

Then 55 µL of cooled electroporation buffer (0.005 M $CaCl_2$, 0.01 M Hepes; pH 7.2, 0.08 M KCl, 0.2 M mannitol, and 0.2 M sorbitol) were transferred into the Eppendorf tubes. It was re-incubated on ice for 1 hour. 1 µg DNA and 10 µg Salmon sperm DNA were added. It was incubated again on ice for 15 minutes. This mixture was transferred to the cooled electroporation cuvette for 20 minutes. Electroporation process was started using Bio Rad Micro Pulser electroporation system. Electricity was given at 2 kV 3.4 ms. The tube was then kept in ice for 10 minutes. At the end of the period, the samples taken out and the Eppendorf tubes were kept in the dark for 24 hours with the help of foil (The pellet, which settled to the bottom every 4 hours, dissolved very slowly).

### 2.13. Selection of PETase3M Positive Transformants in The Presence of Paromamycin Antibiotic on Agar Plates

Transformants were inoculated into TAP agar after 24 hours of incubation at room temperature. 8 ml of 0.8% TAP agar + paromomycin (30 mg/ml) was poured into each petri dish, Immediately after the TAP agar in the petri dishes was frozen, it was cultivated with the help of a loop.

### 2.14. Incubation of Positive Transformants in TAP Medium Over Three Generations

Samples were taken from colonies grown in petri dishes containing paromomycin Transformants were inoculated back into TAP medium containing paromomycin and the fastest growing colonies were selected.

### 2.15. Genomic DNA (gDNA) Isolation From Positive Transformants of PETase

Genomic DNA isolation from *C.vulgaris* 211/11J was made modifying the Cetyltrimethylammonium Bromide (CTAB) method by developed Murray and Thompson 1980; Vimal Kumar et al., 2017.

The modified method is as follows:

- Algae culture in 1.5 ml ×2 Eppendorf was precipitated.
- -20 / + 50 ºC freezing and thawing processes applied (3x)
- 500 µl CTAB + 0.02 g PVPP +2.5 µl Beta mercaptoethanol was added.
- Gently inverted 3-4 times.
- Incubated at +65 ºC for 30 min.
- It was centrifuged for 2 min at the highest speed (14800) rpm
- 500 µl Chloroform isoamyl alcohol added, inverted until homogenized.
- Centrifuged at 14800 rpm for 1 minute
- Steps 6 and 7 were repeated (Upper Phase Taken into New Tube).
- The upper phase was removed and 8% 3M sodium acetate was added.
- 600 µl of isopropanol was added and incubated for 2 hours at -20 ºC.
- Centrifuged at 14800 rpm for 10-15 minutes
- Pellet was washed with 500 µl 70% alcohol.
- The alcohol in Eppendorf was kept at + 37 ºC until it dried ( about 20 minutes)
- Solved in 35 µl.
- Imaging was done on the gel.

After this process, the presence of PETase gene in *C. vulgaris* 211/11J cells was detected by PCR colony with the plasmid we obtained. Colonies were found to be positive for the PETase3M gene.

### 2.16. Ammonium Sulfate Based Precipitation of Culture Supernatants

A selection was made from among algae cultures that have grown. Since all proteins produced extracellularly will be in the medium, algae were first transferred from glass flasks to falcone tubes where they were grown and precipitated with the help of centrifuge at 10000 rpm. This process was repeated (3 times) until no algae residue remained in the falcon. For

obtained supernatant applied 80% ammonium sulfate precipitation. Before this process, our supernatant was slowly transferred to the glass flask. The reason it is slow is to keep our proteins from being damaged.

The flask was placed in an ice-filled container and dropped onto the magnetic stirrer. Later, the amount of ammonium sulfate needed for precipitation of total extracellular proteins was determined using Ammonium Sulfate Calculator (*EnCor Biotechnology Inc.*) program. The magnetic stirrer was set at a speed of about 80 rpm and the ammonium sulfate addition was performed at about 3 grams in for each 2-3 min. After the protein precipitation was finished, the samples were kept at +4 ºC overnight. It was taken back to the flange and precipitated at 10000 rpm for 15 min at +4 ºC and its supernatant was removed.

### 2.17. His Resin-Based Purification of Recombinant PETase Protein from Microalgae Secretome

After the ammonium sulfate precipitation was done, the pellet was dissolved in the wash buffer. Some of it was reserved and saved for the (SDS-PAGE).

Promega MagneHis ™ Protein Purification System was added to the remaining part (30µl for 1ml total protein amount) (*Promega MagneHis*™). The total protein obtained with the Promega MagneHis ™ Protein Purification System was mixed and kept on the spinner at low rpm for 2 hours. After the process was completed, the pellet was collected with the help of a magnet and the supernatant was removed. It was dissolved in 300 µL of elution buffer and protein purification was complete.

### 2.18. SDS-PAGE Based Analysis of *C. vulgaris* Secretome in Culture Supernatants

Protein gel electrophoresis was carried out at 22 mA on a 15% gel. The molecular weight of the PETase protein was determined after performing SDS polyacrylamide gel electrophoresis. The sample buffer was prepared by mixing 0.15 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol and 6%-mercaptoethanol. It was mixed with 15 µl protein and 5 µl loading dye. It was incubated at 95 ° C for 5 minutes. Denatured proteins were loaded on 15% gel. After the SDS was finished, it was stained with Commansie Brillant Blue R-250 (0.125% Commassie Brilliant Blue R-250, 50% methanol, 10% acetic acid) for 45 minutes.

Proteins were immersed in the gel with buffer I (50% methanol, 40% ddH2O, 10% acetic acid) for 45 minutes and finally the gel was washed with buffer II (7% methanol, 10% acetic acid, 83% ddH2O).

### 2.19. PET Plastic Film Degradation Experiment

Generally, experimental groups related to pet degradation were prepared. Purified PETase proteins were combined with PET. Pet films were cut square or rectangular with the help of a scalpel. It was then washed with distilled water against any risk of contamination. It was treated with PETase enzyme in pH 9 buffer. We left each piece of plastic in this buffer for 2 days. We viewed the results under a microscope.

## 3. RESULTS

### 3.1. Homology Modeling Based 3D Structure Prediction of PETase3M Protein

Newly formed PETase3M protein 3D structure prediction was obtained using Phyre2 (Protein Homology/analogY Recognition Engine V2.0) server and visualized through PyMOL visualization program (Figure 16 A and B) (Kelley et al., 2015).

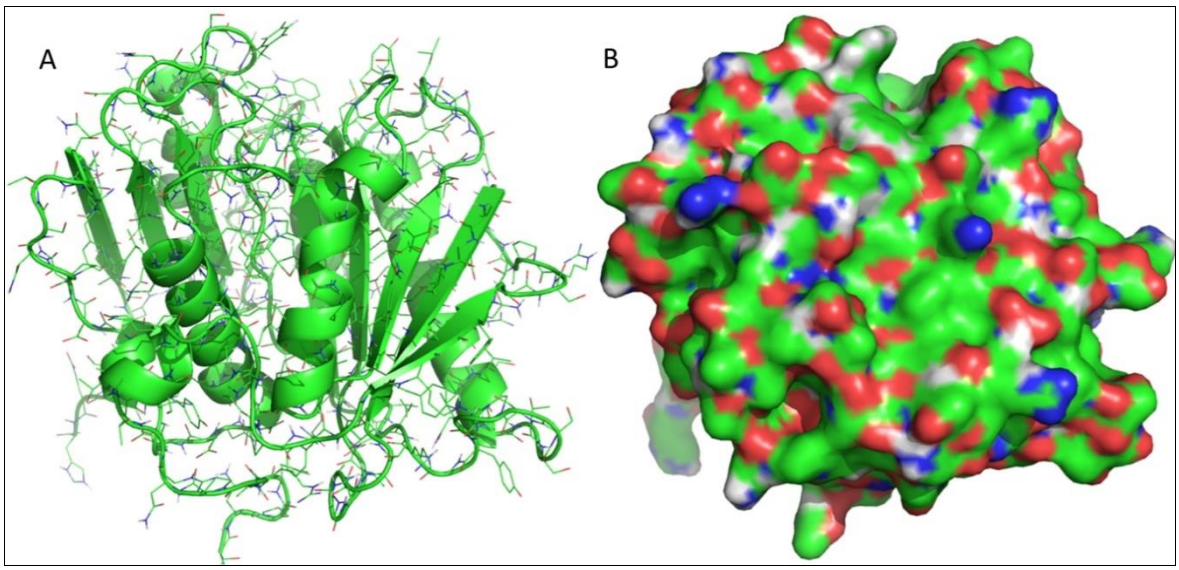



Figure 19. Homology modeled 3D structure of PETase3M mutant. A) 3D structure of PETase3M in Cartoon form. B) 3D structure of PETase3M in surface format.

### 3.2. Comparative Molecular Docking Results

The molecular docking outputs from both wild type PETase and PETase3M mutant were revealed that PETase3M variant displays a higher binding affinity to 2 PET substrate (Table 5).

Table 5: Comparison of the outputs obtained from the docking experiments

| Mode | PETase Affinity (kcal/mol) | PETase3M Affinity (kcal/mol) | PETase dist from rmsd l.b. | PETase3M dist from rmsd l.b. | PETase best mode rmsd u.b. | PETase3M best mode rmsd u.b. |
|---|---|---|---|---|---|---|
| **1** | -6.2 | -7.0 | 0.000 | 0.000 | 0.000 | 0.000 |
| **2** | -6.2 | -6.9 | 5.847 | 2.886 | 8.908 | 4.465 |
| **3** | -6.1 | -6.8 | 1.592 | 1.653 | 10.133 | 2.279 |
| **4** | -6.1 | -6.7 | 1.532 | 1.818 | 2.239 | 10.994 |
| **5** | -6.0 | -6.7 | 1.559 | 1.551 | 2.081 | 2.099 |
| **6** | -6.0 | -6.4 | 1.407 | 1.266 | 10.792 | 10.750 |
| **7** | -6.0 | -6.4 | 1.630 | 1.492 | 9.666 | 10.535 |
| **8** | -5.9 | -6.4 | 6.097 | 1.589 | 9.386 | 10.662 |
| **9** | -5.8 | -6.3 | 1.759 | 3.375 | 10.260 | 3.882 |

For all molecular docking experiments performed on both PETase and PETase3M proteins 2 PET plastic dimer was utilized as major substrate. Related output structures confirming the availability of a higher stabilization of 2 PET molecule within the catalytic cleft of PETase3M were also visualized compararatively (Figure 17 A and B).

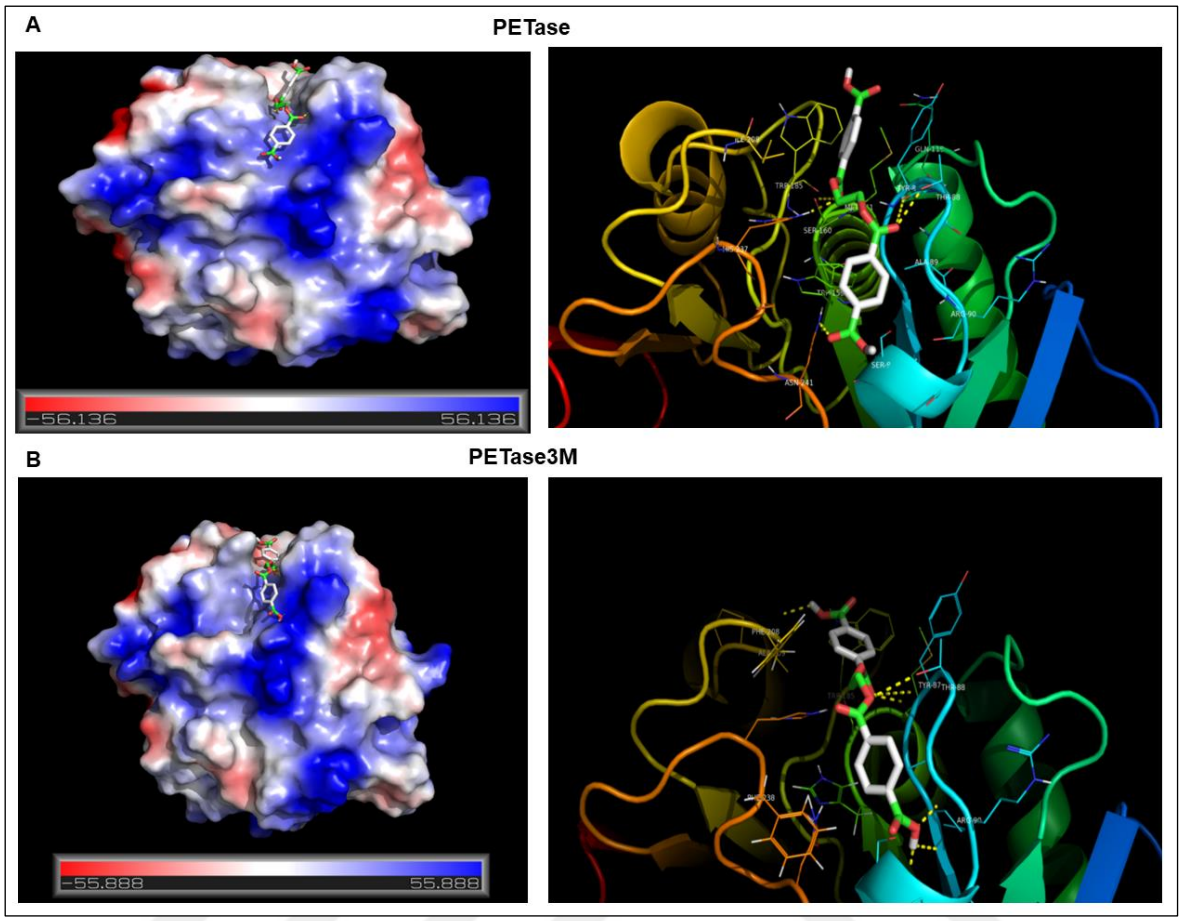



Figure 20. Visualization of comparative molecular docking results from both PETase (6EQE) and PETase3M. A) PETase and 2 PET ligand were visualized together upon obtaining the best docking result with the lowest binding free energy. Wild type PETase can only establish 5 hydrogen bonds bonds between the substrate and its catalytic residues. B) Visualization of the docking result for 2 PET ligand and chimeric PETase3M mutant upon the identification of the best binding affinity value as the lowest binding energy. It is also clear in Figure 17B that PETase3M can perform way better stabilization of 2 PET ligand by establishing 8 hydrogen bonds between the substrate and its catalytic residues.

### 3.3. PCR Amplification of PETase3M Gene

Synthetic PETase3M gene was PCR amplified using Q5 Taq DNA polymerase using the *Applied Biosystem* PCR device. The PCR product was run in 1% agarose gel electrophoresis and visualized with a UV transimulator (Figure 18). The presence of a single band of 810 bp on the gel demonstrated the success of the PCR reaction.

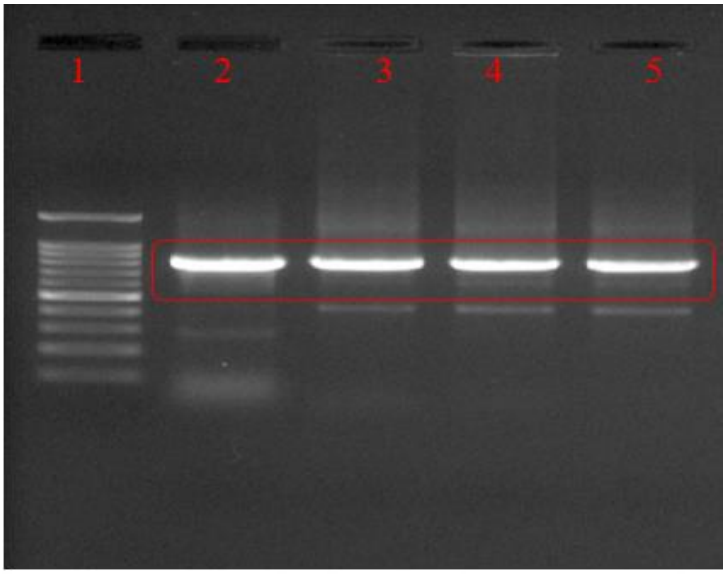



Figure 21. PCR amplification of PETase3M gene encoding a chimeric PETase with three mutations in its active site. 1: DNA ladder (1000 bp) 2, 3, 4 and 5: 810 bp chimeric PETase

### 3.4. Cloning of PETase3M DNA Fragment Into pJET1.2 Vector

Figure 19 reveals the plasmid isolation performed for control after transformation of Jm101 competent cells and to verify the existence of chimeric PETase3M within pJET1.2 cloning vector.

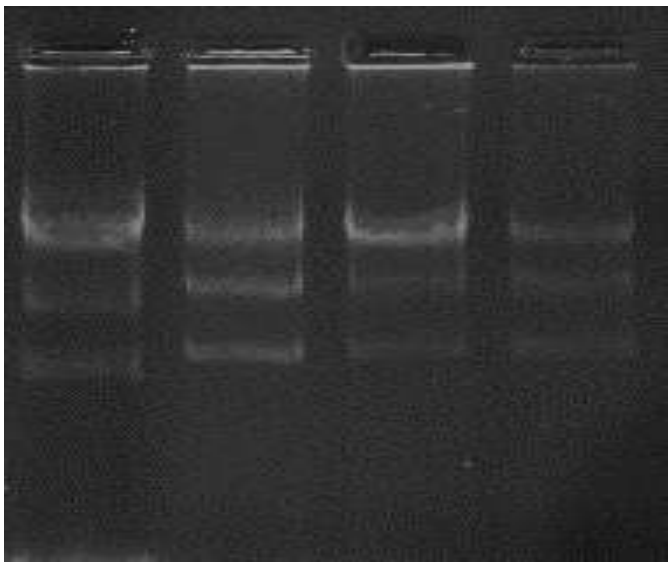


Figure 22. Plazmid isolation after cloning PETase3M into pJET1.2/blunt vector

### 3.6. Restriction Endonuclease Based Confirmation of PETase3M within pOpt_cCa_mRuby2_paro Vector

Upon the ligation of PETase3M DNA fragment into pOpt_cCa_mRuby2_paro expression plasmid, the existence of the related insert within the destination vector was confirmed through double digestion based molecular analysis (Figure 20). For this reaction, the related insert was excised by using *Nde*I and *Eco*RI restriction endonucleases, sequentially.

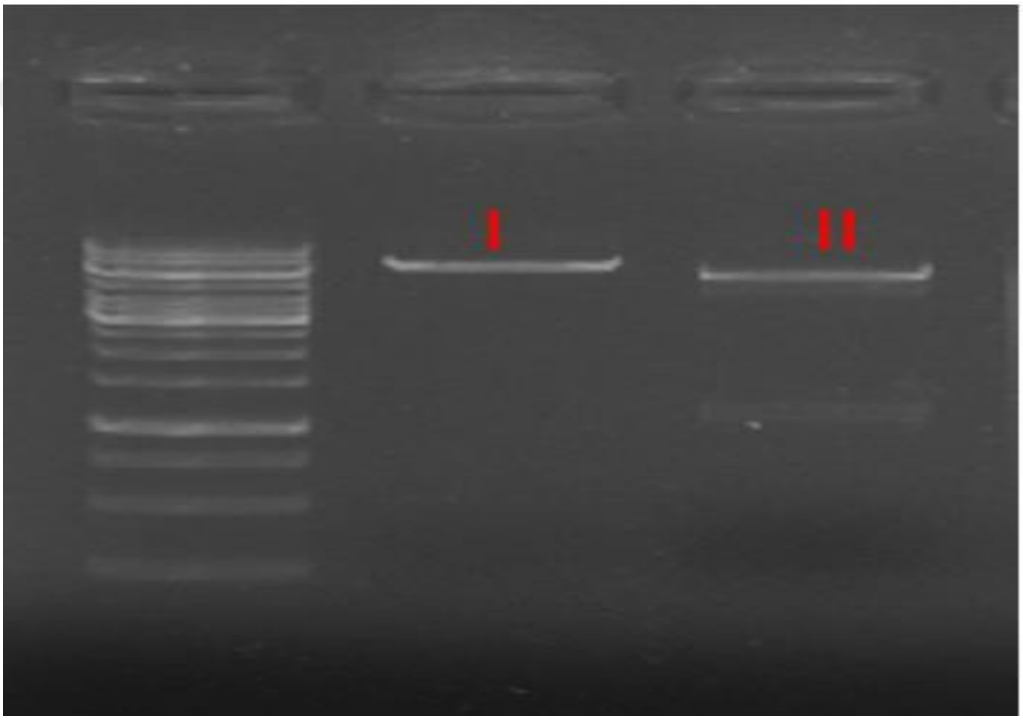


Figure 23. Restriction endonuclease based confirmation of PETase3M DNA insertion within pOpt_cCa_mRuby2_ Paro expression vector. I; First restriction reaction performed on pOpt_cCa_mRuby2_ Paro_PETase3M vector using only *Eco*R1 restriction enzyme. II; Agarose gel based visualization of the double restriction reaction result of pOpt_cCa_mRuby2_ Paro_PETase3M vector with the restriction enzymes *Nde*1and *Eco*R1.

### 3.7. His Resin Based Purification of Recombinant PETase Protein SDS-Page

Thanks to the sequences contained in the vector pOpt_cCa_mRuby2_ Paro. the PETase protein was expressed with 6 additional his amino acids. Thus, PETase protein was isolated thanks to His tail using the "MagneHis Protein Purification System" kit. After the

process to clean the protein, PETase protein was run in SDS-PAGE. The purified protein was visualized by staining with dye (Figure 21).

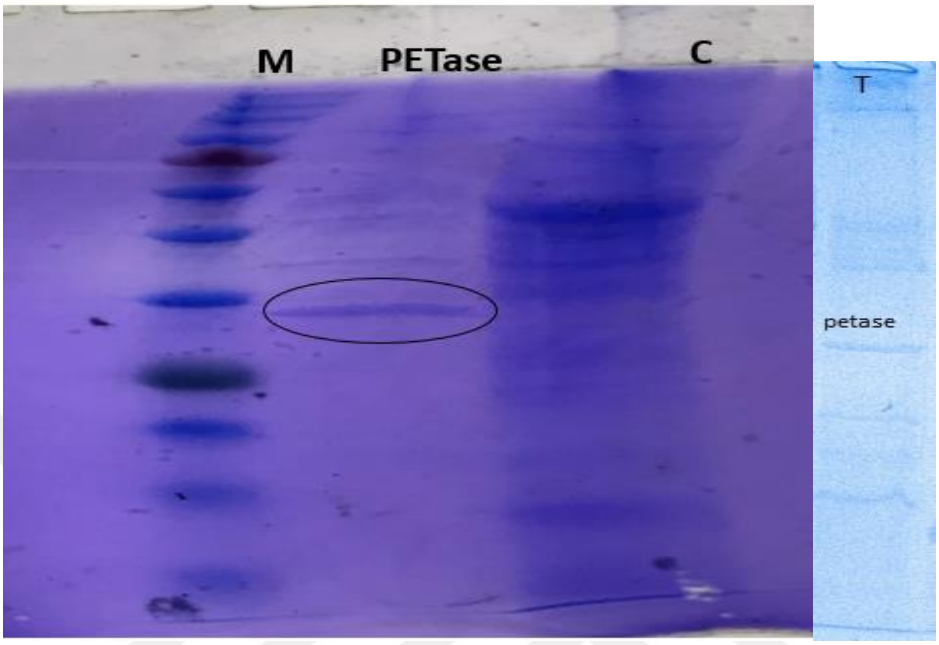



Figure 24. SDS-PAGE analysis of purified PETase3M protein. Total proteins from *C. vulgaris* culture medium were treated with His resins to obtain 6XHis-tagged recombinant PETase3M. M: protein marker, C: control, T: total protein.

### 3.8. PET Plastic Degradation Experiment

Using the purified PETase3M enzyme, PET degradation experiments were performed. The refrigerator bags used for this experiment were first cut into small pieces (2x2) and washed with distilled water. Then the obtained PETase3m was treated with enzyme. In order to confirm the functionality and high specific activity of recombinant PETase3M enzyme, various refrigerator storage bags with differing thicknesses were selected and treated with the varying concentrations of PETase3M. The obtained results were compared over the control PET film light microscope images (Figure 25). Upon 48 hours of incubation at 30 ºC by using 50 nM PETase3M enzyme (pH 7.2) in phosphate buffer, the degraded PET films from different sources were visualized and their surface images confirming the efficient hydrolysis of PET films were taken by utilizing inverted light microscope (Figure 26 A, B,

C, D, E and F). All of the pictures below were taken under the same conditions. All pictures were taken at 40x magnification.

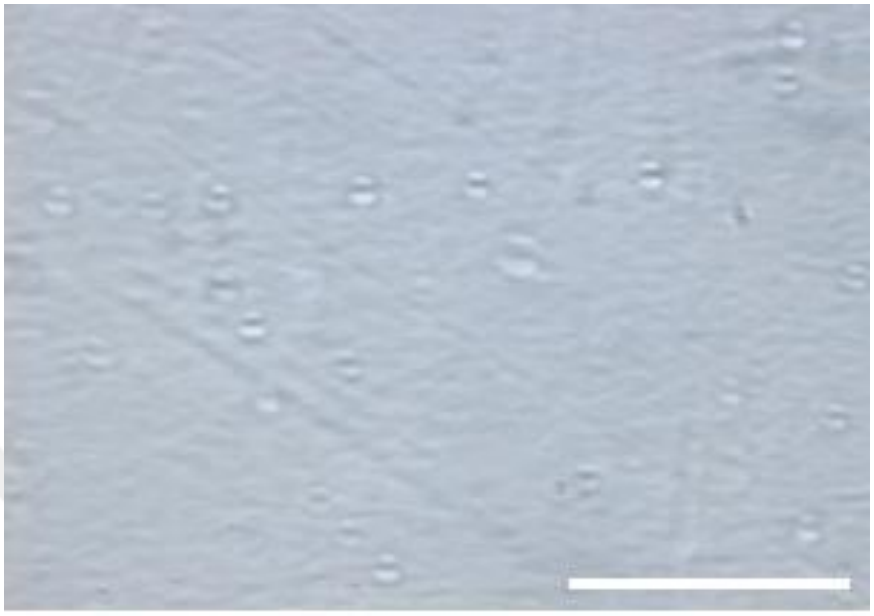


Figure 25 Control: Visualization of untreated PET film surface before any PETase enzyme treatment.

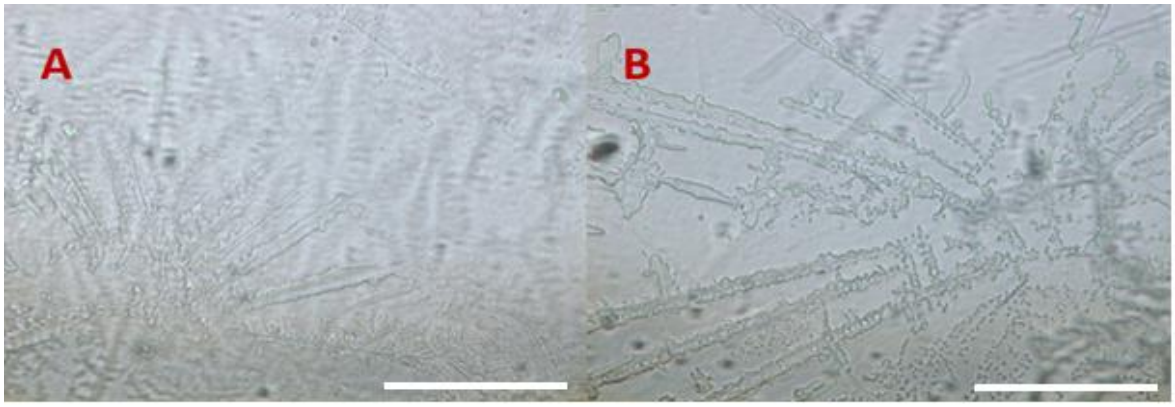
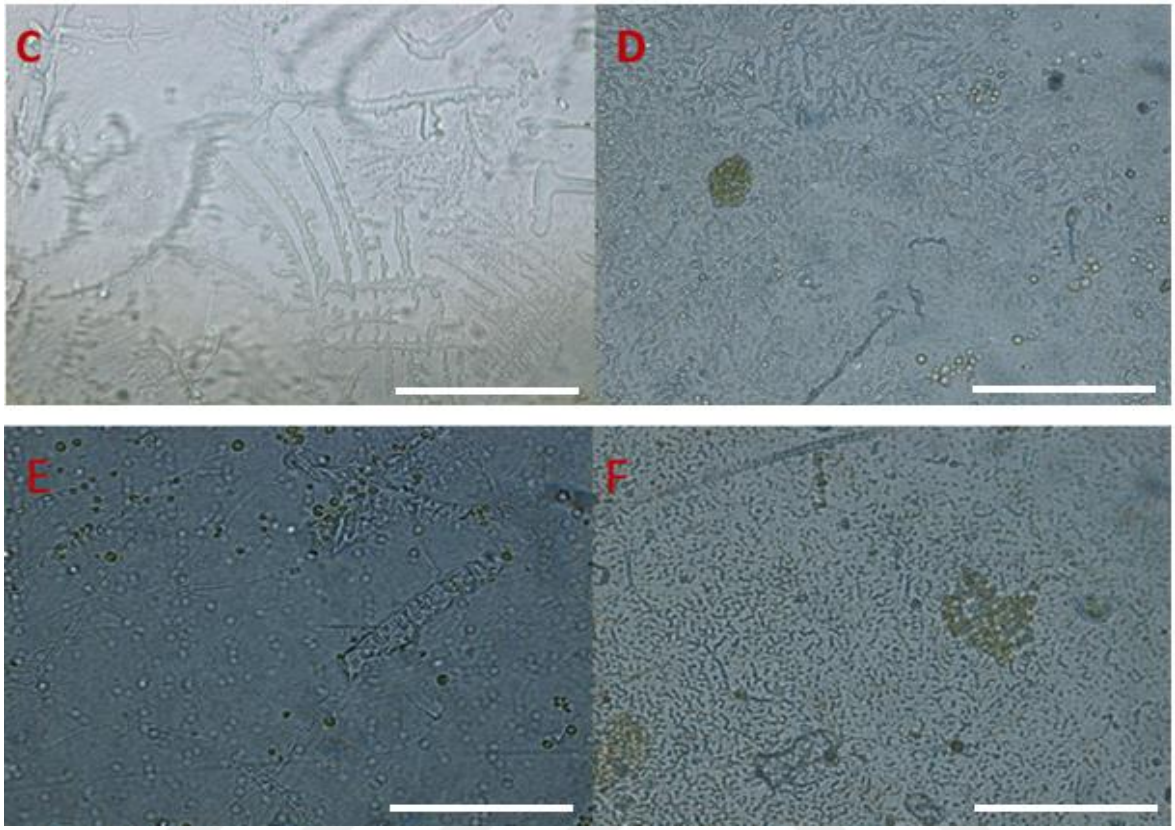



Figure 26. Plastic images after PET degradation under inverted light microscope. A, B and C; Degradations on the PET surface after treatment with purified PETase protein. D, E and F; Degradations on the PET surface after treatment with positive transformant of *C. vulgaris* 211/11J.

Figure 26. Continued



## 4. DISCUSSION

PETase gene sequence was synthesized as synthetic gene fragment. After synthetically rewriting the PETase gene for *C. vulgaris* 211/11J hosts, it was first cloned into pJET1.2 Easy vector for maintenance and storage. After that, the related gene fragment was transferred into *C. vulgaris* 211/11J expression vector pOPT_mRuby2_Paromy by using BamHI and EcoRI restriction sites. In this vector, recombinant PETase fragment was placed under the Hsp70/RbcS2 phusion promoter system. Normally, pOPT_mRuby2_Paromy vector contains RFP coding fragment between Hsp70/RbcS2 phusion promoter and 3'UTR in the related position. Therefore we replace the PETase DNA fragment with RFP region in order to express PETase gene under the related phusion promoter in *C. vulgaris* 211/11J. For this, sequential ligation, restriction reactions, plasmid DNA isolation based screening studies and related all other molecular techniques was performed accordingly.

The transformation of *C. vulgaris* 211/11J microalgae cells with recombinant pOPT_mRuby2_Paromy vector carrying codon-optimized PETase gene was performed through electroporation-based modified processes. The positive transformants of *C. vulgaris* 211/11J cells was selected on TAP agar medium containing 50 ug/ml Paromomycin antibiotics over three consequtive generations. The transformed microalgae cells was further analyzed through genomic DNA isolation and following confirmative PCR analyses to validate the existence of recombinant PETase gene as integrated. Further successful expression, extracellular and photosynthetically production of recombinant PETase enzyme by *C. vulgaris* 211/11J cultures was confirmed through SDS-PAGE analysis.

Plastics are very beneficial materials with extensive ranges of applications and apparently no longer indispensable for our day by day life. Plastic materials, mainly PET, enable economic advantages as they are broadly spread in our every day life, and its worldwide manufacturing is growing rapidly (Lebreton and Andrady 2019). PET, a crude-oil derived synthetic polymer, is presently one of the most significantly used plastics (Liu et al., 2019).

PET is strong and durable, chemically and thermally stable, has low gas permeability and is simple to apply. It is consisting of the building blocks EG and TPA linked via ester bonds. These properties of PET make it an essential product for plastic use worldwide (Falah et al., 2020). PET is mostly used in sheet and film, fabric food and beverage industry

packaging tools, auto parts, home appliances, lighting products, sports equipment, photography equipment, x-ray plates and textile products (Chen et al., 2018; Lu et al., 2009).

However, the features that have made plastic an attractive resource, are similarly as responsible for the harm this is caused as soon as it becomes waste (Song et al., 2019).

Plastic consumption is increasing day by day and plastic pollution is increasing. Around 100 million tons of plastic are produced worldwide each year. It causes adverse effects on the environment. When plastics in water bodies undergo mechanical and chemical deterioration, it causes water pollution and can threaten aquatic life. When plastics come into direct contact with ultraviolet rays from sunlight, they break down into more toxic forms (i.e. micro and nano form) that can quickly enter the food chain and cause environmental and health problems (Falah et al., 2020; Moog et al., 2019). The greatest threat of plastics to aquatic life is the formation and distribution of microplastics. These particles contain harmful substances and can adsorb toxic compounds. This harms living things (Moog et al., 2019). PET recycling describes one of the most a success and extensive examples of polymer recycling. Plastic recycling is important to reduce increasing plastic waste volumes and to produce value added materials by converting from low-cost resources into valuable materials (Jankauskaite, 2016). Plastics can be degraded by several physical, chemical, and biological methods (Karayannidis & Achilias, 2007).

The resulting fibers**,** films and sheets in mechanical recycling are no longer recyclable and often burned and then contribute to increased $CO_2$ emissions. Chemical recycling aims to reduce PET to its basic monomers, which can then be re-polymerized. This method is economically unfavorable because recovered monomers are more expensive than crude oil (Awaja and Pavel, 2005; Hiraga et al., 2019).

In addition, chemical methods toxic as well as high temperature and pressure, ıt contains reagents (Koshti et al., 2018; Wei and Zimmermann, 2017a). Thus, this kind of chemical recycling imposes toxic and environmentally hazardous issues (Chaudhary and Vijayakumar, 2020; Geyer et al., 2014).

While chemical and mechanical processing of PET is already in widespread use, biological recycling is emerging as a more sustainable solution as it can be performed under mild pH and relatively low temperature conditions without the use of hazardous chemicals (Awaja and Pavel, 2005; Webb et al., 2012; Wei and Zimmermann, 2017b).

Recently, different actinomycetes, algae, bacteria and fungi that have the potential to biodegrade various plastic polymers have been investigated. Studies have so far confirmed

27 enzymes that degrade synthetic polymers or oligomers and most of the enzymes were lipase, cutinase, and esterase (Bollinger et al., 2020).

It has been reported that a polyester hydrolase found in *Thermobifida species* degrades PET. In the study using the PET sample, it was observed that there was a weight loss of approximately 10% (Müller et al., 2005).

It has been reported that the cutinase enzyme found in Thermomixers insolents degrades PET in 96 hours (Ronkvist et al., 2009). Another study showed that LC-cutinase enzyme degraded PET at 70 degrees for 24 hours (Sulaiman et al., 2014).

Bacteria and fungi are an important sources of cutinases. It has been show that cutinases from *Aspergilllus oryzae*, *Aspergilllus nidulans* (Bermudez et al., 2017), *Penicillium citrinum* (Liebminger et al., 2007), *Thermobifida fusca* (Brueckner et al., 2008), *Fusarium solani* (Alisch-Mark et al., 2006), *F. solani pisi* (Vertommen et al., 2005) and *Thermobifida cellulolysitica* (Herrero Acero et al., 2011) have hydrolyzing activity toward low-crystallinity PET. Esterase from *Thermobifida* Thh_Est demonstrated effective surface hydrolysis for PET polyester, and its effect was similar to cutinases from the same genus (Ribitsch et al., 2012). The results show that the PET film can cause microbial degradation with the help of an esterase found in *Nocardia* sp. and *Bacillius* sp. (Sharon and Sharon 2012).

Researchers has shown that two polyester hydrolase found can degrade PET in *Thermomonospora curvata*. The authors demonstrated that these enzymes can break down PET nanoparticles at temperatures up to 50°C. However, at higher temperatures, the ability of PET films to degrade efficiently may be limited (Wei et al., 2014).

Researches cloned a cutinase-like polyesterase from *Saccharomonospora viridis*, which can degrade PET film with up to 27% weight loss at 63 °C in 3 days. It has been reported that $Ca^{2+}$ in the reaction mixture increases the enzyme activity (Kawai et al., 2019). Scientist have showed that combined biology with a chemical process by using whole cell biocatalysts consisting of *Camamonas testosterone* F6 under alkaline conditions for the biodegradation of PET (Gong et al., 2018). Similarly, a combined physical and a biological PET degradation process was reported. They have shown that PET and are highly degraded by *Streptomyces* species (Farzi et al., 2019).

Research shows that the ability of bacteria to degrade plastic depends on their natural capacity to degrade long-chain fatty acids, with *Pseudomonas* being the most prominent and studied bacterial strain in terms of plastic polymer degradation (Wilkes and Aristilde 2017).

In another study, researchers discovered a bacterium called *I. sakaiensis* belonging to the genus *Ideonella*. This bacterium provided its degradation by using PET as a carbon source (Yoshida et al., 2016). In a study showed that PET film can be degraded at 30ºC for 48 hours. These results are similar to our results (Ma et al., 2018).

However, bacteria and fungi used in PET degradation have some disadvantages. It is necessary to overcome approaches such as requiring a culture medium for growth and adding carbon sources. In addition, microorganisms such as *I. sakaiensis* are not well-adapted to the marine environment where plastic waste often accumulates. Thus, these organisms are not suitable for bioremediation of PET polluted saltwater (Moog et al., 2019).

In this study, we aimed to examine the effects of *C. vulgaris* on PET biodegradation. There are no studies in the literature showing the effects of *C. vulgaris* on PET biodegradation. There is only one study reporting the heterologous expression of PETase in and physicochemical degradation of PET. Therefore, our study highlights the importance of *C. vulgaris* in PET biodegradation.

It has been shown that algae species such as *Anabaena, Chlorella, Nostoc, Oscillatoria, Spirogyra,* can degrade different plastic surfaces even in terrestrial habitats (Sarmah and Rout, 2018). Studies have demonstrated that the ability of *Scenedesmus dimorphus, Anabaena spiroides,* and *Navicula pupula* to degrade both high-density and low-density PE (Kumar, 2017). Studies have shown that *A. spiroid* degrades 8.18% low density polyethylene after 30 days and *Spirulina sp.* can biodegrades PET and PP (Khoironi et al., 2019). As opposed to bacteria, algae use atmospheric carbon dioxide as their main carbon source and sunlight as their energy source, so it is not surprising to think that they are more effective at degradation (Dineshbabu et al., 2020).

A recent study reported that *Phaeodactylum tricornutum* is a convenient and inexpensive approach to biodegradate PET(Moog et al., 2019). It has been stated that the PETase contained in *C. reinhardtii* CC-124 cells can degrade PET film after 4 weeks of incubation (Kim et al., 2020). These findings showing the effects of microalgae on PET biodegradation also support our results (Figure 22). In a study investigating the PET degradation of *C. vulgaris* by physicochemical methods, *C. vulgaris* has been found to have a pronounced effect on cracking and modification of the plastic polymer. In addition, since the nature of the functional group is different in the presence and absence of pre-treatment, the idea that algae species cannot biologically degrade the plastic structure in the absence of any pre-treatment has emerged (Falah et al., 2020).

These results suggest that algae biodegradation is thought to involve biological degradation, degradation, assimilation and mineralization stages and microalgae are a potential biological treatment strategy for plastic pollution, especially in freshwater and terrestrial environments.

## 5. CONCLUSIONS

The promising and applicable outcomes of the current study are also summarized;

1. A PETase enzyme with improved activity through cognitive rational design strategy was successfully transformed into a totally natural *C. vulgaris* (211/11J) strain for the first time.
2. An electroporation-based transformation protocol for *C. vulgaris* CV211/11J strain was successfully developed and optimized.
3. In this study, PETase3M mutant enzyme was produced extracellularly in *C. vulgaris* and its functionality was confirmed through several degradation experiments performed on intact PET films.
4. The degradative and hydrolytic potentials of recombinant PETase3M enzyme on PET plastics were evaluated through light microscopy.
5. A new C. *vulgaris* strain with versatile tolerance to environmentally harsh conditions was engineered and successfully developed towards large-scale bioremediation of various rural and urban plastics wastes.

## 6. RECOMMENDATION

1. The PETase3M hydrolytic activity on PET films can be further improved by introduction of additional saturation mutations through rational design approach. This is indeed a vital requirement for both PETase and MHETase enzymes revealing reasonably low level thermostability.
2. PETase3M can also be expressed in *C. vulgaris* by utilizing a stronger promoter such as 35S or newly identified inducible promoters including Hsp90A. Using such strong promoters can even improvethe production level of PETase3M enzyme by microalgae hosts. Such advancements may further improve the cost effective bioremediation of PET wastes and related application processes in a timely manner.
3. Concomitant extracellular expression of PETase3M together with MHETase enzyme in the same microalgae host could be the ultimate goal towards complete and efficient hydrolysis of PET films.
4. Specific activity and thermostability analyses could be performed to elucidate the quantitative potentials of recombinant PETase3M enzyme. Furthermore, optimum working temperature of PETase3M enzyme may also be tested towards optimal PET degredation processes.
5. The catalytic activity of recombinant PETase3M could also be tested on various PET substrates to identify its versatility towards the bioremediation processes of different plastic wastes.

## 7. REFERENCES


Ahmad, M. T., Shariff, M., Md. Yusoff, F., Goh, Y. M., and Banerjee, S., 2020. Applications of microalga *Chlorella vulgaris* in aquaculture, Reviews in Aquaculture, 12,1, 328–346.

Alisch-Mark, M., Herrmann, A., and Zimmermann, W., 2006. Increase of the Hydrophilicity of Polyethylene Terephthalate Fibres by Hydrolases from Thermomonospora fusca and Fusarium solani f. Sp. Pisi, Biotechnology Letters, *28*,10, 681–685.

Amobonye, A., Bhagwat, P., Singh, S., and Pillai, S., 2020. Plastic biodegradation: Frontline microbes and their enzymes, Science of The Total Environment, 143536.

Archna, A., Moses, V., Sagar, S., Shivraj, V., and Chetan, S., 2015. A Review on Processing of Waste PET (Polyethylene Terephthalate), Plastics, 1, 1–13.

Austin, H. P., Allen, M. D., Donohoe, B. S., Rorrer, N. A., Kearns, F. L., Silveira, R. L., Pollard, B. C., Dominick, G., Duman, R., Omari, K. E., Mykhaylyk, V., Wagner, A., Michener, W. E., Amore, A., Skaf, M. S., Crowley, M. F., Thorne, A. W., et al., 2018. Characterization and engineering of a plastic-degrading aromatic polyesterase, Proceedings of the National Academy of Sciences, 115,19, E4350–E4357.

Awaja, F., and Pavel, D., 2005. Recycling of PET, European Polymer Journal, 41,7, 1453–1477.

Bal, K., Ünlü, K. C., Acar, I., and Güçlü, G., 2017. Epoxy-based paints from glycolysis products of postconsumer PET bottles: Synthesis, wet paint properties and film properties, Journal of Coatings Technology and Research, 14,3, 747–753.

Barot, A., Panchal, T., Patel, A., and Patel, C., 2019. Polyester the Workhorse of Polymers: A Review, Synthesis to Recycling, 11, 19.

Becker, E. W., 2007. Micro-algae as a source of protein, Biotechnology Advances, 25,2, 207–210.

Berliner, M. D., 1986. Proteins in Chlorella vulgaris, Microbis, 46,188–189, 199–203.

Bermudez, E., Peña, C., Castro-Rodríguez, J., Gonzales Canto, A., Navarro-Ocaña, A., and Farrés, A., 2017. ANCUT2, a Thermo-alkaline Cutinase from Aspergillus nidulans and Its Potential Applications, Applied Biochemistry and Biotechnology, 182.

Bollinger, A., Thies, S., Knieps-Grünhagen, E., Gertzen, C., Kobus, S., Höppner, A., Ferrer, M., Gohlke, H., Smits, S. H. J., and Jaeger, K. E., 2020. A Novel Polyester

Hydrolase From the Marine Bacterium Pseudomonas aestusnigri – Structural and Functional Insights, Frontiers in Microbiology, 11, 114.

Borowitzka, M. A., 2018. Biology of Microalgae, Ed., Levine I. A., and Fleurence, J., Microalgae in Health and Disease Prevention, Academic Press, 23-72.

Brennan, L., and Owende, P., 2010. Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products, Renewable and Sustainable Energy Reviews, 14,2, 557–577.

Brueckner, T., Eberl, A., Heumann, S., Rabe, M., and Guebitz, G. M., 2008. Enzymatic and chemical hydrolysis of poly(ethylene terephthalate) fabrics. Journal of Polymer Science Part A: Polymer Chemistry, 46,19, 6435–6443.

Champenois, J., Marfaing, H., and Pierre, R., 2014. Review of the taxonomic revision of Chlorella and consequences for its food uses in Europe, Journal of Applied Phycology, 27.

Chaudhary, A. K., and Vijayakumar, R. P., 2020. Studies on biological degradation of polystyrene by pure fungal cultures, Environment, Development and Sustainability, 22,5, 4495–4508.

Chen, G. Q., and Chen, F., 2006. Growing Phototrophic Cells without Light, Biotechnology Letters, 28,9, 607–616.

Chen, S., Hassanzadeh-Aghdam, M. K., and Ansari, R., 2018. An analytical model for elastic modulus calculation of SiC whisker-reinforced hybrid metal matrix nanocomposite containing SiC nanoparticles, Journal of Alloys and Compounds, 767, 632–641.

Choix, F. J., de-Bashan, L. E., and Bashan, Y., 2012. Enhanced accumulation of starch and total carbohydrates in alginate-immobilized Chlorella spp. induced by Azospirillum brasilense: II. Heterotrophic conditions, Enzyme and Microbial Technology, *51*,5, 300–309.

Chu, W. L., 2012. Biotechnological applications of microalgae, International e-Journal of Science Medicine and Education, 6, 2,14.

Cooper, T. A., 2013. Developments in plastic materials and recycling systems for packaging food, beverages and other fast-moving consumer goods, Beverages and Other Fast-Moving Consumer Goods (FMCG), 58-107

Daliry, S., Hallajsani, A., Mohammadi Roshandeh, J., Nouri, H., and Golzary, A., 2017. Investigation of optimal condition for Chlorella vulgaris microalgae growth, Global Journal of Environmental Science and Management, 3,2, 217-230.

Dineshbabu, G., Uma, V. S., Mathimani, T., Prabaharan, D., and Uma, L., 2020. Elevated CO2 impact on growth and lipid of marine cyanobacterium Phormidium

valderianum BDU 20041– towards microalgal carbon sequestration, Biocatalysis and Agricultural Biotechnology, 25, 101606.

Dodbiba, G., and Fujita, T., 2004. Progress in Separating Plastic Materials for Recycling, Physical Separation in Science and Engineering, 13, 165-182.

Faheed, F., and Fattah, Z., 2008. Effect of *Chlorella vulgaris* as Bio-fertilizer on Growth Parameters and Metabolic Aspects of Lettuce Plant, Journal of Agriculture and Social Research, 4, 165–169.

Falah, W., Chen, F. J., Zeb, B., Hayat, T., Mahmood, Q., Ebadi, A., Toughani, M., and Li, E. Z., 2020. Polyethylene Terephthalate Degradation by Microalga Chlorella vulgaris Along with Pretreatment, Materiale Plastice, 57, 260–270.

Farzi, A., Dehnad, A., and Fotouhi, A. F., 2019. Biodegradation of polyethylene terephthalate waste using Streptomyces species and kinetic modeling of the process, Biocatalysis and Agricultural Biotechnology, 17, 25–31.

Fischer-Colbrie, G., Heumann, S., Liebminger, S., Almansa, E., Cavaco-Paulo, and A., Guebitz, G., 2004. New enzymes with potential for PET surface modification, Biocatalysis and Biotransformation, *22*, 341-346.

Frumento, D., Aliakbarian, B., Casazza, A. A., Converti, A., Al Arni, S., da Silva, M. F., 2016. *Chlorella vulgaris* as a lipid source: Cultivation on air and seawater-simulating medium in a helicoidal photobioreactor, Biotechnology Progress, 32,2, 279–284.

Garcia, L.C., 2012. The promises of Chlorella vulgaris as the best alternative for biodiesel: A review, Journal of Nature Studies, 11, 103–123.

Geyer, B., Röhner, S., Lorenz, G., and Kandelbauer, A., 2014. Synthesis of ethylene terephthalate and ethylene naphthalate (PET-PEN) block- *co* -polyesters with defined surface qualities by tailoring segment composition, Journal of Polymer Science, 131,17.

Goje, A. S., and Mishra, S., 2003. Chemical Kinetics, Simulation, and Thermodynamics of Glycolytic Depolymerization of Poly(ethylene terephthalate) Waste with Catalyst Optimization for Recycling of Value Added Monomeric Products, Macromolecular Materials and Engineering, 288,4, 326–336.

Gong, J., Kong, T., Li, Y., Li, Q., Li, Z., and Zhang, J., 2018. Biodegradation of Microplastic Derived from Poly(ethylene terephthalate) with Bacterial Whole-Cell Biocatalysts, Polymers, 10,12, 1326.

Gouveia, L., Batista, A. P., Miranda, A., Empis, J., and Raymundo, A., 2007. Chlorella vulgaris biomass used as colouring source in traditional butter cookies, Innovative Food Science & Emerging Technologies, 8,3, 433–436.

Gouveia, L., Raymundo, A., Batista, A. P., Sousa, I., and Empis, J., 2005. Chlorella vulgaris and Haematococcus pluvialis biomass as colouring and antioxidant in food emulsions, European Food Research and Technology, 222,3, 362.

Güçlü, G., and Acar, I., 2015. Tüketici Kaynaklı Atık Pet Şişelerinin Alkid Reçinesi Üretiminde Kullanımı, Boya Türk, 14-22.

Guedes, A. C., Amaro, H. M., and Malcata, F. X., 2011. Microalgae as Sources of Carotenoids, Marine Drugs, 9,4, 625.

Herrero Acero, E., Ribitsch, D., Steinkellner, G., Gruber, K., Greimel, K., Eiteljoerg, I., Trotscha, E., Wei, R., Zimmermann, W., Zinn, M., Cavaco-Paulo, A., et al., 2011. Enzymatic Surface Hydrolysis of PET: Effect of Structural Diversity on Kinetic Properties of Cutinases from Thermobifida, Macromolecules, 44,12, 4632–4640.

Hiraga, K., Taniguchi, I., Yoshida, S., Kimura, Y., and Oda, K., 2019. Biodegradation of waste PET: A sustainable solution for dealing with plastic pollution. EMBO Reports, 20,11, e49365.

Jankauskaite, V., 2016. Recycled Polyethylene Terephthalate Waste for Different Application Solutions, Environmental Research, Engineering and Management, 72,1, 1-14.

Joo, S., Cho, I. J., Seo, H., et al., 2018. Structural insight into molecular mechanism of poly(ethylene terephthalate) degradation, Nature Commun, 9,1, 382

Karayannidis, G. P., and Achilias, D. S., 2007. Chemical Recycling of Poly(ethylene terephthalate), Macromolecular Materials and Engineering, 292,2, 128–146.

Kawai, F., Kawabata, T., and Oda, M., 2019. Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields, Applied Microbiology and Biotechnology, 103,11, 4253–4268.

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., and Sternberg, M. J. E., 2015. The Phyre2 web portal for protein modeling, prediction and analysis, Nature Protocols, 10,6, 845–858.

Khoironi, A., Anggoro, S., and Sudarno, S., 2019. Evaluation of the Interaction Among Microalgae Spirulina sp, Plastics Polyethylene Terephthalate and Polypropylene in Freshwater Environment, Journal of Ecological Engineering, 20,6, 161–173.

Kim, J. W., Park, S. B., Tran, Q. G., Cho, D. H., Choi, D. Y., Lee, Y. J., and Kim, H. S., 2020. Functional expression of polyethylene terephthalate-degrading enzyme (PETase) in green microalgae, Microbial Cell Factories, 19,1, 97.

Knott, B. C., Erickson, E., Allen, M. D., Gado, J. E., Graham, R., Kearns, F. L., Pardo, I., Topuzlu, E., Anderson, J. J., Austin, H. P., Dominick, G., Johnson, C. W., Rorrer, N. A., et al., 2020. Characterization and engineering of a two-enzyme system for

plastics depolymerization, Proceedings of the National Academy of Sciences, 117,41, 25476–25485.

Kong, W., Song, H., Cao, Y., Yang, H., Hua, S., and Xia, C., 2011. The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of Chlorella vulgaris under mixotrophic cultivation, African Journal of Biotechnology, 10,55, 11620–11630.

Koshti, R., Mehta, L., and Samarth, N., 2018. Biological Recycling of Polyethylene Terephthalate: A Mini-Review, Journal of Polymers and the Environment, 26,8, 3520–3529.

Kottuparambil, S., Thankamony, R. L., and Agusti, S., 2019. Euglena as a potential natural source of value-added metabolites. A review, Algal Research, 37, 154–159.

Krienitz, L., Huss, V. A. R., Bock, C., 2015. Chlorella: 125 years of the green survivalist, Trends in Plant Science, 20,2, 67–69.

Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F. X., and van Langenhove, H., 2010. Enhanced $CO_2$ fixation and biofuel production via microalgae: Recent developments and future directions, Trends in Biotechnology, 28,7, 371–380.

Kumar P, S., 2017. Cultivation and Chemical Composition of Microalgae Chlorella vulgaris and its Antibacterial Activity against Human Pathogens, Journal of Aquaculture & Marine Biology, 5,3,00119.

Lebreton, L., and Andrady, A., 2019. Future scenarios of global plastic waste generation and disposal, Palgrave Communications, 5,1, 6.

Li, Y., Horsman, M., Wu, N., Lan, C. Q., and Dubois-Calero, N., 2008. Biofuels from Microalgae, Biotechnology Progress, 24,4, 815–820.

Liebminger, S., Eberl, A., Sousa, F., Heumann, S., Fischer-Colbrie, G., Cavaco-Paulo, A., and Guebitz, G. M., 2007. Hydrolysis of PET and bis-(benzoyloxyethyl) terephthalate with a new polyesterase from *Penicillium citrinum*, Biocatalysis and Biotransformation, 25,2–4, 171–177.

Liu, C., Shi, C., Zhu, S., Wei, R., and Yin, C. C., 2019. Structural and functional characterization of polyethylene terephthalate hydrolase from Ideonella sakaiensis, Biochemical and Biophysical Research Communications, 508,1, 289–294.

Lopez, G., Artetxe, M., Amutio, M., Bilbao, J., and Olazar, M., 2017. Thermochemical routes for the valorization of waste polyolefinic plastics to produce fuels and chemicals. A review, Renewable and Sustainable Energy Reviews, 73, 346–368.

Lu, D. R., Xiao, C. M., and Xu, S. J., 2009. Starch-based completely biodegradable polymer materials, Express Polymer Letters, 3,6, 366–375.

Raziyafathima, M., Praseetha, P. K., and Isaac, R., 2016. Microbial Degradation of Plastic Waste: A Review, Journal of Pharmaceutical, Chemical and Biological Sciences, 4,231–242

Ma, Y., Yao, M., Li, B., Ding, M., He, B., Chen, S., Zhou, X., and Yuan, Y. 2018. Enhanced Poly(ethylene terephthalate) Hydrolase Activity by Protein Engineering, Engineering, 4,6, 888–893.

Maliwat, G. C., Velasquez, S., Robil, J. L., Chan, M., Traifalgar, R. F., Tayamen, M., and Ragaza, J. A., 2017. Growth and immune response of giant freshwater prawn Macrobrachium rosenbergii (De Man) postlarvae fed diets containing Chlorella vulgaris (Beijerinck), Aquaculture Research, 48,4, 1666–1676.

Maruyama, I., Nakao, T., Shigeno, I., Ando, Y., and Hirayama, K., 1997. Application of unicellular algae Chlorella vulgaris for the mass-culture of marine rotifer Brachionus, Hydrobiologia, 358,1, 133–138.

Mata, T. M., Martins, A. A., and Caetano, Nidia. S., 2010. Microalgae for biodiesel production and other applications: A review, Renewable and Sustainable Energy Reviews, 14,1, 217–232.

Mohd Yusof, Y. A., 2011. Fatty acids composition of microalgae Chlorella vulgaris can be modulated by varying carbon dioxide concentration in outdoor culture. African Journal of Biotechnology, 10, 13536–13542.

Moog, D., Schmitt, J., Senger, J., Zarzycki, J., Rexer, K.-H., Linne, U., Erb, T., and Maier, U. G., 2019. Using a marine microalga as a chassis for polyethylene terephthalate (PET) degradation, Microbial Cell Factories, 18,1, 171.

Morris Quevedo, H. J., Quintana Cabrales, M. M., Almarales Arceo, A., and Hernández Nazario, L., 1999. Composición bioquímica y evaluación de la calidad proteica de la biomasa autotrófica de chlorella vulgaris, Revista Cubana de Alimentacion Nutrition, 13, 123–128.

Mostafa, S., 2012. Microalgal Biotechnology: Prospects and Applications, Plant Science, 275–314

Müller, R. J., Schrader, H., Profe, J., Dresler, K., and Deckwer, W. D., 2005. Enzymatic Degradation of Poly(ethylene terephthalate): Rapid Hydrolyse using a Hydrolase from T. fusca, Macromolecular Rapid Communications, 26,17, 1400–1405.

Nkwachukwu, O. I., Chima, C. H., Ikenna, A. O., and Albert, L., 2013. Focus on potential environmental issues on plastic world towards a sustainable plastic recycling in developing countries, International Journal of Industrial Chemistry, 4,1, 34.

Poulikakos, L. D., Papadaskalopoulou, C., Hofko, B., Gschösser, F., Cannone Falchetto, A., Bueno, M., Arraigada, M., Sousa, J., Ruiz, R., Petit, C., Loizidou, M., and Partl, M.

N., 2017. Harvesting the unexplored potential of European waste materials for road construction, Resources, Conservation and Recycling, 116, 32–44.

Pratt, R., Daniels, T. C., Eiler, J. J., Gunnison, J. B., Kumler, W. D., Oneto, J. F., Strait, L. A., Spoehr, H. A., Hardin, G. J., Milner, H. W., Smith, J. H., and Strain, H. H., 1944. Chlorellin, An Antibacterial Substance from Chlorella, Science, 99,2574, 351–352.

Radmer, R. J., and Parker, B. C., 1994. Commercial applications of algae: Opportunities and constraints, Journal of Applied Phycology, 6, 2, 93–98.

Raymundo, A., Gouveia, L., Batista, A. P., Empis, J., and Sousa, I., 2005. Fat mimetic capacity of Chlorella vulgaris biomass in oil-in-water food emulsions stabilized by pea protein, Food Research International, 38,8, 961–965.

Ribitsch, D., Herrero Acero, E., Greimel, K., Dellacher, A., Zitzenbacher, S., Marold, A., Rodriguez, R. D., Steinkellner, G., Gruber, K., Schwab, H., and Guebitz, G. M., 2012. A New Esterase from Thermobifida halotolerans Hydrolyses Polyethylene Terephthalate (PET) and Polylactic Acid (PLA), Polymers, 4,1, 617–629.

Ronkvist, A., Xie, W., Lu, W., and Gross, R., 2009. Cutinase-Catalyzed Hydrolysis of Poly(ethylene terephthalate), Macromolecules, 42, 5128–5138.

Safi, C., Charton, M., Pignolet, O., Silvestre, F., Vaca-Garcia, C., and Pontalier, P. Y., 2013. Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors, Journal of Applied Phycology, 25, 523–529

Safi, C., Zebib, B., Merah, O., Pontalier, P. Y., and Vaca-Garcia, C., 2014. Morphology, composition, production, processing and applications of Chlorella vulgaris: A review, Renewable and Sustainable Energy Reviews, 35, 265–278.

Sarmah, P., and Rout, J., 2018. Algal colonization on polythene carry bags in a domestic solid waste dumping site of Silchar town in Assam, Pyhkos, *48*,1, 67–77.

Sathasivam, R., Radhakrishnan, R., Hashem, A., and Abd_Allah, E. F., 2019. Microalgae metabolites: A rich source for food and medicine, Saudi Journal of Biological Sciences, 26,4, 709–722

Shaaban, M., 2001. Green Microalgae Water Extract as Foliar Feeding to Wheat Plants, Pakistan Journal of Biological Sciences, 4, 628-632.

Shaaban, M. M., 2001. Nutritional Status and Growth of Maize Plants as Affected by Green Microalgae as Soil Additives, Journal of Biological Sciences, 1,6, 475–479.

Sharma, R., 2012. Effects of Culture Conditions on Growth and Biochemical Profile of Chlorella Vulgaris, Journal of Plant Pathology & Microbiology, *3*,5, 1-6.

Sharon, C., and Sharon, M., 2012. Studies on Biodegradation of Polyethylene terephthalate: A synthetic polymer, Journal of Microbiology and Biotechnology Research, 2, 248–257.

Sigamani, S., and Natarajan, H., 2016. Bioactive compounds from Microalgae and its different applications-A review, Advances in Applied Science Research, 7, 153-158.

Sinha, V., Patel, M., and Patel, J., 2010. Pet Waste Management by Chemical Recycling: A Review, Journal of Polymers and the Environment, 18, 8–25.

Smith, P., 2013. Trends in Biobased Chemistry, Plastics Engineering, 69, 44–48.

Song, Y., Cao, C., Qiu, R., Hu, J., Liu, M., Lu, S., Shi, H., Raley-Susman, K. M., and He, D., 2019. Uptake and adverse effects of polyethylene terephthalate microplastics fibers on terrestrial snails (Achatina fulica) after soil exposure, Environmental Pollution, 250, 447–455.

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A., 2006. Commercial applications of microalgae, Journal of Bioscience and Bioengineering, 101,2, 87–96.

Sulaiman, S., You, D.-J., Kanaya, E., Koga, Y., and Kanaya, S., 2014. Crystal Structure and Thermodynamic and Kinetic Stability of Metagenome-Derived LC-Cutinase, Biochemistry, 53,11, 1858–1869.

Tanasupawat, S., Takehana, T., Yoshida, S., Hiraga, K., and Oda, K., 2016. Ideonella sakaiensis sp. Nov., isolated from a microbial consortium that degrades PET, International Journal of Systematic and Evolutionary Microbiology, 66, 2813–2818.

Taniguchi, I., Yoshida, S., Hiraga, K., Miyamoto, K., Kimura, Y., and Oda, K., 2019. Biodegradation of PET: Current Status and Application Aspects, ACS Catalysis, 9,5, 4089–4105.

Tiong, I., Yeong, yik sung, Jusoh, M., Wahid, E., and Nagappan, T., 2020. Chlorella vulgaris: A perspective on its potential for combining high biomass with high high value bioproducts, Applied Phycology, 1, 1–10

Tomaselli, L., 2007. The Microalgal Cell, Ed., Richmond, A., In Handbook of Microalgal Culture, Biotechnology and Applied Phycology, Oxford, UK: Blackwell Science Ltd. 1-19.

Vertommen, M. A., M. E., Nierstrasz, V. A., et al., 2005. Enzymatic surface modification of poly(ethylene terephthalate), Journal of Biotechnology, 120,4, 376–386.

Vitova, M., Bisova, K., Kawano, S., and Zachleder, V., 2015. Accumulation of energy reserves in algae: From cell cycles to biotechnological applications, Biotechnology Advances, 33,6, 2, 1204–1218.

Webb, H., Arnott, J., Crawford, R., and Ivanova, E., 2012. Plastic Degradation and Its Environmental Implications with Special Reference to Poly(ethylene terephthalate), Polymers, 5,1, 1–18.

Wei, R., Oeser, T., Then, J., Kühn, N., Barth, M., Schmidt, J., and Zimmermann, W., 2014. Functional characterization and structural modeling of synthetic polyester-degrading hydrolases from Thermomonospora curvata, AMB Express, 4, 44.

Wei, R., and Zimmermann, W., 2017a. Biocatalysis as a green route for recycling the recalcitrant plastic polyethylene terephthalate, Microbial Biotechnology, 10,6, 1302–1307.

Wei, R., and Zimmermann, W., 2017b. Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: How far are we?, Microbial Biotechnology, 10,6, 1308–1322.

Wijffels, R. H., Barbosa, M. J., and Eppink, M. H. M., 2010. Microalgae for the production of bulk chemicals and biofuels, Biofuels, Bioproducts and Biorefining, 4,3, 287–295.

Wilkes, R. A., and Aristilde, L., 2017. Degradation and metabolism of synthetic plastics and associated products by *Pseudomonas* sp. Capabilities and challenges, Journal of Applied Microbiology, 123,3, 582–593.

Wyeth, N. C., 1988. Inventıng the Pet Bottle, Research Technology Management, 31,4, 53–55.

Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., and Takriff, M. S., 2014. An overview: Biomolecules from microalgae for animal feed and aquaculture. Journal of Biological Research, 21,1, 6.

Yamamoto, M., Fujishita, M., Hirata, A., and Kawano, S., 2004. Regeneration and maturation of daughter cell walls in the autospore-forming green alga Chlorella vulgaris (Chlorophyta, Trebouxiophyceae), Journal of Plant Research, 117,4, 257–264.

Yeh, K. L., Chang, J. S., and Chen, W., 2010. Effect of light supply and carbon source on cell growth and cellular composition of a newly isolated microalga Chlorella vulgaris ESP-31, Engineering in Life Sciences, 10,3, 201–208.

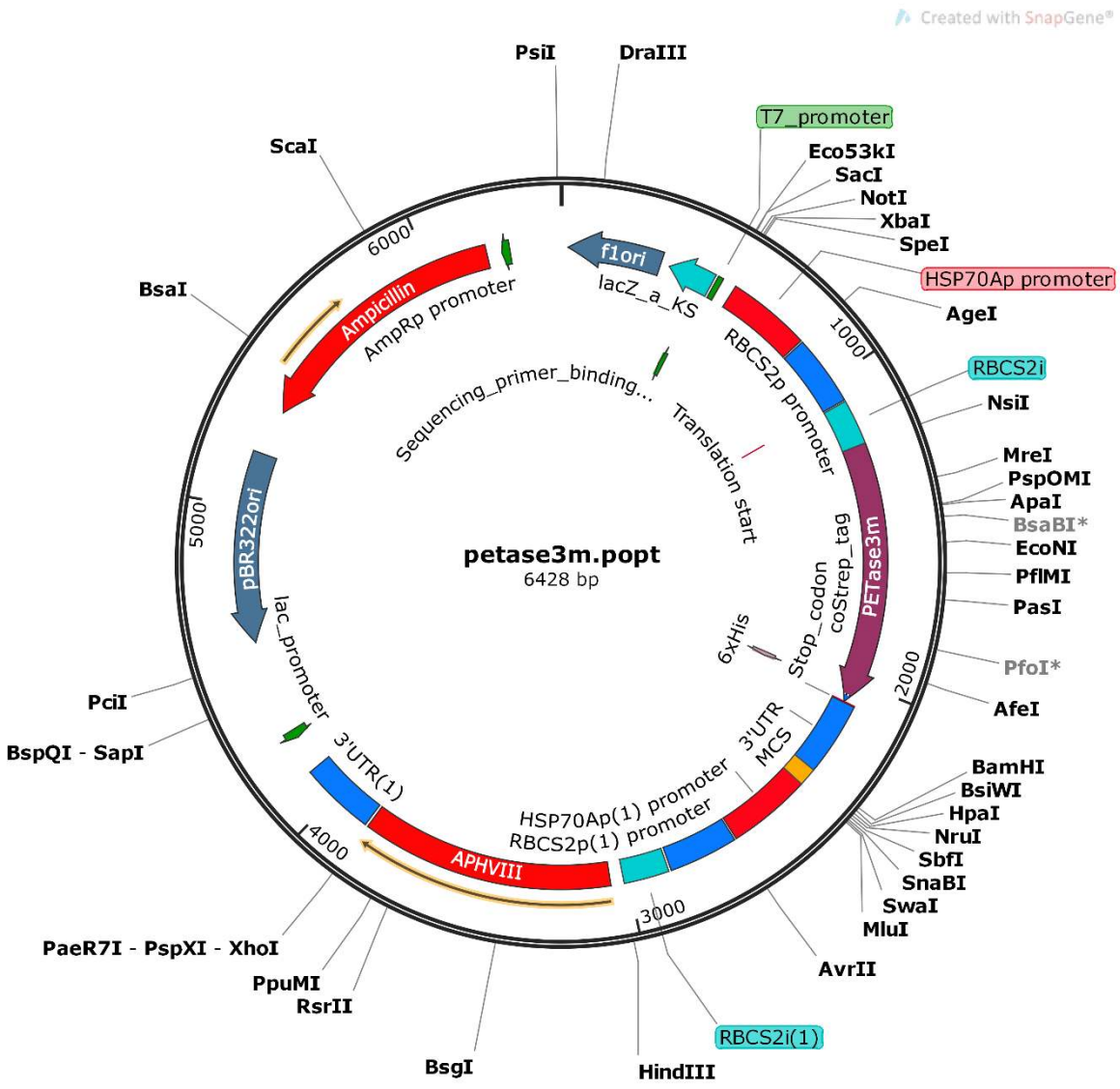
Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., and Oda, K., 2016. A bacterium that degrades and assimilates poly(ethylene terephthalate), Science, 351,6278, 1196–1199.

Zein, R., Konswa, A., Ibrahim, M., El-mittiny, E., Abdullah, H., Saeed, M., Saber, M., Mohsen, M., Fathy, M., Ismail, R., Metwally, R., and Esam, R., 2010. PET Production, Master Thesis, Cairo University, Egypt, Giza.

Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., and Dou, C., 2011. Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: A comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves, Applied Biochemistry and Biotechnology, 164,7, 1215–1224.

# 8. APPENDIX

## 8.1. PETase3M.Popt.Cca



## 8.2. Sequence

```
LOCUS       EMBOSS_001              6495 bp    DNA     linear   UNC 18-FEB-2021
ORIGIN
       1  ATAGACCGAG ATAGGGTTGA GTGTTGTTCC AGTTTGGAAC AAGAGTCCAC TATTAAAGAA
      61  CGTGGACTCC AACGTCAAAG GGCGAAAAAC CGTCTATCAG GGCGATGGCC CACTACGTGA
     121  ACCATCACCC TAATCAAGTT TTTTGGGGTC GAGGTGCCGT AAAGCACTAA ATCGGAACCC
     181  TAAAGGGAGC CCCCGATTTA GAGCTTGACG GGGAAAGCCG GCGAACGTGG CGAGAAAGGA
     241  AGGGAAGAAA GCGAAAGGAG CGGGCGCTAG GGCGCTGGCA AGTGTAGCGG TCACGCTGCG
     301  CGTAACCACC ACACCCGCCG CGCTTAATGC GCCGCTACAG GGCGCGTCCC ATTCGCCATT
     361  CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT TACGCCAGCT
     421  GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA ACGCCAGGGT TTTCCCAGTC
     481  ACGACGTTGT AAAACGACGG CCAGTGAGCG CGCGTAATAC GACTCACTAT AGGGCGAATT
     541  GGAGCTCCAC CGCGGTGGCG GCCGCtctag aactagtGCT GAGGCTTGAC ATGATTGGTG
     601  CGTATGTTTG TATGAAGCTA CAGGACTGAT TTGGCGGGCT ATGAGGGCGG GGGAAGCTCT
     661  GGAAGGGCCG CGATGGGGCG CGCGGCGTCC AGAAGGCGCC ATACGGCCCG CTGGCGGCAC
     721  CCATCCGGTA TAAAAGCCCG CGACCCCGAA CGGTGACCTC CACTTTCAGC GACAAACGAG
     781  CACTTATACA TACGCGACTA TTCTGCCGCT ATACATAACC ACTCAGCTAG CTTAAGATCC
     841  CATCaccggt GCATGCCGGG CGCGCCAGAA GGAGCGCAGC CAAACCAGGA TGATGTTTGA
     901  TGGGGTATTT GAGCACTTGC AACCCTTATC CGGAAGCCCC CTGGCCCACA AAGGCTAGGC
     961  GCCAATGCAA GCAGTTCGCA TGCAGCCCCT GGAGCGGTGC CCTCCTGATA AACCGGCCAG
    1021  GGGGCCTATG TTCTTTACTT TTTTACAAGA GAAGTCACTC AACATCTTAA AATGGCCAGG
    1081  TGAGTCGACG AGCAAGCCCG GCGGATCAGG CAGCGTGCTT GCAGATTTGA CTTGCAACGC
    1141  CCGCATTGTG TCGACGAAGG CTTTTGGCTC CTCTGTCGCT GTCTCAAGCA GCATCTAACC
    1201  CTGCGTCGCC GTTTCCATTT GCAGGATGca tatgGCGCGT ACTGGCGCTC TACTCCTGGT
    1261  CGCGCTGGCG CTTGCGGGCT GCGCGCAGGC TTGCaGATCC CAGACCAACC CCTACGCCCG
    1321  CGGCCCGAAC CCCACCGCCG CCTCCCTGGA GGCCAGCGCC GGCCCCTTCA CCGTGCGCTC
    1381  GTTCACCGTG AGCCGCCCGA GCGGCTACGG CGCCGGCACC GTGTACTACC CCACCAACGC
    1441  CGGCGGCACC GTGGGCGCCA TCGCCATCGT GCCGGGCTAC ACCGCGCGCC AGTCGAGCAT
    1501  CAAGTGGTGG GGCCCCCGCC TGGCCTCGCA CGGCTTCGTG GTGATCACCA TCGACACCAA
    1561  CTCCACCCTG GACCAGCCGT CCAGCCGCTC GTCGCAGCAG ATGGCCGCCC TGCGCCAGGT
    1621  GGCCTCGCTG AACGGCACCA GCAGCAGCCC GATCTACGGC AAGGTGGACA CCGCCCGCAT
    1681  GGGCGTGATG GGCCACTCGA TGGGCGGCGG CGGCTCGCTG ATCTCGGCGG CCAACAACCC
    1741  CTCGCTGAAG GCCGCGGCGC CCCAGGCCCC CTGGGACAGC TCGACCAACT TCTCGTCGGT
    1801  GACCGTGCCC ACCCTGATCT TCGCCTGCGA GAACGACAGC TTCGCCCCCG TGAACTCGTC
    1861  CGCCCTGCCC ATCTACGACA GCATGTCGCG CAACGCCAAG CAGTTCCTGG AGATCAACGG
    1921  CGGCTCGCAC TTCTGCGCCA ACAGCGGCAA CAGCAACCAG GCCCTGATCG GCAAGAAGGG
    1981  CGTGGCCTGG ATGAAGCGCT TCATGGACAA CGACACCCGC TACTCCACCT TCGCCTGCGA
    2041  GAACCCCAAC AGCACCCGCG TGTCGGACTT CCGCACCGCG AACTGCAGCc accaccaCca
    2101  ccaccactaa GaattcTGGA GCCACCCGCA GTTCGAGAAG TAACCGCTCC GTGTAAATGG
    2161  AGGCGCTCGT TGATCTGAGC CTTGCCCCCT GACGAACGGC GGTGGATGGA AGATACTGCT
    2221  CTCAAGTGCT GAAGCGGTAG CTTAGCTCCC CGTTTCGTGC TGATCAGTCT TTTTCAACAC
    2281  GTAAAAAGCG GAGGAGTTTT GCAATTTTGT TGGTTGTAAC GATCCTCCGT TGATTTTGGC
    2341  CTCTTTCTCC ATGGGCGGGC TGGGCGTATT TGAAGCGgga tccttcgaac gtacggttaa
    2401  ctcgcgacct gcaggtacgt aatttaaata cgcgtGCTGA GGCTTGACAT GATTGGTGCG
    2461  TATGTTTGTA TGAAGCTACA GGACTGATTT GGCGGGCTAT GAGGGCGGGG GAAGCTCTGG
    2521  AAGGGCCGCG ATGGGGCGCG CGGCGTCCAG AAGGCGCCAT ACGGCCCGCT GGCGGCACCC
    2581  ATCCGGTATA AAAGCCCGCG ACCCCGAACG GTGACCTCCA CTTTCAGCGA CAAACGAGCA
    2641  CTTATAGGTA CCCGACTATT CTGCCGCTAT ACATAACCAC TCAGCTAGCT TAAGATCCCA
    2701  TCcctaggGC ATGCCGGGCG CGCCAGAAGG AGCGCAGCCA AACCAGGATG ATGTTTGATG
    2761  GGGTATTTGA GCACTTGCAA CCCTTATCCG GAAGCCCCCT GGCCCACAAA GGCTAGGCGC
    2821  CAATGCAAGC AGTTCGCATG CAGCCCCTGG AGCGGTGCCC TCCTGATAAA CCGGCCAGGG
    2881  GGCCTATGTT CTTTACTTTT TTACAAGAGA AGTCACTCAA CATCTTAAAA TGGCCAGGTG
    2941  AGTCGACGAG CAAGCCCGGC GGATCAGGCA GCGTGCTTGC AGATTTGACT TGCAACGCCC
    3001  GCATTGTGTC GACGAAGGCT TTTGGCTCCT CTGTCGCTGT CTCAAGCAGC ATCTAACCCT
    3061  GCGTCGCCGT TTCCATTTGC AGGaagcttA CTCCGCCCTC CCCGGTGCTG AAGAATTTCG
    3121  AAGCATGGAC GATGCGTTGC GTGCACTGCG GGGTCGGTAT CCCGGTTGTG AGTGGGTTGT
    3181  TGTGGAGGAT GGGGCCTCGG GGGCTGGTGT TTATCGGCTT CGGGGTGGTG GGCGGGAGTT
    3241  GTTTGTCAAG GTGGCAGCTC TGGGGGCCGG GGTGGGCTTG TTGGGTGAGG CTGAGCGGCT
    3301  GGTGTGGTTG GCGGAGGTGG GGATTCCCGT ACCTCGTGTT GTGGAGGGTG GTGGGGACGA
    3361  GAGGGTCGCC TGGTTGGTCA CCGAAGCGGT TCCGGGGCGT CCGGCCAGTG CGCGGTGGCC
    3421  GCGGGAGCAG CGGCTGGACG TGGCGGTGGC GCTCGCGGGG CTCGCTCGTT CGCTGCACGC
    3481  GCTGGACTGG GAGCGGTGTC CGTTCGATCG CAGTCTCGCG GTGACGGTGC CGCAGGCGGC
```

```
3541  CCGTGCTGTC GCTGAAGGGA GCGTCGACTT GGAGGATCTG GACGAGGAGC GGAAGGGGTG
3601  GTCGGGGGAG CGGCTTCTCG CCGAGCTGGA GCGGACTCGG CCTGCGGACG AGGATCTGGC
```

Ek 8.2'nin devamı

```
3661  GGTTTGCCAC GGTCACCTGT GCCCGGACAA CGTGCTGCTC GACCCTCGTA CCTGCGAGGT
3721  GACCGGGCTG ATCGACGTGG GGCGGGTCGG CCGTGCGGAC CGGCACTCCG ATCTCGCGCT
3781  GGTGCTGCGC GAGCTGGCCC ACGAGGAGGA CCCGTGGTTC GGGCCGGAGT GTTCCGCGGC
3841  GTTCCTGCGG GAGTACGGGC GCGGGTGGGA TGGGGCGGTA TCGGAGGAAA AGCTGGCGTT
3901  TTACCGGCTG TTGGACGAGT TCTTCTGAct cgagTGACCG CTCCGTGTAA ATGGAGGCGC
3961  TCGTTGATCT GAGCCTTGCC CCCTGACGAA CGGCGGTGGA TGGAAGATAC TGCTCTCAAG
4021  TGCTGAAGCG GTAGCTTAGC TCCCCGTTTC GTGCTGATCA GTCTTTTTCA ACACGTAAAA
4081  AGCGGAGGAG TTTTGCAATT TTGTTGGTTG TAACGATCCT CCGTTGATTT TGGCCTCTTT
4141  CTCCATGGGC GGGCTGGGCG TATTTGAAGC GGACCCggta ccCAGCTTTT GTTCCCTTTA
4201  GTGAGGGTTA ATTGCGCGCT TGGCGTAATC ATGGTCATAG CTGTTTCCTG TGTGAAATTG
4261  TTATCCGCTC ACAATTCCAC ACAACATACG AGCCGGAAGC ATAAAGTGTA AAGCCTGGGG
4321  TGCCTAATGA GTGAGCTAAC TCACATTAAT TGCGTTGCGC TCACTGCCCG CTTTCCAGTC
4381  GGGAAACCTG TCGTGCCAGC TGCATTAATG AATCGGCCAA CGCGCGGGGA GAGGCGGTTT
4441  GCGTATTGGG CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT
4501  GCGGCGAGCG GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA
4561  TAACGCAGGA AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC
4621  CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG
4681  CTCAAGTCAG AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG
4741  AAGCTCCCTC GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT
4801  TCTCCCTTCG GGAAGCGTGG CGCTTTCTCA TAGCTCACGC TGTAGGTATC TCAGTTCGGT
4861  GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG
4921  CGCCTTATCC GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT
4981  GGCAGCAGCC ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT
5041  CTTGAAGTGG TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT
5101  GCTGAAGCCA GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC
5161  CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC
5221  TCAAGAAGAT CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG
5281  TTAAGGGATT TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA
5341  AAAATGAAGT TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA
5401  ATGCTTAATC AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT CCATAGTTGC
5461  CTGACTCCCC GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC
5521  TGCAATGATA CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC
5581  AGCCGGAAGG GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT
5641  TAATTGTTGC CGGGAAGCTA GAGTAAGTAG TTCGCCAGTT AATAGTTTGC GCAACGTTGT
5701  TGCCATTGCT ACAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC
5761  CGGTTCCCAA CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG
5821  CTCCTTCGGT CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT
5881  TATGGCAGCA CTGCATAATT CTCTTACTGT CATGCCATCC GTAAGATGCT TTTCTGTGAC
5941  TGGTGAGTAC TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG
6001  CCCGGCGTCA ATACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT
6061  TGGAAAACGT TCTTCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC
6121  GATGTAACCC ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC
6181  TGGGTGAGCA AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA
6241  ATGTTGAATA CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG
6301  TCTCATGAGC GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG
6361  CACATTTCCC CGAAAAGTGC CACACTAAAT TGTAAGCGTT AATATTTTGT TAAAATTCGC
6421  GTTAAATTTT TGTTAAATCA GCTCATTTTT TAACCAATAG GCCGAAATCG GCAAAATCCC
6481  TTATAAATCA AAAGA
//
```

## CURRICULUM VITAE

He completed him primary school education in Kayseri. He finished his primary school between in 2007 and he continued to his high school education at Kayseri Mustafa Eraslan High School between 2007-2011. He completed his undergraduate education in Biological Sciences at Karadeniz Technical University, in 2016.

In 2017, he started his masters education at the Department of Molecular Biotechnology at Karadeniz Technical University (KTU).