Production and Characterization of Cutinase from Polyethylene Terephthalate Degrading Bacterial Strain Isolated from Compost Soil

By

Laiba Raees

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad, 2024

Production and Characterization of Cutinase from Polyethylene Terephthalate Degrading Bacterial Strain Isolated from Compost Soil

A thesis submitted in partial fulfillment of the requirements for the Degree

of

Master of Philosophy in

Microbiology

By

Laiba Raees

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad, 2024

Dedication

This thesis is dedicated to my beloved Parents and family, Thank you for all your endless love, sacrifices, prayers, and support.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Laiba Raees

Certificate

This thesis submitted by Laiba Raees titled, "*Production and Characterization of Cultinase from Polyethylene Terephthalate Degrading Bacterial Strain Isolated from Compost Soil*" is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

Supervisor: _____
(Prof. Dr. Aamer Ali Shah)

External Examiner: _____
(Prof. Dr. Azra Khanum)

Chairman: _____
(Prof. Dr. Naeem Ali)

Dated: 12-03-2024


بسم الله الرحمن الرحيم

List of Contents

List of Tables

List of Figures

List of Abbreviations

˚C	Degree Celsius
%	Percentage
AEG	Applied Environmental Geomicrobiology
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CaCl2	Calcium chloride
CaSO4	Calcium sulphate
CCD	Central composite design
Cu	Copper
CuSO4	Copper sulfate
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
EC	Enzyme Commission
EG	Ethylene glycol
FeSO4	Iron Sulphate
HCl	Hydrochloric acid
hrs	Hours
i-e	That is
KCL	Potassium Chloride
kDa	Kilo Dalton
Km	Michaleous-Menton constant
K_2HPO_4	Dipotassium phosphate Monopotassium phosphate
LDPE	Low density polyethylene
$MgSO_4$	Magnesium Sulphate
MHET	mono(2-hydroxyethyl) terephthalic acid
M	Molar
Km	Michaleous-Menton constant
K_2HPO_4	Dipotassium phosphate
KH_2PO_4	Monopotassium phosphate
Vmax	Maximum velocity
$MgSO_4$	Magnesium Sulphate

$ZnSO_4$	Zinc Sulphate
$CuSO_4$	Copper Sulphate
Min	minutes
mM	milli Molar
ml	Milli liter
µl	microliter
DNS	3,5-Dinitrosalicylic acid
CFS	Cell free supernatant
SDS	Sodium dodecyl sulphate
nm	Nanometer
M	Molar
MgSO4	Magnesium sulfate
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NiSO4	Nickel Sulfate
(NH4)2SO4	Ammonium Sulphate
OD	Optical Density
pH	Paviour of hydrogen
RPM	Revolutions per minutes
UV	Ultra Voilet
ELISA	Enzyme-linked immunosorbent assay
SA	Specific Activity

ACKNOWLEDGEMENTS

*All the praises and appreciations are for **Allah**, **The Lord of the Alamine (mankind, jinn's and all that exists)** whose blessing enables me to complete this hard job. The deepest respect and love for the **Holy Prophet, Hazrat Muhammad (P.B.U.H)**, who enlightened our lives with Islam, saved us from the dark and helped us to recognize our only and true Creator. I express my deepest gratitude and sense of obligation to my research supervisor, **Professor Dr. Aamer Ali Shah**, Department of Microbiology, Quaid-i-Azam University, Islamabad, for his supervision and support. His constructive comments and suggestions throughout the experimental and thesis work have contributed to the success of this research. Words are insufficient to express my thanks to **Dr. Salah Ud Din** whose constant presence during my research work made it possible for me to complete this hard job quite smartly. It was because of their inspiring guidance and dynamic cooperation during the entire study program that I could complete this manuscript. I am extremely grateful to the entire faculty of at the Department of Microbiology, Quaid-i-Azam University, Islamabad. I would like to express my appreciations to all my seniors, lab fellows and colleagues for their help. I appreciate all my friends and research fellows especially Salahuddin junior, Alamzeb Khan, Zia ul Haq, Ubaid Khan, Muhammad Ismail, Mehmoona, Tayyaba Alam, Afaq Ahmed, Haris Siraj, Syed Khalid Shah and Inam Ur Rehman Zafar for their kindness, care, and moral support during my work. I would also like to thank my friends and colleagues Asma Mukhtiar, Kainat misal khan, Nimrah shah, Hira Shah for their encouragement and support in my academic and personal life. Special thanks to **Syed Yawar Saeed**, who has been sincerely supportive throughout my MPhil research. Last but not the least a non-payable debt to my loving Parents as well as Family Members for prayers, love, teaching, support, and encouragement without which I would not be the one I am and without them, this study would have been impossible. I would like to mention my brothers Uzair Afridi and Salman Afridi for their support and encouragement. Finally, I express my gratitude and apology to all those who provided me the opportunity to achieve my endeavors, but I missed mentioning them personally.*

Laiba Raees

Abstract

Poly (ethylene terephthalate) (PET), a petroleum-derived synthetic plastic is considered chemically inactive and highly resistant to microbial attack. The accumulation of plastic waste in the environment not only is a source of pollution but also threatens the ecosystem through the release of microplastic. In the current study compost soil was screened out for isolation of polyethylene terephthalate degrading bacterial strain. A bacterium designated as CS7 possesses the ability to utilize PET as a sole carbon source at mesophilic temperature ~35°C. Strain CS7 showed biofilm formation ability over PET pieces and exhibited surface hydrophobicity. Gram staining, biochemical, and molecular characterization results indicated that the strain CS7 was similar to *Bacillus subtilis*. The degradation of PET was further confirmed through weight loss and Fourier-transform infrared spectroscopy (FTIR). During PET biodegradation, the *Bacillus subtilis* CS7 exhibited cutinase activity. Various physicochemical conditions and nutritional factors were statistically analyzed and optimized for maximum cutinase production using the Plackett-Burman design and Central Composite Design software. Cutinase was purified to homogeneity by column chromatography technique using Sephadex G-100 gel resin. The specific activity of the purified cutinase from strain CS7 was found to be 7.58510 U/mg, achieving a 6.1062-fold purification and a yield of 64.01%. The kinetic parameters for this enzyme, *Km* and *Vmax*, were determined to be 2.87 mg/ml and 64.102 µmol/mg/min, respectively. The stability of purified enzyme was observed over a wide range of temperatures from 30 to 40°C and pH values from 8.0 to 10.0. The cutinase exhibited stability towards mesophilic temperature, and various pH ranges, and could further be tested for its resistance to solvents, metal ions, and detergents that will confirm its efficiency for various biotechnological applications under mesophilic conditions. This study suggests that *Bacillus subtilis* CS7 possesses the ability to biodegrade PET and could be further explored for enzymatic hydrolysis of plastic waste.

1. Introduction

Plastic pollution is increasing day by day and is causing serious issues worldwide. The main challenges like global warming, water pollution, and deforestation are also significant environmental problems (Geyer et al., 2017). The total plastic production from 1950 to 2015 was 8300 million tons, and the yearly production keeps rising, which reached 359 million tons in 2018 (Team, 2019). A growing crisis is predicted, with the amount of plastic debris in the ocean increasing as compared to the total fish amount (800 million tons) by 2050 (Chu et al., 2021). This growing plastic waste creates a serious threat to human health and is a major source of environmental pollution. Various technologies have been developed to tackle these problems and try to treat plastic waste chemically and physically. However, the byproducts from these processes also contribute to environmental pollution (Kawai, 2021).

The processing of plastics involves different methods like landfilling, waste incineration, and recycling. If the plastic waste is not disposed of properly, these plastics can become a significant source of environmental pollution over time (Kawai, 2021). Common ways to dispose of plastic include landfilling, recycling, and incineration. However, it is crucial to ensure proper disposal, as incomplete incineration can lead to the production of persistent organic pollutants (POPs), such as furans and dioxins, which are harmful to the environment (Ragaert et al., 2017).

In 2020, the use of plastics for energy recovery was approximately 12.4 million tons, and the use of plastic in the incineration process plays a crucial role in managing municipal solid waste (Tokiwa et al., 2009). However, incineration for energy recovery can result in the release of harmful substances like dioxins, furans, heavy metals, and sulfides, contributing to environmental pollution (Marten et al., 2005). If these plastics are not appropriately recycled, they pose a challenge in treatment as they essentially become waste. Studies on biobased and biodegradable materials have been ongoing since 1980, as these materials are less susceptible to microbial attacks (Danso et al., 2019). Despite their benefits, the adoption of these materials in industries has been limited due to their high cost. Consequently, scientists are actively researching and developing more cost-effective degradable polymers to safeguard plastics from

environmental erosion, photo-degradation, and thermal degradation (Koshti et al., 2018). The most widely employed petroleum-based polymers for the production of single-use plastic materials encompass five main types: low-density polyethylene (LDPE), high-density polyethylene (HDPE), polypropylene (PP), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) (Hiraga et al., 2019).

Polyethylene terephthalate (PET or PETE) is among the versatile linear semicrystalline thermoplastic polymers within the polyester family. Significantly important for its exceptional combination of mechanical, thermal, and chemical resistance, along with dimensional stability, PET has a chemical formula of $C_{10}H_8O_4^n$. This semicrystalline, colorless, and hygroscopic resin reveals important qualities, including high wear and tear resistance, elevated tensile strength, and transparency (Carr et al., 2020).

The synthesis of polyethylene terephthalate involves polycondensation of terephthalic acid (TPA) and ethylene glycol (EG) or transesterification of dimethyl terephthalate and EG, resulting in a semi-aromatic polyester polymer (Sarioğlu & Kaynak, 2017). Ester linkages connect the TPA and EG monomeric units in PET, rendering it chemically inert and hydrophobic, leading to a nearly non-soakable surface. These structural attributes confer upon PET high crystallinity, a substantial melting point, rigidity, and chemical stability (Sharma & Chatterjee, 2017). The global utilization of PET is present across diverse applications, including personal protective equipment, food production, medical treatment, beverage bottles, synthetic textile fibers, films, and resins. PET fibers find application in cloth, technical, and medical textiles, as well as furnishings (Geyer et al., 2016).

PET is recognized as a stable and versatile material, widely used for its safety in medical applications. The disposal of plastic waste offers several methods, including incineration, recycling, and landfills (Rhodes, 2018). In recent years, the global demand for PET has been consistently raised, reaching an annual production of up to 82 million metric tons. Unlike other plastics like polyethylene (PE), PET poses greater challenges in processing and handling, primarily due to its unique chemical structure (Ronkvist et al., 2009). Despite its processing challenges, PET significantly contributes to the presence of microplastics, particularly during laundry cycles. Microfibers, mainly

composed of PET, are released from polyester textiles, commonly known as microplastics. Reports indicate that approximately 1900 microfibers are released into wastewater with each wash, with the majority falling in the ≤5 μm range (48.64%) (Ronkvist et al., 2009).

These microplastics find their way into natural water systems through wastewater discharge, eventually reaching coastal areas and marine environments. Not only do they stay and accumulate within aquatic ecosystems, leading to ecological burdens and risks (Marten et al., 2005), but they also enter the food chain, posing a potential threat to human health. Consequently, the disposal of PET waste has appeared as a major global concern (Yeung et al., 2021). Effectively addressing PET pollution in the environment requires significant attention to proper PET processing methods.

Currently, the processing of PET depends primarily on certain methods like physical, chemical, and biological methods. Physical recycling results in the fragmentation of PET molecular chains, which results in a reduction in the molecular weight of PET, and the presence of some factors such as challenging-to-remove impurities. This significantly reduce the overall performance and economic capability of PET (Kim et al., 2022). Chemical processing, on the other hand, is energy-intensive, expensive, and may generate toxic secondary pollutants. In contrast to physical and chemical methods, the biodegradation approach stands out for its environmental friendliness and low energy consumption, making it potentially the most effective PET degradation method (Jadaun et al., 2022).

Among biological techniques, microbial enzymes can be utilized for degradation of PET, especially PET hydrolase (PETase), plays a very crucial role in breaking down PET through ester bond hydrolysis, resulting in simpler forms of the polymer (Šudomová et al., 2023). Due to the aromatic components in PET's structure, direct entry into microbial cells for biodegradation is not possible for microorganisms. Consequently, these microbes secrete extracellular enzymes that initially break down PET macromolecular polymers into water-soluble small molecules. These smaller molecules can subsequently be absorbed into microbial cells for additional digestion, ultimately leading to hydrolysis into CO2 and H2O (Gao et al., 2021).

Bioremediation methods utilize the capabilities of diverse microorganisms to decrease the concentrations and toxicity of various chemical pollutants, including polycyclic aromatic hydrocarbons, phthalate esters, and plastics (Taghavi et al., 2021). The microorganisms initiate the process by colonizing the plastic, initially adhering to surface of PET. Later, the hydrolytic enzymes that are released by these microbes work together to degrade the polymer (Jachimowicz et al., 2022). The effectiveness of biodegradation depends on certain factors which include the molecular weight of the plastic, its composition (e.g., presence of plasticizers, dyes, flame retardants), the availability of microorganisms, and environmental conditions (e.g., soil pH, temperature) (Janczak et al., 2018).

Two primary types of enzymes are actively involved in the biological breakdown of polymers since most polymers are too big to pass through cell membranes two types of depolymerases: intracellular and extracellular. Exoenzymes from microbes break down complex polymers during this process, producing simpler molecules like oligomers, dimers, and monomers. The process known as "depolymerization" occurs when these smaller molecules get penetrate the semi-permeable outer bacterial membranes and function as a source of energy and carbon. (Sarmah & Rout, 2018). In environments with low or unavailable nutrition, microorganisms use plastics as a source of carbon.

Bacteria involved in process of biodegradation of PET include *Bacillus sp., Pseudomonas sp., and Serratia sp.* (Bano et al., 2017). In the world of biodegradation research, fungi are also receiving substantial attention, particularly from genera such as *Sporotrichum, Talaromyces, Candida, Penicillium* (Bubpachat et al., 2018). These microorganisms show a remarkable ability to colonize even extreme environments, enhancing their enzyme production capabilities (including lactases, hydrolases, esterases, and dehydrogenases) to degrade various chemical compounds with different functional groups. Notably, fungi produce specific proteins called hydrophobins, facilitating their adherence to diverse surfaces, including plastics (Janczak et al., 2018). The biodegradation of a polymer and its rate are influenced by a combination of physical and chemical factors. Key elements include the surface condition of the plastic,

the first-order structure, and the high-order structure, all of which play vital roles in the biodegradation process (Lucas et al., 2008).

Polyesters with side chains reveal a lower assimilation compared to those without side chains. Another key factor that is influencing the degradability of the polymer is its molecular weight (Lucas et al., 2008). Polymers with higher molecular weights are less prone to degradation by microorganisms than those with lower molecular weights. Additionally, the polymer's morphology plays a role, where the degree of crystallinity is a significant factor. Microorganisms tend to target more amorphous polymers, so a higher crystalline nature in a polymer reduces the likelihood of microbial degradation (Tymiński et al., 2019).

The hydrolase family of enzymes, which is known for catalyzing reactions in the presence of water and breaking down substrate chemical bonds, frequently includes all of the enzymes involved for PET degradation. (Tournier et al., 2023). Important enzymes involved in PET degradation include cutinase, lipase, esterase, and PETase. These polyethylene terephthalate hydrolytic enzymes have garnered significant attention in the field of biotechnology due to their potential applications in biorecycling, biocatalysis, waste treatment, and sustainable polymer modifications (Sarioğlu & Kaynak, 2017).

Cutinases have been identified as significant contributors to the hydrolysis of polyesters and polyethylenes, as indicated by different research findings. Various studies have been conducted on the substrate specifications, aiming to know the structure and nature of the binding sites (Berselli et al., 2021). Additionally, research efforts have been made for understanding mutations in the enzyme structure. Several studies are specifically focusing on the hydrolysis of PET through the activity of cutinases

In the current study, *Bacillus subtilis* CS7 Strains was isolated from compost soil. The CS7 strain was screened out for the PET degradation using analytical techniques i.e., Fourier transform infrared (FTIR) of *Bacillus subtilis* CS7 was also screened out for the isolation of the enzyme, cutinase which is widely used for PET degradation. The

cutinase report in this research was a stepwise optimization policy for maximum enzyme production, including elucidation of environmental constituents and medium that produce the enzyme in much higher quantity, using statistical tools such as Central Composite design and Placket Burman. Additionally, creating a numerical model communicates the connection between optimized factors for the elevated production of cutinase as well as purifying and characterizing it.

Aim and Objectives

Aim

The aim of this study is production and characterization of cutinase from polyethylene terephthalate degrading bacterial strain isolated from compost soil.

Objectives

1. Isolation and screening of PET-degrading bacteria from compost soil.
2. To assess the biodegradation of PET by isolated strain using analytical techniques.
3. To optimize physicochemical parameters for cutinase production in bulk using Plackett burmen and Central composite designs.
4. To produce cutinase in bulk and its purification using column chromatography.
5. Characterization of purified cutinase and degradation of PET by purified enzyme.

2. Literature Review

2.1 Brief overview on plastic

Plastics Europe (2018) reported that about 350 million tonnes of plastic are produced each year, and 90% of this comes from burning fossil fuels. In the last sixty years, polymeric materials have become popular due to their low cost, light weight, and resistance to various effects like mechanical, chemical, biological, and physical impacts (Singh et al., 2023).

The term "Plastikos," of Greek origin, means "ready to be transformed into a variety of different materials." Initially, materials like starch and protein, as well as their derivatives like wood and leather, were commonly studied. With the passage of time, humans realized the need to chemically modify these materials to create certain products, leading to significant growth in various product types (Hossain et al., 2022). This chemical transformation resulted in the increased production of synthetic polymers, influencing every aspect of life. The strength and stability of artificial polymers had both pros and cons, providing strength against chemical and physical factors but also posing a challenge due to their high resistance to the degradation process. The primary problem of man-made polymers was their persistence in the environment, leading to many troubles. Plastic, widely used in medicine, pharmacology, and the food industry, becomes inefficient and turns into waste after a short period of time. The global production of petroleum-derived polymers is increasing at a rate of 150 million tons annually, which mainly involves the food, packaging, pharmaceutical, cosmetic, and chemical industries (Shah et al., 2008). The polymeric material is being used more frequently, with a growth rate of 12% annually. These numbers guarantee that a sizable number of plastic wastes contaminates the environment.

In 2014, global plastic production was observed to increase by 38.20%, compared to 2004, as shown in (Fig. 2.1) (Shah et al., 2008). The 21st century showed a substantial increase in plastic production due to its increased demand, resulting in a threefold rise in plastic waste generation over two decades. Presently, approximately 0.3 billion plastic wastes are generated, with a surprising 90% ending up in the oceans (Khoaele

et al., 2023). Since the 1950s, a massive amount, 8300 million plastic wastes have been generated, and if the current trend continues with same ratio, this figure is expected to reach double-digit billion by the end of 2050 (R. Geyer et al., 2017). Plastics are preferred in numerous industrial applications due to their resistance to organic solvents, oxidation, and ionizing radiation. Notably, 33% of the total plastic production is dedicated to packaging (Hurley et al., 2018). Polyethylene terephthalate (PET) stands out among different plastics, especially utilized in the packaging industry, due to its strength and ability to resist high temperatures.

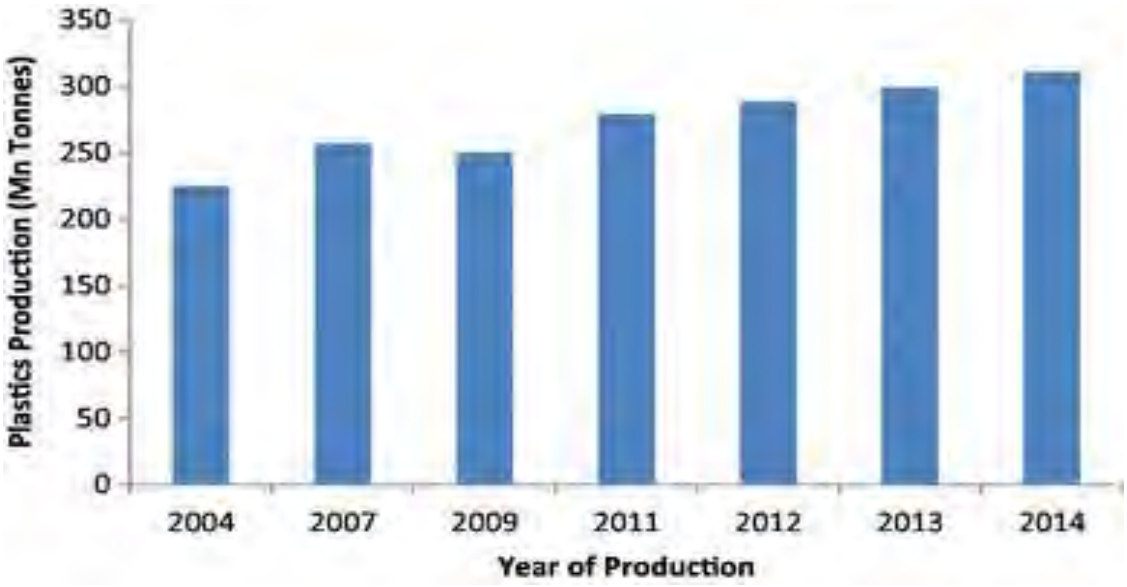

Figure 2.1: Annual production of plastics.

2.2 Plastics in Environment

In 2017, per annum growth in the production of polyethylene terephthalate (PET) has crossed 30 million tons. About forty percent of plastic production belongs to the packaging industry (PlasticsEurope, 2018). This packing material adds to the urban solid waste and synthetic plastic in the oceans, which is the most alarming situation on a global level for the environment (Sharma & Chatterjee, 2017). Plastic is dumped on land and is in direct contact with oxygen and sunlight which causes breaking and fragmentation and eventually forming microplastics. These microplastics in water bodies are of major concern (B. Geyer et al., 2016). These microplastics are taken up the aquatic life and birds as food. The presence of plastic in the digestive tracts of

aquatic life has been brought to light by many researchers. Studies have shown that the ingestion of microplastic by the animals (land and aquatic) and birds is due to their scent, size and shape as food. This is a serious health risk that we are consuming microplastic contaminated with environmental toxins (Rhodes, 2018).

2.3 Production and use of Plastic

2.3.1 Production

Plastic production has hit an all-time high of 350 million tons (de Castro et al., 2017). By 2030, it is expected that it will rise to 590 million tons, plastic materials or PMs have become part of daily life due to their increased usage and versatile nature. During the years 2011 to 2015, the usage of polymers has drastically increased from 300 million tons to 350 million tons. By 2050, the consumption of plastic is set to hit 12,000 million tons which is almost double the production in 2015, which was approximately 6300 million tons (Mohsin et al., 2017). Out of this figure, nine percent is reused, and twelve percent of the rest is burned, or seventy-nine percent is dumped in landfills (Fig. 2.2). The distribution of plastic production globally is described as follows; Asia, Japan, 3.9%; China, 29.4% and Rest of Asia, 16.8%, Europe at 18.5%, NAFTA at 17.7%, Middle East and Africa at 7.1%, Latin America at 4%, and CIS at 2.6%, which is the least of the numbers (Furukawa et al., 2019). Another aspect to consider is that half production of polymers is for single use. This means that they become waste after being used for the initial purpose. Petro chemistry and fossil feedstock are constantly linked with the production of plastics. Furthermore, studies show that 99 percent of the raw material to produce plastic is fossil fuel based. Out of this, 8–9% accounts for global oil and gas consumption. It is further estimated that 4–5% of this is used as raw material and 3–4% as energy (Zekriardehani et al., 2017). However, Global consumption and production for PMs are going to increase as the years go by. Petrochemical companies have the plan to have massive investments in new production facilities. PMs can be classified into two major groups: thermoplastics and thermoset. Formers are mostly branched and liner polymeric material. They are remolded easily and can also survive in a wide range of temperatures. On other hand, the thermosets are crossed linked polymer. This means that they are rigid and cannot be molded easily (Ronkvist et al., 2009). These two groups are divided into 7 classes (Table 2.1).

Table 2.1: Different types of synthetic plastics

Types	Plastics	Abbreviation
Type 1	Polyethylene Terephthalate	PET
Type 2	High density polyethylene	HDPE
Type 3	Polyvinyl Chloride	PC
Type 4	Low Density Polyethylene	LDPE
Type 5	Polypropylene	PP
Type 6	Polystyrene	PS
Type 7	Other Plastic such as Polycarbonate, acrylonitrile, styrene, and nylon etc	

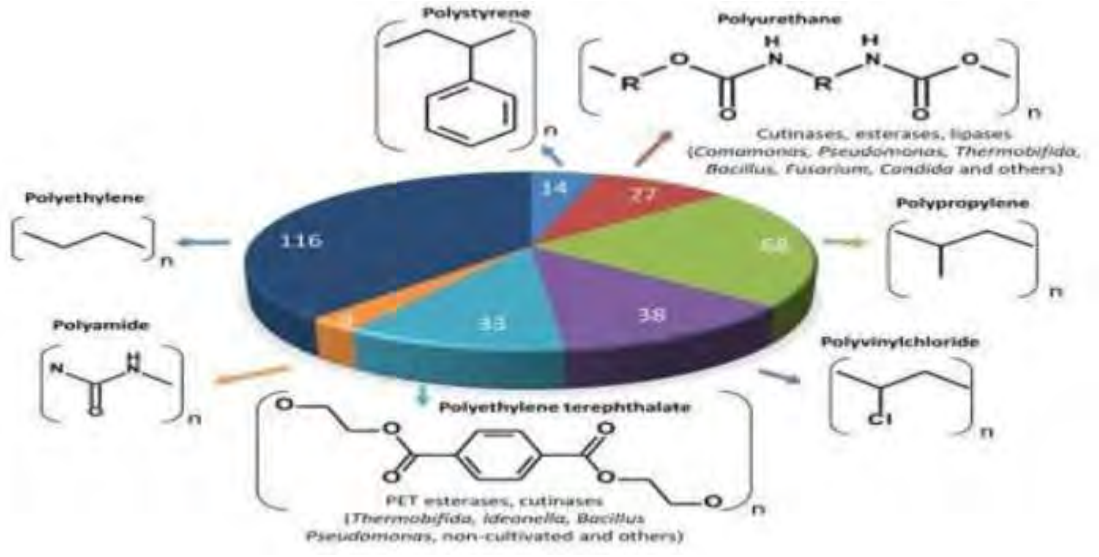

Figure.2.2: In 2017, the most common synthetic polymers were developed around the world. The numbers in the graph represent the global annual output of the mentioned synthetic polymer (in millions of tons) (AlMaadeed et al., 2020).

2.3.2 Challenges Posed by PET Waste

The term "drivers" refers to human activities that are primarily caused by anthropogenic actions, impacting socioeconomic factors. Unfortunately, these activities have severe adverse effects on the environment (Taniguchi et al., 2019). In recent years, there has been a significant increase in the use of plastics, leading to a corresponding rise in plastic waste. This surge has resulted in numerous global issues, particularly exacerbating the problem of municipal solid waste (MSW). Despite the continuous increase in plastic waste, recycling efforts have not kept pace. Littering of plastic waste on streets, beaches, and public spaces has become a common sight in many countries. The wind often carries plastic waste from its disposal site to nearby areas and water bodies, posing a threat to mammals and marine life, leading to fatalities (Taniguchi et al., 2019). One of the main reasons for the alarming increase in marine issues is that sea animals mistakenly recognize plastic waste as food. Ingesting plastic leads to severe consequences, such as starvation, as it becomes stuck in their alimentary canal. Plastic waste debris scattered around beaches, lakes, and ponds has caused significant harm to marine life (R. Geyer et al., 2017). It's important to note that most plastics are non-biodegradable, adding to the persistence of this environmental problem.

The extensive presence of plastic debris in our environment poses a significant challenge. Although some plastics do degrade, it's a slow process that releases byproducts into the soil and water over time (Scalenghe, 2018). Additionally, many plastics contain additives like plasticizers and catalyst residues, which are commonly included during manufacturing. These substances tend to stick to the soil and are not easily disposed of effectively (Salvador et al., 2019). As a result, plastic waste (PW) emerges as a major source of persistent organic pollutants (POPs), known for their severe detrimental effects on both animal and human endocrine and reproductive systems (McKeen, 2018).

The additives used in the manufacturing process, such as antimicrobials, flame inhibitors, preservatives, and plasticizers, contribute to health issues. Take, for example, bisphenol A (BPA), a plasticizer used as a monomer for polycarbonates and epoxy resins. Its leaching qualities make it resistant to degradation, especially when containers

are washed and reused. BPA is harmful to the human body, disrupting natural hormonal signals and damaging the endocrine system (Ji, 2013). This can lead to various disorders and diseases, including obesity, type-2 diabetes, early puberty, chromosomal ovarian damage, prostate cancer, reduced sperm production, changes in the immune system, increased incidences of breast and metabolic disorders, frequent miscarriages, and cardiovascular disorders (Demirel et al., 2011).

2.3.3 Accumulation in landfill

Plastics within the global ecosystem can be classified into three main categories: plastics that are currently in use, mismanaged plastic waste (MPW), which includes urban litter, and post-consumer managed plastic waste. The composition of plastic waste is primarily made up of thermoplastic varieties. Polypropylene (PP) constitutes 21%, low-density and linear low-density polyethylene (LDPE and LLDPE) account for 18%, polyvinyl chloride (PVC) makes up 17%, and high-density polyethylene (HDPE) represents 15%. Other plastic kinds include 5% thermosetting plastic polyurethane, 7% polyethylene terephthalate (PET) (without including PET fibres), 8% polystyrene (PS) and expanded PS (EPS) (Hahladakis et al., 2018).

The kinds of microplastics (MPs) that are produced and discharged vary according to the structure of the plastic trash that they are derived from. The synthesis of these variations is achieved by gathering dispersed data on the production, utilization, and end-of-life management of polymer resins, synthetic fibers, and additives. As of 2015, an estimated 6300 Mt of plastic waste has been generated, with approximately 9% undergoing recycling, 12% being incinerated, and 79% accumulating in landfills or the natural environment. If current trends in production and waste management persist, projections suggest that around 12,000 Mt of plastic waste will be present in landfills or the natural environment by the year 2050 (Ashby & Johnson, 2013).

2.3.4 Impact on ecosystems and wildlife

Due to the large amount of plastic trash produced, the ecosystem is seriously threatened. Landfilling, ocean dumping, and open dumping all cause pollution of the soil and ocean, as well as air pollution. (Kibria et al., 2023). These problems accumulate over

the duration of plastic's life cycle, posing a danger to ecological balance. In the first scenario, especially challenging for undeveloped countries, collecting, and sorting plastic waste from other solid waste for special recycling or disposal processes is a complex task.

In addition, smaller and less developed nations frequently turn to incineration or open burning to minimize the mass and volume of plastics, either due to space constraints or a lack of appropriate dumping sites. However, these activities release excessive amounts of greenhouse gases (GHGs), COx, NOx, and various poisonous gases into the environment. Open dumping also raises concerns about spreading odors and infectious diseases (Ferronato & Torretta, 2019). Thirdly, when dumped on land, plastic waste renders soil infertile and contaminates groundwater; When plastic is dumped into the ocean, marine life is drastically affected since it may remain for hundreds of years. Approximately 80% of this waste is estimated to end up in land disposal (Lepoittevin et al., 2002). Moreover, improper plastic waste management systems commonly result in the clogging of drainage systems, natural disasters, soil infertility, and water contamination (Dombre et al., 2014).

Plastics have application across nearly every sector, including domestic use, food packaging, and industrial settings. This extensive usage contributes significantly to the generation of plastic waste, which, in turn, impacts the ecosystem. According to a report by the World Bank group, plastics make up approximately 5–12% of the world's total waste generation, accounting for 20–30% by weight. Furthermore, approximately 60% of plastics are released into the environment as PW.) (Demirel, 2017).

The considerable volume of plastic waste has notable implications on the ecosystem, manifesting in soil pollution through landfill disposal, marine pollution via ocean dumping, and air pollution through open dumping. Notably, these plastics are not integrated into our food chain (Morris, 2022). The main causes of plastic pollution are poor management, inadequate regulation, inappropriate dumping methods, and a lack of infrastructure (Lepoittevin et al., 2002).

2.3.5 Soil

Plastics are usually taken to secondary transfer stations where appropriate arrangement occurs after being collected from homes, workplaces, business centres, and enterprises. (Foschi et al., 2021). Based on the existing infrastructure and technologies, plastics are either subjected to recycling or reuse processes. Alternatively, plastic waste (PW) might be disposed of in open spaces, sent to landfill sites, or incinerated in open areas. When plastics become embedded in the soil matrix, they can impact soil porosity and binding properties, influencing soil aggregation and water dynamics. Plastic wastes, especially microplastics (MPs), have the potential to interrelate with various soil properties, as depicted in Fig. 2.3 (Lozano et al., 2021). Hazardous pollutants and toxic additives, such as polybrominated diphenyl ether (PBDE), perfluorochemicals (PFOS), and heavy metals including copper, zinc, and lead, are present in microplastics. The remarkable dispersion capacities of microplastics let them to blend with soil, contributing to the degradation of soil fertility (Šujica & Smole, 2003).

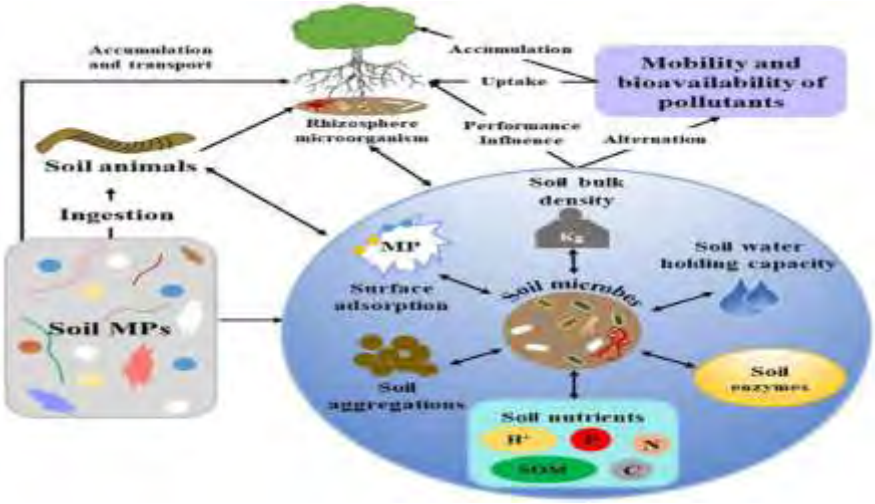

Figure 2.3: Interrelation of microplastics with soil.

2.4 Living Beings and Environment

When plastic is dumped on open landfills, microplastics (MPs) are formed through interactions with environmental factors like pressure, humidity, and temperature (Bracco et al., 2005). These MPs easily infiltrate and blend with the surroundings, including soil, water, and storms, accumulating in the human body through various exposures such as food consumption, dust inhalation, and water contamination. It is

estimated that adults and children consume 1063 and 3223 MPs, respectively, from dust. (Çınar & Kar, 2018).

Moreover, various sea species, such as crabs, oysters, mussels, sea cucumbers, and more, ingest microplastics, and these species are subsequently consumed by humans. Moreover, some have chemicals like artificial pigments and UV stabilisers, which can lead to major health problems like cancer, endocrine disruption, weight gain, and insulin resistance. These health hazards pose significant risks for human beings (Gorrasi et al., 2018).

2.5 Plastic in Marine Environment

Approximately 8 million tons (MT) of plastic waste is added annually, but according to UNEP predictions, the rate at which plastic enters the sea is estimated to be closer to 10–20 MT (Durgashyam et al., 2019). This substantial accumulation of plastic waste poses a severe threat to marine life, leading to damage to the marine ecosystem. Given that microorganisms, such as copepods and zooplanktons, in oceans play a crucial role in absorbing 30% of CO2 from the environment and contribute to mitigating greenhouse gas (GHG) emissions, it becomes imperative to ensure their survival in the marine environment (Hiraga et al., 2019).

The ingestion of copepods by MPs, even at concentrations as low as 75 MPs per milliliter, has adverse effects. The release of smaller eggs with a very low possibility of successfully hatching is evidence of this impact. It highlights the importance of directing the presence of MPs in marine environments for the well-being of copepods and, subsequently, the broader marine ecosystem (Gregory, 2009).

2.6 Plastic Disposal Methods

Landfilling, chemical recycling, incineration, thermal treatment, and biodegradation are some common plastic disposal methods.

2.6.1 Landfill

Approximately 60% of waste, comprising both plastic and other materials, is commonly disposed of in landfills. This prevalent practice is observed not only in third-world

countries but also in some developed nations. The primary reason for this high percentage is the ease associated with landfill disposal for solid waste (Magnan et al., 2016). However, this practice is problematic because waste disposed of in landfills does not readily degrade. The waste in landfills, particularly plastic waste, exhibits UV resistance and incorporates materials during manufacturing that prevent solar radiation from having degradable effects. Furthermore, the anaerobic conditions prevailing in landfills pose challenges for the degradation of materials, including plastics (Cox et al., 2019).

2.6.2 Thermal Treatment

Incineration is the process of heating plastic at high temperatures in the presence of oxygen, converting gases into heat that can further be utilized as an energy source (Barboza et al., 2020). Conversely, pyrolysis involves converting plastic into specific chemicals by heating the material at a specific high temperature while keeping oxygen absent. This method is considered highly effective in Japan, where land limitations drive its popularity. However, a significant challenge of this method is the emission of hazardous dioxins and furans during incineration. Another obstacle is the purification of emitted gases, including the need for desulphurization. Both of these processes require substantial investment, making them time-consuming and taking several years to implement (Macko, 2019).

2.6.3 Recycling

Materials such as glass or aluminum undergo recycling processes, contributing to the creation of reusable products and promoting a concept known as "closing the loop." These processes not only benefit the environment but also reduce the need for resource extraction or mining, ultimately saving energy. However, this concept is not fully applicable in the recycling of plastics, as the loops are not effectively closed. The limited ability of plastics to be recycled is attributed to factors such as the mechanical strength of the material and challenges in degrading and reheating. These recycling processes may result in delays in disposal. Nonetheless, PET recycling can be partially achieved through various methods, including chemical, mechanical, and biological processes (Bharadwaj et al., 2015).

2.6.4 Mechanical Process

Plastic goods undergo specific steps during mechanical processes when recycled. These steps include collection, handling, sorting, melting. This procedure is classified into two types: primary recycling, which involves waste from manufacturing factories, and secondary recycling, where the recycled product may have slightly reduced properties compared to new ones (Encarnação et al., 2019). For instance, instead of disposing of plastic waste, it is demolished and reused in the production of raw plastic to create items like dustbins or tables/chairs.

2.6.5 Chemical Process

The depolymerization of plastic waste followed by chemical processes is gaining considerable attention in current approaches. Chemical processes involved in this context include glycolysis, hydrolysis, and others. Some studies explore various approaches within the degradation procedure, aiming to streamline the process and reduce the need for labor and external efforts (Ramakrishna et al., 2021).

2.6.6 Biological Process

An emerging and economically favorable recycling method focuses on the enzymatic degradation of PET under specific conditions. PVC contamination might happen during the recycling process of PET bottles because the two polymers have comparable densities and appearances. (Danso et al., 2019). Separating them using techniques like floating sink or hydro clones becomes nearly impossible. Consequently, one PVC bottle can compromise the recycling melting process of a bundle containing 100,000 PET bottles. The concerns regarding the degradation technique are a topic of global discussion, with barriers including energy consumption, resource availability, financial investment, and time. Potential solutions involve implementing techniques such as filtration, temperature management, distillation, or crystallization (Wojnowska-Bary\la et al., 2022).

2.7 Synthetic Biopolymer Degradation Mechanisms and Pathways

The word "biodegradation" refers to a biological process. However, it's essential to consider both biotic and abiotic factors when decomposing organic matter. Abiotic degradation takes place before assimilation by microbes, and it must be taken into account when describing degradation methods (E. Wang et al., 2022). The mechanism

of polymer biodegradation involves multiple steps, each of which can be influenced or prevented. Figure 2.4 illustrates the stages of deterioration.

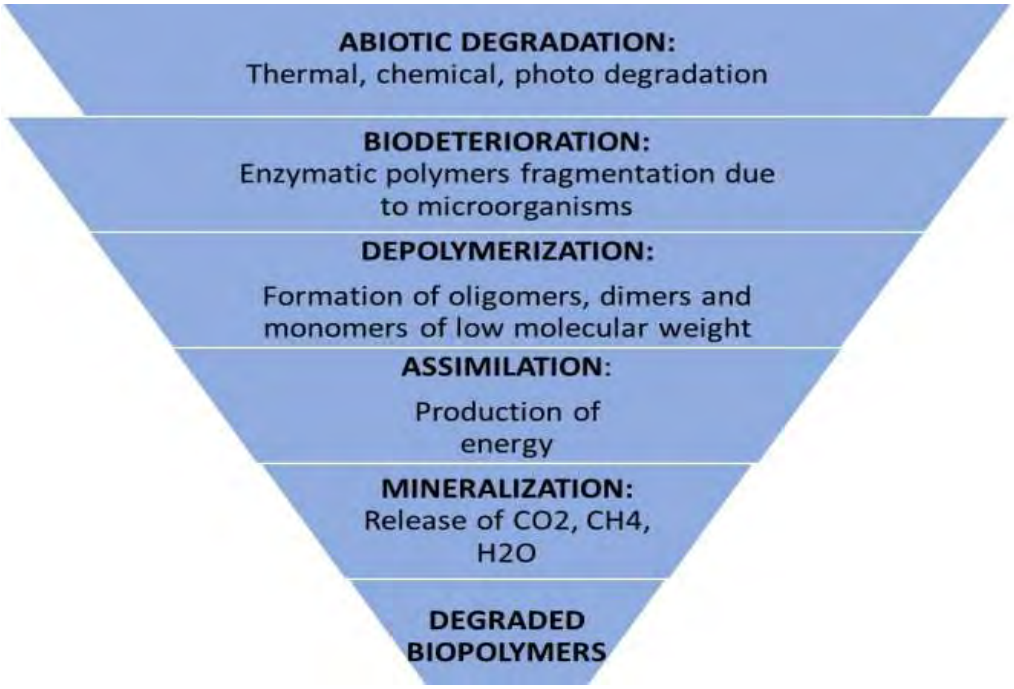

Figure 2.4: Various stages of polymer degradation

The biodegradation of plastic waste is a crucial area of research with the potential to address various environmental issues related to plastic pollution. Microbial activity plays a key role in the complex process of plastic degradation, involving multiple stages (Masud et al., 2019). The biodegradation process begins with the development of microbial biofilms, where bacteria bind to plastic surfaces, creating what is known as the plastisphere. This plastisphere serves as a habitat for diverse bacterial species, fostering an active microbiota that contributes to the degradation of plastic (Landon-Lane, 2018). In aquatic ecosystems, this biofilm production process facilitates the breakdown of plastic polymers by microbial colonies.

Biofilms form efficiently on plastic surfaces, significantly altering their buoyancy and hydrophobic nature. For instance, Rhodococcus ruber invasion leads to the formation of a 3-D structure resembling mushrooms on the developed biofilm. Studies highlight

the remarkable capacity of Alcanivorax borkumensis to create dense biofilms and its ability to break down low-density polyethylene in marine microbial environments (Carpenter & Wolverton, 2017).

2.8 Degradation by Abiotic means

External factors like temperature, aging, sunlight, soil burial, and water can accelerate the degradation activity. Consequently, polymers undergo thermal, chemical, mechanical, and photochemical degradation, making them influential forces in the biodegradation method. The macromolecular matrix organization is significantly affected by temperature (Carpenter & Wolverton, 2017). Thermoplastic polymeric substances generally have melting points higher than ambient temperature, whereas thermoplastic polymers with melting temperatures close to ambient temperature, like polycaprolactone (PCL) at 60°C, exhibit a rubber-like low rigidity, promoting the degradation process (Kumar et al., 2018).

Polymorphism in the crystal region of certain polymers, like polybutylene adipate (PBA), can influence their interaction with microbes. The presence of crystals within the temperature range of 27 to 32°C can yield different effects on the degradation process, depending on the surrounding temperature. Degradation may occur due to exposure to environmental toxins, agrochemicals in the soil, oxygen (O2), and water (H2O). Oxygen serves as an effective agent for abiotic degradation, attacking covalent bonds and generating free radicals (Kumar et al., 2018). The polymer structure, including features like unsaturated links or branched chains, can either obstruct or facilitate degradation, leading to cross-linked reactions (Diggle & Walker, 2022).

Additionally, the polymer amorphous phase promotes oxidative and hydrolytic degradation, while crystalline domains can act as barriers to O2 and H2O diffusion, limiting chemical degradation. Factors such as water activity, pH, temperature, and time can be regulated to encourage hydrolytic degradation. For instance, polymers like PLA, PCL, and PPC degrade slowly in a neutral environment but rapidly in basic or acidic environments (Ogwueleka & B p, 2021). Hydrolysis of ester bonds releases low molecular weight molecules, contributing to polymer degradation.

At the molecular level, mechanical damage caused by compression and strain can trigger the degradation process. While this abiotic parameter may not be influential on its own, it becomes synergistic when combined with temperature, chemicals, and light (Dissanayake et al., 2022). Another abiotic factor affecting biodegradation is photodegradation, induced by exposing polymer materials to light (Hodson et al., 2017).

2.9 Biodeterioration

The widespread presence and enduring nature of microplastics in the marine environment create an ideal space for microbes to establish their niche. The progression of biodeterioration is guided by the proliferation of microorganisms, ultimately leading to biofilm production (Siddiqua et al., 2022). Bacteria produce enzymes, both endo and exo-enzymes, as the initial responders to break down plastic polymers. Exopolysaccharides (EPS) act as a glue, facilitating attachment and bonding between the biofilm and the plastic layer. The interplay of exopolysaccharides and enzymes significantly influences the process of biodeterioration (Dehghani et al., 2017).

2.10 Bio-fragmentation

The process of fragmentation involves converting complex polymers into simpler forms through assimilation. Enzymes play a critical role in stimulating fragmentation process, weakening the structure of existing polymeric substance. Polymers undergo decomposition into oligomers, dimers, and finally into monomers, disrupting the carbon polymer structure and facilitating the fragmentation of plastic (Kibria et al., 2023). Enzymatic depolymerization breaks down polymers into monomers, which are rapidly absorbed by microbes, leading to a substantial increase in bacterial biomass. Research has demonstrated the involvement of enzymes such as laccases, oxidases, and peroxidases in the degradation of polymers (Mazhandu et al., 2020).

2.11 Mineralization

Mineralization marks the endpoint in the degradation of plastic by microorganisms. In the process biodegradation, the plastic polymers are fully deteriorated into CO_2, H_2O,

and CH4. The rate of degradation can be quantified based on the levels of mineralization (Mazhandu et al., 2020).

2.12 Different types of synthetic plastic

2.12.1 Polyurethanes

Polyurethanes (PURs) are produced through various methods, one of which involves using polyether or polyester polyols. PUR is a polymeric material composed of organic units and connected by carbamate linkages. The physio-chemical characteristics of polymers can be influenced by the incorporation of aromatic ring structures. Polyurethane is commonly utilized in the manufacturing of products such as polyurethane foam, thermal insulation, garments, solvents, and paints. Biodegradation of PUR has been explored using bacteria or fungi as agents (Cole & Galloway, 2015).

2.12.2 Polyethylene

Polyethylene (PE) is composed of repeated linking of ethylene polymers and is produced in two main forms: high-density polyethylene (HDPE) or low-density polyethylene (LDPE). It is manufactured through chemical means, polymerizing ethane, and its properties are highly variable due to the diverse side-chain structures obtained through different manufacturing processes (Lebreton et al., 2017). These variations impact the molecular weight and crystallinity of the material. Polyethylene is the primary and most widely used packaging material in the packaging industry. The global annual production of polyethylene is approximately 100 million tons. Various bacterial genera have been associated with potential polyethylene degradation, including Gram-negative genera like Ralstonia, Stenotrophomonas, and Pseudomonas, as well as several Gram-positive taxa. Fungi such as Cladosporium, Aspergillus, and Penicillium are also known to contribute to polyethylene degradation (Chen & Patel, 2012).

2.12.3 Polyamide

Polyamide (PA) refers to repeating units of semi-aromatic, aromatic, or aliphatic molecules linked through amide bonds. There are various types of synthetic polyamides, as they are composed of versatile monomers. Some popular examples include nylon and Kevlar. Polyamides find applications in various industries such as automotive, textiles, sportswear, and carpets. Interestingly, proteins and natural silk are

also examples of polyamides. Given this foundation, it can be expected that enzymes have naturally evolved to act on these polymers. However, fully degrading intact high molecular weight polymer, especially by microorganisms, has not yet been discovered (Gricajeva et al., 2022).

2.12.4 Polystyrene

Polystyrene is a polymer composed of styrene monomers. In addition to its widespread use as a synthetic plastic in the packaging industry, polystyrene is also employed in the manufacturing of everyday items such as CD cases, Petri dishes, plastic cutlery, and various other products. Approximately 14 million tons of polystyrene were produced in the year 2016. In studies examining the degradation of polystyrene, the focus has primarily been on weight loss, and as of these studies, no enzyme has been identified to be directly associated with the assumed depolymerization process (Carr et al., 2020).

2.12.5 Polyvinylchloride PVC and polypropylene PP

Polyvinyl chloride (PVC) and polypropylene (PP) are considered highly significant polymers and are produced in greater quantities compared to the polymers mentioned earlier. PVC is composed of repeating units of chloroethyl, while PP is made up of repeating units of propane-1,2-diyl. Both PVC and PP are manufactured in large quantities on a global scale (Kawai et al., 2019).

2.13. Polyethylene terephthalate (PET)

The flexible linear semicrystalline thermoplastic polymer known as polyethylene terephthalate, or PETE, is a member of the polyester family of polymers. It is renowned for its impressive combination of properties, exhibiting significant strengths in mechanical, thermal, and chemical resistance, as well as dimensional stability. The chemical formula for PET is $(C_{10}H_8O_4)_n$ (Fig. 2.5). PET is a semicrystalline, colorless, and hygroscopic resin that is known for its exceptional qualities, (Koczoń et al., 2022). These properties make PET significantly utilized in the packaging industry, with remarkable applications in plastic bottles for soft drinks, food jars, and plastic films.

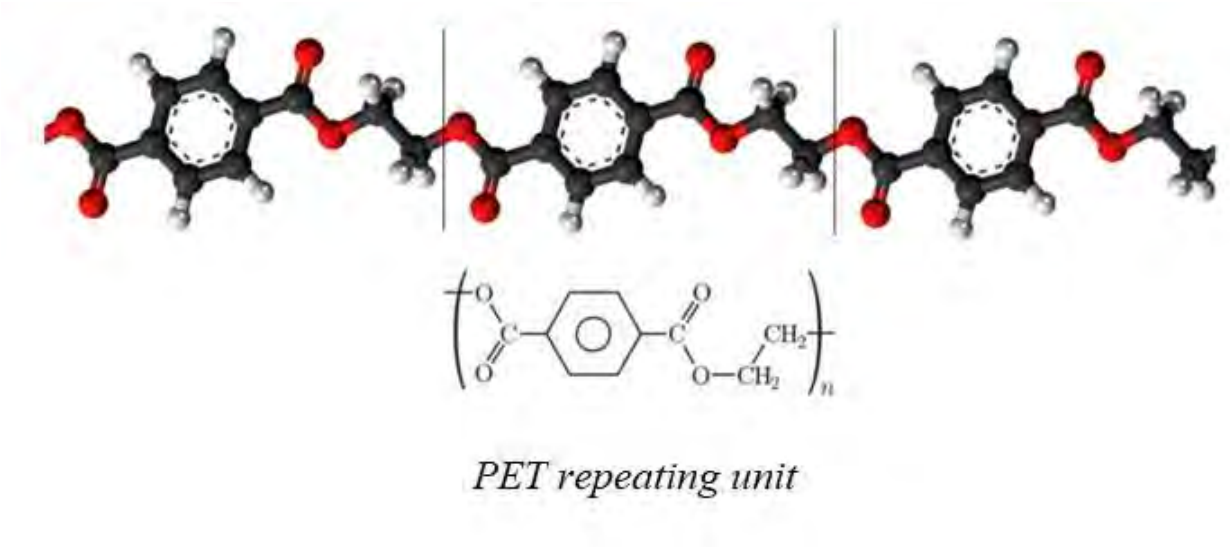

Figure 2.5: Repeating units of PET

The melting temperature (Tm) of PET is observed to fall within the range of 240–250°C, indicating commendable hydrolytic stability. The substantial demand for PET-based plastics, particularly in the packaging industries, resulted in a total production of 18.8 million tons in 2015 out of the overall 269 million tons of total plastic production (Fan et al., 2022). Only 28.4% of the PET produced gets recycled into fibre, sheets, films, and bottles; the other PET is discarded in the environment. Like other plastics, PET is typically non-biodegradable and poses many challenges in their decomposition, exceptionally those with high crystallinity. Therefore, a common practice involves either incinerating or depositing most PET-based plastic wastes in landfill sites (Müller et al., 2001).

2.13.1 Significance of polyethylene terepthalate in the environment

Because of its great transparency, which is comparable to that of glass, amorphous PET is used extensively in the manufacturing of bottles and packaging. The extensive use of PET's in various industries is due to its promising mechanical properties such as abrasion resistance, dimensional stability, resistance, ease of processing for details and surfaces, and high impact strength, even at low temperatures (<−70 °C) (Panowicz et al., 2021). These qualities make PET widely prevail in large industries including machinery, automotive, electromechanical, and electronics. Food industry, food

packaging, and household appliances frequently use it for their applications because of its resistant to external variables and lack of dangerous low molecular weight components. (Müller et al., 2001).

Polyethylene terephthalate (PET) and other plastics have been observed to have a diverse range of applications in many sectors, including personal protective equipment, food production, and medical treatment. PET, recognized for its stability and durability, is a commonly used and safe material for a wide range of medical purposes. As of 2015, the global production of polymers, including PET, reached 322 million metric tons, with major production observed in areas situated in Asia, Europe, and the United States. Plastics, including PET, play an important role in reducing transportation expenditures, prolonging the shelf life of consumable items, and contributing significantly to increase in economy (Yoshida et al., 2016).

2.14. Synthesis of polyethylene terephthalate (PET)

The conventional manufacture process of PET involves the use of ethylene glycol (EG) and terephthalic acid, or its dimethyl ester known as DMT, both derived from crude oil through traditional oil refinery methods (Lucas et al., 2008).

2.15. Properties of Polyethylene Terephthalate (PET)

PET stands out as a recyclable thermoplastic polymer of significant universal importance, resulting in its versatile applications in the domain of plastics. It serves as an environmentally friendly alternative to widely used thermoplastics like polyethylene and other polymers. PET is available in both amorphous (transparent) and semi-crystalline forms, with the latter showing creditable mechanical properties (Kawai et al., 2020). The chemical structure of PET is represented in Fig. 2.6, where 'n' corresponds to the number of repeat units. The intrinsic viscosity, an essential property of PET is dependent upon the polymer chain length, a factor that can be controlled during the polymerization process of PET (Juers et al., 2012).

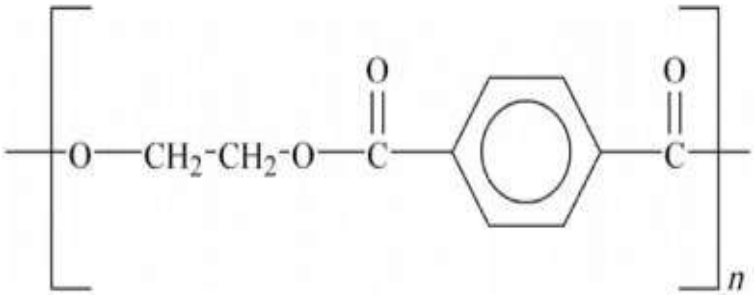

Figure 2.6: The chemical structure of PET

2.15.1 Density: The density values of PET are higher than that of water, indicating that this polymer is denser than water. Consequently, PET sinks in water, regardless of its crystalline degree.

2.15.2 Optical properties: The optical properties of PET are affected by its degree of crystallinity. Specifically, amorphous PET is transparent, while semi-crystalline PET appears opaque. (Rhodes, 2018).

2.15.3 Thermal properties: The presence of aromatic rings and polar groups within the main chain of polyethylene terephthalate involves enhancing the thermal stability and overall toughness of the polymer (Qi et al., 2020).

2.15.4 Mechanical and physical properties: The mechanical properties of PET are significantly affected by the degree of crystallization, which, in turn, depends on many factors such as molecular weight, degree of chain orientation, nucleating agents, and more.

2.16. Uses of polyethylene terephthalate in the environment

PET plays a significant role in the European market, forming approximately 7.7% (around 4 million tonnes), making it the sixth most important polymer, following PP, LDPE, HDPE, PVC, and PUR (Chamas et al., 2020). PET is predominantly used in formation of bottles and containers for beverages. Additionally, PET is employed in the form of sheets/films (about 14%), in the food industry (around 9%), and in non-food applications such as cosmetics (approximately 6%) (Auta et al., 2018) . Other important applications include the manufacture of fibers for textiles, biomedicine, composites (Puglisi et al., 2019). The convenience of PET, recognised for its lightweight nature,

durability, and moldability, makes it a versatile alternative for containers, films, fibers, and bottles, both in terms of production and utility (Pujic et al., 2024). PET acquires important characteristics that make it a valued material in the packaging industry. Due to these beneficial properties, PET will continue to be one of the primary polymeric materials widely utilized in the packaging industry (Gilan & Sivan, 2013).

2.16.1 Electrical and Electronics: Due to its prominent thermal stability, PET is widely used in various electrical and electronic applications. It is used in manufacturing electrical connectors, insulators, and circuit boards, benefiting from its high dielectric strength and resistance to heat.

2.16.2 Automotives: In the automotive industry, PET can be used for various applications due to its lightweight nature and outstanding mechanical properties. It is used in the production of interior elements such as seatbelts, headliners, and door panels. PET's durability and resistance make it compatible for automotive parts where strength and reliability are necessary (Hwang et al., 2020). **2.16.3 Medical and Healthcare:** PET plays a vital role in the medical and healthcare sector, where it is utilized for packaging pharmaceuticals, medical devices, and diagnostic equipment. Its chemical resistance and barrier properties make it appropriate for sustaining the integrity of medical products. PET films are also used in medical imaging devices like X-ray and MRI machines, because of their clarity and ability to effectively transmit electromagnetic waves (Hussain et al., 2022).

2.16.4 Construction and Building Materials: PET has many applications in the construction industry. It is used in insulation materials, roofing membranes, and composite panels, primarily because of its thermal insulation properties, durability, and resistance to moisture. Additionally, the lightweight nature of PET contributes to feasibility of installation and transportation in construction applications (Ahmad et al., 2022).

2.16.5 Sustainable Solutions: The recyclability and environmental sustainability of PET have made it a key player in the formation of sustainable solutions. PET is recycled into new products, helping reduce dependence on virgin materials and minimizing waste. Furthermore, PET is used in the production of eco-friendly packaging, including compostable or biodegradable containers and films, contributing to environmentally conscious practices (Benyathiar et al., 2022).

Polyethylene terephthalate (PET) is an amazingly useful material with a wide range of applications. Its unique properties have made it a key material across various industries, driving technological advancements, promoting sustainability, and enhancing everyday convenience. PET is increasingly replace with glass and metal containers due to its durability, transparency, lightweight nature, non-reactivity, shatterproof quality, thermal stability, cost-effectiveness, higher pressure resistance, mechanical strength, and superior barrier properties against liquids and gases (Zhang et al., 2020).

However, the extraordinary resistance of PET to hydrolytic and enzymatic degradation is a challenge. This resistance makes PET difficult to decompose, leading to the heap of considerable quantities of end-of-life PET plastics in landfills, global oceans, and natural ecosystems. This accumulation has adverse effects on both terrestrial and aquatic life, raising concerns about environmental impact (Sánchez, 2020).

2.17. Biodegradation of Polyethylene terephthalate

The incorporation of plastics into current society has become indispensable, exceeding an annual production of 350 million metric tons (Sarmah & Rout, 2018). Among these plastics, polyethylene terephthalate (PET), derived from crude oil, stands out as one of the most extensively used synthetic polymers. PET is made up of ethylene glycol (EG) and terephthalic acid (TPA) repeating units. While synthetic polymers were initially observed as resistant to microbial degradation, recent studies have revealed that specific microbes produce hydrolase enzymes, enabling them to break down or modify PET. This shows the microbial evolution of various hydrolytic enzymes capable of degrading and processing polymers prevalent in nature.

Enzymes with PET hydrolyzing capabilities consists of carboxylic ester hydrolase enzymes (EC 3.1.1), including cutinases, lipases, and esterases (Moog et al., 2019). These hydrolases have been identified in bacterial sources such as *Ideonella sakaiensis* and *Thermobifida fusca*, as well as in fungi like *Fusarium solani, insolens*, and *Aspergillus oryzae* (Arribas Arias, 2021). Lipases primarily act on medium to long-chain acyl esters (>C10), cutinases target short to medium-chain acyl esters (up to C8–C10), while esterases focus on short-chain acyl esters.

Polyethylene terephthalate hydrolytic enzymes have gained significant attention in the field of biotechnology (Alvarez et al., 2017).

2.18. Mechanism of biodegradation of Polyethylene terephthalate

Biodegradation, characterized by the utilization of living microorganisms to depolymerize plastics and utilize the resulting monomers for their growth, offers a relatively cost-effective alternative to other treatment technologies. This is attributed to the fact that harsh operating conditions are not necessary. Furthermore, the process of biodegradation offers a promising and eco-friendly approach to tackling plastic waste by preventing the release of harmful gases and toxic compounds. The microbial mechanism of biodegradation involves the transformation of insoluble long-chain polymers (plastic) into soluble monomers through biodeterioration, depolymerization, assimilation, and mineralization (Puglisi et al., 2019). Microbial adhesion and colonization on the polymer surface initiate the biodegradation process, leading to surface deterioration. This degradation enables microbes to penetrate the surface, facilitating internal colonization. Microbial extracellular enzymes play a crucial role in breaking down polymers into monomers with diverse molecular weights (Gajendiran et al., 2016).

Upon complete biodegradation, the final mineralization products depend on microbial respiration, resulting in the production of CO2, H_2O, and CH_4 (Usman et al., 2020). The efficiency of biodegradation is primarily associated with the secretion of microbial exoenzymes on the surface of the plastic (Fig. 2.7) (Zhang et al., 2020). Microbial enzymes serve as crucial catalysts in breaking down high-weighted and long-chain polymers into monomers, lowering activation energy and increasing reaction rates. The monomers, dimers, and oligomers thus formed are then converted into carbon dioxide and water through mineralization (Sánchez, 2020). Under aerobic conditions, oxygen acts as an electron acceptor, leading to the synthesis of carbon dioxide and water as the final products.

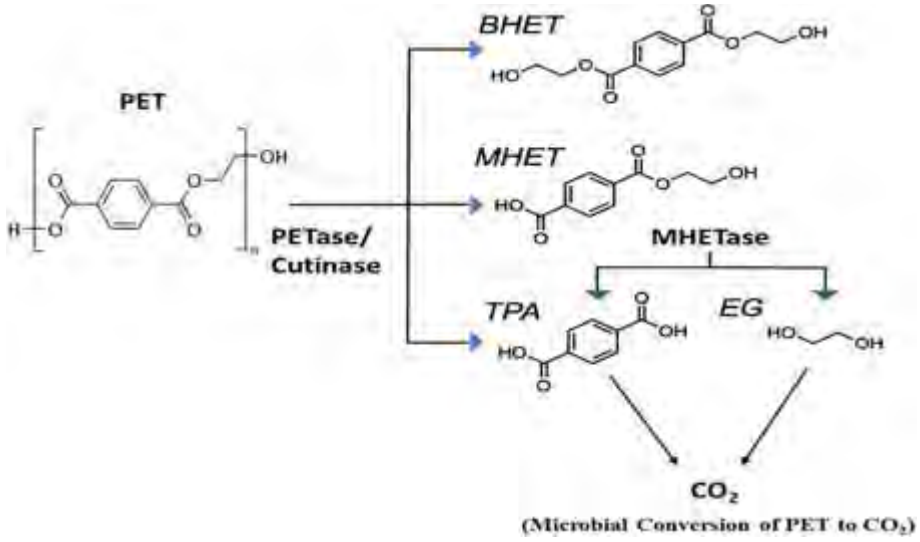

Figure 2.7: Enzymatic degradation of PET

2.19. Factors affecting PET biodegradation.

Several factors affect the biodegradation process, including the diversity of strains in the consortium, size of the microbial community, the type of respiration (aerobic or anaerobic), pH levels, and the extent of expansion on/in plastic.

In aerobic environments, microbes utilize plastic as the sole carbon source and oxygen for the electron transport chain (ETC), ultimately producing CO2 and H2O as final products. (Khoironi et al., 2019). Aerobic respiration generates more ATP compared to anaerobic respiration, promoting higher microbial survival rates and therefore, greater biodegradation efficiency (Arribas Arias, 2021). The inoculum, representing the number of microorganisms, is another important factor affecting biodegradation. Additionally, the physiochemical characteristics of plastics play important roles in either favoring or hindering the biodegradation process (Khoironi et al., 2019).

2.20. Microbial Sources of PET Hydrolyzing Enzymes

PET hydrolases derived from carboxylic ester hydrolases include lipases, cutinases, and carboxylesterase. Recent discoveries in this field introduced PETase and MHETase. Both MHETase and PETase are classified under the α/β hydrolase superfamily enzymes and share related structures, particularly in the central domain and catalytic properties (Dąbrowska et al., 2021).

2.20.1 Actinomycetes

Actinomycetes, a diverse group of filamentous bacteria found in soil, plant tissues, and marine environments, exhibit significant potential for plastic biodegradation. Various actinomycetes, including the *Streptomyces* groups, *Rhodococcus ruber.* and thermophilic *Thermoactinomyces* species, have been isolated from different ecological zones (Avendaño Toledo & Castro Velazco, 2020a). These microorganisms generate a diverse array of hydrolytic enzymes. Moreover, *actinomycetes* produce extracellular polymers like dextran, glycogen, and N-acetylglucosamine-rich slime polysaccharides, facilitating their attachment to plastic surfaces for subsequent microbial action (Khairul Anuar et al., 2022). Similar to bacteria, biofilm formation is also an important factor in the actinomycetal colonization of plastics (Maurya et al., 2020).

2.20.2 Bacteria

Bacteria are known as the driving force behind Earth's nutrient cycles, playing a very important role in the transformation and cycling of nutrients throughout the environment. Extensively studied for their significant contributions to bioremediation, bacteria have revealed the ability to degrade various materials, including antibiotics, metal compounds, petroleum, and plastics, among others (Yang et al., 2013). Importantly, bacterial species from genera such as *Pseudomonas*, *Escherichia*, and *Bacillus* maintain significant potential in the degradation of plastic polymers. Bacteria capable of degrading plastics have been isolated from various ecological niches, encompassing dumpsites, recycling sites, landfills, cold marine environments, and the digestive tracts of insects. (Ma et al., 2012). The formation of biofilms has been observed as an important factor in the bacterial decomposition of plastics. The formation of biofilms encourages bacterial colonies to adhere to plastic surfaces, thereby enhancing their persistence in the degradation process. Moreover, bacteria from several classes, including *Bacillus*, *Pseudomonas*, and *Micrococcus*, have been shown to degrade various thermoplastics, particularly polyurethane (Ma et al., 2012).

2.20.3 Fungi

Fungi, in comparison with bacteria, plays vital roles in maintaining biogeochemical cycles and essential nutrients on Earth. (de Castro et al., 2017). Aspergillus species such as *A. clavatus, A. fumigatus*, and *A. niger* have been isolated from diverse terrestrial habitats and demonstrated their capability to degrade PE, PU, and PP, respectively (Gao

et al., 2021). Other fungal species exhibiting significant plastic degradability include *Fusarium solani*, *Alternaria solani., Penicillium spp.,* among others (Koshti et al., 2018). Fungal hyphae's penetration and dispersion become crucial components in their first colonization and subsequent depolymerization, as increases their capacity to release hydrophobins for improved hyphal adhesion to hydrophobic substrates. (Ribitsch et al., 2011).

Till now, a wide range of enzymes involved in the breakdown of polymers have been found and isolated from a variety of microorganisms, including bacteria, fungus, actinomycetes, and algae. The breakdown of PET is attributed to different groups, with bacteria contributing 56.3%, fungi accounting for 32.4%, a combined effort of both (bacteria and fungi) at 7.0%, microalgae contributing 1.4% (Khairul Anuar et al., 2022). Most importantly, the genera *Aspergillus* and *Fusarium sp.* are frequently reported among fungi, while *Bacillus sp., Rhodococcus spp.,* and *Pseudomonas spp*. dominate among bacteria (Yoshida et al., 2016). Wild-type microorganisms showcasing the capability to degrade PET have primarily been isolated from soil, constituting 22.6%, particularly forest soil, followed by aquatic habitats at 12.4%, encompassing both salt and fresh water. Landfills contribute to 8.0% of the isolates (Joo et al., 2018).

2.21. PET Biodegradation enzymes

PET degrading enzymes, especially those from the polyethylene family, are usually classified as hydrolases. When catalytic processes occur in the presence of water, hydrolase enzymes help to break down the chemical bonds in the substrate. (Avendaño Toledo & Castro Velazco, 2020b). Among the common enzymes involved in PET degradation are cutinase, lipase, and PETase, with PETase being specifically associated with PET breakdown. These enzymes exhibit hydrolytic cleavage capabilities in the long carbon chain of PET, and their actions extend to other plastics as well. This process produces monomers that are broken down by enzymes inside the microbial cell, releasing metabolic products in the process. A range of hydrolases, including lipases, cutinases, esterases, PETase, and MHETase, has been identified for their ability to degrade PET (Fecker et al., 2018).

2.21.1 Cutinase

Cutinase is mainly produced by saprophytic microorganisms that use cutin as a carbon source or by phytopathogenic microorganisms to overcome the cutin barrier when it enters host plants. (Dhawan et al., 2019). Cutin and similar synthetic substances with a large molecular weight can be absorbed by its active site. It exhibits hydrolytic activity on synthetic polymers such as PET, polycaprolactone, and polystyrene (Temporiti et al., 2022). Cutinase is commonly found in fungal and bacterial species (Nikolaivits et al., 2018). Since cutinases have large substrate binding pockets without lid structures that allow PET to interact with their active centres, they exhibit a strong ability to hydrolyze PET. Notably, cutinases typically degrade PET at elevated temperatures, ranging from 50 to 70 °C (Berselli et al., 2021).

2.21.2 Lipase

It has been reported that the successful degradation of PET nanoparticles using lipase from Candida cylindracea and Pseudomonas sp. (Tamoor et al., 2021). Furthermore, it has also been observed that Aspergillus oryzae-induced lipase can hydrolyze PET through BHET/TPA. It is important to remember that lipase and cutinase have the same surface hydrophobicity. (X. Wang et al., 2008). Interestingly, lipases show lower PET hydrolysis activity, primarily due to lid structures covering their catalytic centers. This covering restricts the interaction and catalytic efficacy of lipases with the PET substrate. It is important to mention that lipases primarily act on medium to long-chain acyl esters (>C10) (Gao et al., 2021).

2.21.3 Esterase

The monomers of PET are connected through ester linkages, a feature that can be broken down by esterases present in nearly all living organisms. According to a study, PET was hydrolyzed into TPA and MHET [mono(2-hydroxyethyl)] TPA using Bacillus subtilis nitrobenzylesterase (BsEstB). There is little evidence that esterase activity can effectively hydrolyze hydrophobic PET, and it is typically restricted to short-chain acyl esters (de Queiros Eugenio et al., 2022).

2.21.4 PETase

The enzyme PETase was first recognized in the bacterium I. sakaiensis 201-F6 by Yoshida et al. in 2016. PETase and cutinases exhibit significant structure similarity, suggesting the presence of important structural features involved in substrate binding (Taniguchi et al., 2019). PETases belong to the esterase class of enzymes and are

responsible for catalyzing the hydrolysis of PET into mono-2-hydroxyethyl terephthalate (MHET), along with minor amounts of terephthalic acid (TPA) and bis(2-hydroxyethyl) TPA (BHET). Moreover, these enzymes can further hydrolyze BHET into MHET (da Costa et al., 2020).

2.22. Application of Cutinase in Industry

Cutinases show significant versatility, proving capabilities in hydrolysis, esterification, and transesterification. These diverse functions open up significant potential applications across various industries, including chemical, food, detergent, and textile. In the food industry, cutinases serve as effective precursors for processing vegetables, milk products, and essential fatty acids like EPA and DHA. They contribute to the manufacturing of biodiesel, phenolic substances, surfactants, and other chiral chemicals in the chemical sector. In polymer recycling and surface modification of polymers, cutinases show promise (Martínez & Maicas, 2021). Particularly, they play a crucial role in the detergent industry as lipolytic enzymes for dishwashing and laundry detergent. Cutinases exhibit stable performance in a temperature range of 20°C to 50°C and operate effectively within a pH range of 8 to 11 (“Enzymes Revolutionize the Bioproduction of Value-Added Compounds,” 2020). They can function in the presence of other enzymes like H_2O_2 and are particularly efficient in extracting triacylglycerols and degrading fats in the absence of calcium, outperforming commercial lipase. In the environmental sector, cutinase contributes to waste breakdown, including pesticide and polymer waste. The significance of cutinase has seen a notable increase in its utilization as a biocatalyst in the textile industry in recent years (Dimarogona et al., 2015).

Material and methods

The research was performed at the Applied Environmental and Geo-microbiology (AEG) Laboratory of the Microbiology Department, Quaid-i-Azam University Islamabad Pakistan. All the experimentation for the study was conducted using standard protocols for microbiology.

3.1. Collection of Sampling

A known quantity (20 kg) of soil sample was collected aseptically from compost soil (NARC Islamabad) in sterile zipper bag. During sampling, the materials that were used, sterile zipper bag, distilled water, thermometer, cotton, ethanol (90%), gloves, spatula and pH strips. Temperature and pH were measured by means of thermometer and pH strips. Sample was transported carefully to Department of Microbiology, Quaid-I-Azam University Islamabad and keep at 4°C for further analysis.

3.2. Isolation of bacteria

3.2.1 Colony forming Unit (CFU) or the Viable cell count

To isolate types and numbers of bacteria, a compost soil sample was tenfold diluted ranging from 10^{-1} to 10^{-10}. At the end of dilution, 100µl of sample was shifted aseptically with the help of a micropipette to the nutrient agar plates and by using sterile glass spreader. Afterward, plates were stored at static incubator for 24 hrs at 37°C. After incubation, the types and number of colonies were counted and examined.

3.2 Preparation of inoculum

The bacterial strain Bacillus subtilis was sub-cultured in nutrient broth to prepare the inoculum. The inoculated broth was then incubated for 24 hours at 37°C. After incubation cultures were preserved on nutrient agar plates for further use.

3.3 Microscopic Identification, Biochemical Characterization, and Molecular Identification

PET degrading bacterial strain CS7 was identified by means of biochemical and morphological characteristics. In order to carry out molecular identification, 16S rRNA gene sequencing was used.

3.3.1 Gram's Staining Principle

The following procedure was done for the gram staining.

On a sterile glass slide, a normal saline drop was placed. A thin smear was formed by means of spreading the whole bacterial colony on a slide. To heat fix bacteria the slide was passed about 3-4 times through the flame of 3-4 times; then allowed to cool to the touch before applying the stain. The smear was first stained using crystal violet (dye) for 2 min and gently washed with ionized water for 2 seconds. Afterward, slide was flooded with Grams iodine for upto 2 min and then washed gently with tap water. Ethanol was added drop by drop for 10-15 sec to decolorize the slide and run clear. Then using the safranin counter stain, the slide was flooded for 2 minutes and washed with tap water until no appearance of color in the effluent and blot dry with absorbent paper. The slide was then observed by microscope using different magnifications of 4X, 10X, 40X, and 100X.

3.3.2 Biochemical identification

3.3.2.1 Catalase test

In order to check catalase enzyme activity, hydrogen peroxide was used as catalase reagent. A loopful strain CS7 colony was taking s mixed along with a drop of hydrogen peroxide in the middle of the sterile glass slide. The formation of bubbles indicated the presence of catalase which was observed as the positive strain.

3.3.2.2 Oxidase test

In order to check oxidase enzyme activity, N,N-dimethyl-p-phenylenediamine oxalate was used as agent. 3 drops of freshly prepared reagent were transferred on a sterile filter paper. Through sterilized tooth pick the isolated bacterial strain CS7 colony was picked up and touched over the filter paper surface. 20 sec later, the positive results were noted as color changed to purple to dark purple from pink one.

3.3.2.3 Amylase test

In order to confirm amylase production, the CS7 strain was inoculated on nutrient agar supplemented with 1% starch by incubating at 37˚C for 24 hrs. The plate was swamped with crystal iodine after incubation. The plate was observed for clear zone around the colony that produced amylase.

3.3.2.4 Protease test

The bacterial strain CS7 was directly streaked on 1% casein supplemented nutrient agar by incubating for 24 hrs at 37˚C. After incubation for 24 hrs the plate was swamped with 10% glacial acetic acid then kept for 30 min at 35˚C. After incubation clear zone around CS7 colony was observed.

3.3.2.5 Triple sugar iron test (TSI)

TSI agar was prepared aseptically and slant was formed in test tube. Pure colony of strain CS7 was picked by mean of sterilized loop and stabbed the slant butt and then streaking was done on surface of sloop by incubating for 24 hrs at 37˚C. Slant was observed after incubation by color change to yellow from red which indicated the carbohydrate fermentation.

3.3.2.6 Urease test

The main purpose of urease test is to check the ability of bacteria and whether it can degrade urea. Urea degradation results in the formation of ammonia and carbon dioxide. Prepared 40% urea solution, then added urea to urease broth. Inoculated with selected strain CS7 and incubated for 24 hrs at 37˚C. Phenol red (indicatore) was used. Based on ammonium carbonate formation strain CS7 showed positive result in pink red color.

3.3.3 Molecular characterization of bacterial strain CS7 (DNA analytical methods)

3.3.3.1 Extraction of genomic DNA

The phenol-chloroform method was used for extraction of bacterial strain CS7 DNA extraction from isolated bacterial strain. The bacterial culture of 2ml was centrifuged in Eppendorf tubes for 4 min at 10,000rpm in order to collect the cells (pellets). The pellet was then washed with 600µl TE buffer two times while discarding the supernatant. of 10% sodium dodecyl sulphate (SDS) 30µl and Proteinase-K (PK) 3µl were added and incubated for 1 hr at 37˚C in water bath. After incubation, 5M NaCl (100µl) and CTAB (80µl) were added then mixed thoroughly and again incubated at 65˚C for 10 min. After this, 700µl phenol/chloroform/ isoamyl alcohol at a ratio 25:24:1 was gently mixed. The mixture was centrifuged for 15 min at 10,000rpm. The upper layer was transferred to a fresh Eppendorf tube. In this aqueous solution, equal amount of isoamyl alcohol, phenol and chloroform were added. Mixed the solution well and

then centrifuged for 15 min at 10,000rpm. The aqueous layer was formed again and transferred to the new Eppendorf tube. 600µl of chilled isopropanol was added to the same Eppendorf, mixed well, and again centrifuged at the same conditions. The obtained pellet was washed with 500µl ethanol (70%) and allowed to dry. The dry pellet was dissolved in 60-70 µ*l* TE buffer containing RNase. The obtained pellet was first air dried and then dissolved in 100µl TE buffer and stored overnight at -20˚C.

3.3.3.2 Preparation of agarose gel

Agarose (1%) was added to TBE buffer (1X) and boiled for 2 min. ethidium bromide 3µl was the added in same solution. Afterward, gel was transferred to the gel tray and walls was made by an injecting comb in gel. When gel solidification was completed, 3µL of the sample was loaded and provided (110V and 400mA) voltage for 30 min.

3.3.3.3 Confirmation of DNA

The bacterial DNA was confirmed by running on agarose gel. The band was then visualized on a UV trans-illuminator**.**

3.4. Preparation of media for Degradation and Cultivation

3.4.1 Preparation of inoculum

The inoculum was prepared in nutrient broth. *Bacillus subtilis* strain CS7 was sub-cultured in nutrient broth. At 35ºC for 24 hours, the inoculated broth was incubated. After the completion of the incubation period, 2ml of the freshly grown culture was subjected to centrifugation for ten minutes at 8,000rpm. When the centrifugation cycle was complete, supernatant was discarded, and pellet formed at bottom of the Eppendorf was washed with normal saline (500 µL) to eliminate all the nutrient broth from the pellet. To determine the capability of bacterial strains to break down PET by using a minimal salt medium (MSM).

3.4.2 Quantitative assay

A 4-week shake flask experiment was set up to determine the ability of cutinase production from *Bacillus subtilis* strain CS7 during biodegradation of PET. A freshly prepared 1 mL bacterial strain inoculum was centrifuged for 10 minutes at 8,000 rpm. The pellets were washed twice with normal saline and then resuspended in one milliliter MSM. Separate flasks, each containing MSM and pieces of PET as a carbon source, were inoculated with approximately 108 cells/ml of both bacterial strains for

the degradation of PET. These flasks were then incubated for 28 days or 4 weeks at 37°C in a shaking incubator set to150 rpm. Samples were collected from each flask after 48 hours, and they were centrifuged for 10 minutes at 4°C at 8,000 rpm. The supernatant was assayed for cutinase activity. Additionally, a stock solution of p-Nitrophenyl Butyrate (p-NPB) at a concentration of 10 mM was prepared using isopropanol.

For the cutinase enzyme assay 20 µL substrate p-NPB, 200 µL triton X-100 was added to 1384 µL phosphate buffer with a pH 7.0. For the removal of turbidity. A 200-microliter enzyme solution was added, and it was incubated for 30 minutes at 37 °C in a water bath.

To slow down the reaction rate, the reaction mixture was cooled to approximately -20°C for 10-15 minutes. The absorbance of this mixture was then measured at 410 nm. A reaction mixture without the enzyme solution was used as blank for comparison. The standard curve for p-nitrophenol (pNP) was used to determine the amount of p-nitrophenol released. One unit of enzyme activity was defined as the amount of enzyme needed to convert 1 micromole of p-nitrophenol per minute.

3.4.3 PET degradation screening of *Bacillus subtilis* CS7

To determine the ability to degrade PET, *Bacillus subtilis* CS7 was screened. PET bits used in the experiment were obtained from disposable Nestle water bottles. These plastic pieces were cut into small sizes of approximately 2cm x 2cm. To clean and sterilize them, 70% ethanol was used. After sterilization, any residual ethanol was removed by washing the pieces with autoclaved distilled water. These prepared PET pieces were then aseptically introduced into Erlenmeyer flasks, each containing 100ml of Minimal Salt Medium (MSM). The media is composed of the following components, (gram/Liter); K_2HPO_4, 1.0; KH_2PO_4, 0.2; NaCl, 1.0; $CaCl_2$. $2H_2O$, 0.002; $(NH_4)_2$ SO4, 1.0; $MgSO_4$. $7H_2O$, 0.5; $FeSO_4.7$ H_20, 0.01. The PET plastic pieces in the media were the only source of carbon for the bacteria Bacillus subtilis. 1ml is taken from the prepared inoculum and added to the MSM with the PET pieces. A bacterium free medium with PET pieces was used as a control medium. The flasks were incubated for four weeks at 37°C in a shaking incubator running at 150 rpm. After one week, a new flask containing a fresh Minimal Salt Medium (MSM) was used to inoculate the PET

pieces with 1 ml of initially prepared MSM. These flasks were then incubated again at 37°C in a shaking incubator at 150 rpm for the next seven days. This process was repeated each week for the duration of the four-week experiment.

3.5 Biofilm assay for PET degrading *Bacillus subtilis* CS7

3.5.1 Microtiter Plate Assay

To determine the *Bacillus subtilis* CS7 ability to develop biofilms on the PET surface, a biofilm assay was conducted via a Microtiter plate (Satti et al., 2017). For this experiment sterilized PET pieces were placed in the wells of a microtiter plate. The wells of the microtiter were then inoculated with bacterial strain. *Pseudomonas aeruginosa*, known for its biofilm-producing capabilities, was used as the positive control. For negative control, the wells were left uninoculated and contained only nutrient broth. After inoculation, the microtiter plate was placed in an incubator set at 37°C for a period of 48 hours. When incubation time was complete, the PET pieces were transferred to clean Eppendorf tubes for processing, along with the biofilm formed on the PET surface. The nutrient broth was first removed from the microtiter plate, and then the wells with the PET pieces were washed three times with autoclaved deionized water. To stain the biofilm in the wells, an 800-microliter solution with a 0.5 percent concentration of crystal violet was used, which is then washed three times with water. Following the staining and washing steps, the plate is left to air-dry. After that, 800 microliters of 30 percent acetic acid are added to each well, and the plate is then incubated for 15 minutes. Once the incubation is complete and the biofilms are transferred from the wells, their absorbance is measured at 600nm using an ELISA reader. The absorbance of removed CH_3COOH from PET pieces were measured at 600 nm using clean microtiter plates. The formation of biofilm was calculated by removing the cognate controls' absorbance (average) from inoculated samples' average absorbance.

3.5.2 Bacterial Hydrophobicity Evaluation

BATH (Bacterial adherence to hydrocarbons) process focuses on the potential of bacterial cells to adhere to the hydrocarbons like hexadecane. The stronger the bacterial cells' affinity towards hydrocarbons, the greater will be their hydrophobicity. As a

result, the bacteria transition from the aqueous phase's suspended condition to the organic phase, which lowers the culture's turbidity or cloudiness. (2).

For BATH assay, *Bacillus subtilis* CS7 was inoculated in nutrient broth till the log phase. After this, the culture was centrifuged, the resulting bacterial pellet was washed two times with PUME buffer. The composition of the PUME buffer as follows as [gram/Liter); K_2HPO_4, 17; KH_2PO_4, 7.26; Urea, 1.8; $MgSO_4 \cdot 7H_2O$, 0.2]. When the cell was washed with PUME buffer they were re-suspended PUME buffer to attain the OD 1.0 at 600nm. 1.2ml volume of suspension was poured into test tubes. In the test tubes, varying amounts of hexadecane, ranging from 0.08 to 0.4 ml, were added in ascending order. These tubes, containing the hexadecane, were then placed on a shaker for 15 minutes, followed by a resting period of 5 minutes to facilitate phase separation. After the phases had separated, the optical density of each sample was measured at 400nm. A cell-free buffer was used as the blank for these measurements.

3.6. Biodegradation Analysis

3.6.1 Dry weight evaluation of residues of PET pieces

The layer of biofilm that formed on the PET pieces was removed by mixing the culture with a water solution containing 2 percent sodium dodecyl sulfate (SDS). The experiment was incubated at 50ºC for 4 hours. After incubation, it was washed with warm distilled water (Hickson et al., 2004). PET pieces were gathered on filter paper, washed with distilled water and they were placed at 60oC for drying so that they could be weighed (Kyaw et al., 2012).

% of loss of weight = {(Final weight - Initial weight)/ original weight} *100

3.6.2 FTIR – Fourier Transform Infrared Spectroscopy

To identify the alteration in the functional groups in the chemical structure of treated PET films, an FTIR spectrophotometer (BRUKER, Tensor 27, Germany FTIR series) was utilized. PET pieces were placed on a sample plate of FTIR for this purpose. For each sample, a single spectrum of 500-4000 wave numbers cm-1 was used. Abiotic samples were taken as a control with test samples.

3.7. Qualitative Cutinolytic activity screening on Poly Caprolactone Agar Plates

To test for the presence of cutin-degrading enzymes, assays were performed using plates made of polycaprolactone (PCL). In the PCL assay, 0.1g of PCL was dissolved in 30ml of acetone and was combined with autoclaved nutrient agar. The combination of acetone and PCL was sonicated for 15 minutes to achieve uniformity. Subsequently, the resulting mixture was poured into Petri plates to create Poly caprolactone Agar Plates. The plates were then inoculated with a bacterial strain using a sterile loop and were left to incubate at 37°C for 24 hours. On the PCL agar plates, the appearance of a clear zone around the colonies during their growth indicated cutinolytic enzyme production. To assess this activity, the diameter of the clear zone and the distance between the bacterial colony and the edge of the clear zone were measured.

3.8 Cutin Extraction from tomato peels.

Chemicals used for cutin extraction were Oxalate buffer (pH 3.5): Oxalic acid 4g/L, Ammonium oxalate 16g/L, Chloroform: Methanol (2:1) tomatoes was purchased from Barakahu Islamabad. The tomato was initially washed with distilled water to remove the pulp and contaminations. Tomatoes were peeled and were dried at 60°C for 12 hours in an oven. The dried peels were placed in boiling oxalate buffer at pH 3.5 for one hour to extract any pectin-like substances. After this process, the peels were filtered using cheesecloth. The material obtained was then repeatedly washed with distilled water to ensure the complete removal of any remaining pectin-like residues. It is again dried at 60°C. The dried material was treated with a chloroform and methanol solution in a 2:1 ratio. This mixture was then placed in a shaking incubator, set at 180 rpm, and maintained at 30°C overnight. Following this, the material was dried again at 60°C. The dried product was then ground using a mortar and pestle, and converted into a fine powder ready for use.

3.9. Determination of Cutinase activity

3.9.1 Reagents for Cutinase assay

In cutinase assay the following reagents were used; 100 mM phosphate buffer with a pH 7.0, Triton X-100 0.4%, 12mM 4-nitrophenyl butyrate (pNPB), culture supernatant.

3.9.2 Stock Preparation of Substrate

For the enzyme assay, a stock solution of p-Nitrophenyl Butyrate (p-NP-B) was prepared using isopropanol. To make a 12mM stock solution of p-NP-B, 100 microliters of p-NB-P were added to 100 ml of isopropanol. This substrate was then stored in the refrigerator at 4°C.

3.9.3 Sodium Phosphate Buffer

A phosphate buffer (100mM) was prepared by dissolving 6.8g of KH_2PO_4 and 8.7g of KH_2PO_4 in distilled water, reaching a final volume of 500ml. The solution's pH was adjusted to 7.0.

3.9.4 p-Nitrophenol Standard Curve

10-100 µM solution of PNP in phosphate buffer with a pH 7.0 of different PNP concentrations was used to formulate a standard curve for PNP, and absorbance was measured at 410 nm.

3.9.5 Determination of Cutinase Activity

3.9.5.1 Enzyme production media

The bacterial strain is screened for cutinase activity in nutrient broth media with 0.4% Cutin. 30 ml nutrient broth media (Glucose 3g/L, peptone 5g/L, Yeast extract 3g/L, $NaNO_3$ 2g/L, K_2HPO_4 2.5 gram/Liter, $(NH_4)_2SO_4$ 5.5 gram/Liter $MgSO_4.7H_2O$ 0.5 g/L, KCL 0.5 g/L, NaH_2OH 4g/L, $FeSO_4.7H_2O$ 0.1 g/L and cutin 3 g/L) was prepared for the strain. After the media is autoclaved, it is inoculated using a sterile loop full of isolated bacterial strain. It is incubated for 48 hours in a shaking incubator at 150rpm at 37°C.

3.9.5.2 Quantitative assay for Cutinase activity

After incubation, a 2ml sample was taken from the inoculated broth and the inoculated broth was subjected to centrifugation for 10 minutes at 10,000rpm. The resulting supernatant, which is free of cells, was then assessed for both cutinase activity and total protein content. The working solution consisted of 0.4% Triton X100; 200 microliters, 100mM Phosphate buffer; 1380 microliter, 12mM p-Nitrophenyl Butyrate; 20 microliters, supernatant; 200 microliters. The reaction mixture was incubated in a water bath at 37°C for one hour. Once the incubation period was completed, the mixture was then cooled to a low temperature of -20°C for 15 minutes to slow down reaction. The

activity of cutinase was measured using a spectrophotometer, by measuring the absorbance at 410 nm. (3)

To identify the unknown concentration of p-NP-B produced, the standard curve was used. For negative control, a reaction mixture without enzyme was utilized. According to Lowry's method, protein concentration was estimated using bovine serum as standard. To specify one unit of enzyme activity, the volume required for cutinase assay release 1 mole of p-NPB per minute at $37^{O}C$.

3.9.5.3 Enzyme Activity per Unit

One-unit enzyme activity can be characterized under standard assay condition as enzyme concentration needed to produce 1 µM of para- nitrophenol per minute per ml.

3.9.6 Enzyme Specific Activity

Protein concentrations were divided by PET cutinase activity to determine the specific activity of the enzyme. Specific Activity = Enzyme Activity/ Sample protein contents.

3.10 Determination of total Protein

3.10.1 Reagents Used for Protein Estimation.

Following is the composition of Solutions:

Solution A

Solution A consists of a mixture containing 2% sodium carbonate (Na_2CO_3) and 0.5% sodium hydroxide (NaOH).

Solution B

Solution B is composed of 1% potassium sodium tartrate.

Solution C

Solution C is composed of 0.5% $CuSO_4$. $5H_2O$.

Solution D

Solution D is prepared by combining Solution A, Solution B, and Solution C in a ratio of 48:1:1. **Solution E**

Solution E is made by combining Folin-Phenol reagent and distilled water in a 1:1 ratio.

3.10.1.1 Preparation of Solution A

Solution A was formed by thoroughly mixing 2g of NaOH with 500 mL of distilled water. 10g Na_2CO_3 was mixed with the solution to prepare final volume of 500ml.

3.10.1.2 Preparation of Solution B

For the preparation of solution B, 0.5g potassium sodium tartrate was added into 20ml distilled water and mixed well. By adding more distilled water, the final volume was brought to about 500ml.

3.10.1.3 Preparation of Solution C

To prepare solution C, 0.25g of $CuSO_4 \cdot 5H_2O$ was introduced into 20 ml of distilled water and thoroughly mixed. By adding more distilled water, the final volume was brought to about 500ml.

3.10.1.4 Preparation of Solution D

Solution D is prepared by mixing solutions A, B, and C in a 48:1:1 ratio.

Solution D is always freshly prepared to be used for the protein estimation.

3.10.1.5 Preparation of Solution E

To prepare solution E, Folin-Ciocalteu reagent (often referred to as Folin-Phenol) and distilled water are mixed in a 1:1 ratio. Like solution D, solution E should also be freshly prepared just before it is used.

3.10.2 Development of Standard Curve BSA

1mg/ml concentration with multiple dilutions was made to prepare Bovine serum albumin (BSA) standard solution to examine protein estimation. The determination of total protein was conducted as previously described. The absorbance was measured at 650nm by a spectrophotometer. The absorbance recorded was plotted against BSA concentration (Table 3.1). The graph was made using Microsoft excel. An array line was applied to the graph to evaluate the uncertain estimation of released sugars, and an equation was calculated from the graph's slant.

Table 3.1: Stock preparation of BSA standard curve

No. of Test Tube	Distilled Water ml	BSA Stock Solution ml	Protein Conc. mg/ml	Absorbance
1	0.00	1	1	0.987
2	0.1	0.9	0.9	0.266
3	0.2	0.8	0.8	0.307
4	0.3	0.7	0.7	0.423
5	0.4	0.6	0.6	0.564
6	0.5	0.5	0.5	0.632
7	0.6	0.4	0.4	0.746
8	0.7	0.3	0.3	0.836
9	0.8	0.2	0.2	0.901
10	0.9	0.1	0.1	0.987
11	1	0.00	0.00	0

3.10.3 Total protein estimation

The protein concentration is determined in the sample through Lowry's Method (Lowry et al., 1951). Lowry's method involves two reactions that lead to the formation of a color complex. In the initial step, within an alkaline environment, the peptide bond in proteins reacts with copper ions (Cu^{2+}), causing the Biuret reaction. This reaction ultimately results in the reduction of Cu^{2+} to Cu^{+}. According to Lowry's reaction, the combination of the protein with the Folin-Ciocalteu reagent (often referred to as Folin-Phenol, mixed with distilled water in 1:1 ratio) results in the formation of blue color. This color can be measured by determining the absorbance at 650 nm.

3.10.3.1 Procedure

For protein estimation, 1ml of enzyme solution was added into a test tube and 1ml of solution D. The solution was mixed well and stood at room temperature for 10 minutes. After this incubation period, add 100 µL of Folin-Ciocalteu reagent (often referred to as Folin-Phenol and distilled water mixed in a 1:1 ratio) to the mixture using a pipette

mix thoroughly after adding solution E. Then incubate the test tubes for 30 minutes in a dark environment. The development of a blue color indicates the presence of protein, and its concentration can be quantitatively measured using a spectrophotometer at 650 nm.

3.11 Optimization of Standard Conditions for Cutinase Assay

3.11.1 Enzyme Production Media

The media used for enzyme production was nutrient broth media with 0.4% Cutin. 30 ml nutrient broth media (Glucose 3g/L, peptone 5g/L, Yeast extract 3g/L, $NaNO_3$ 2g/L, K_2HPO_4 2.5 gram/Liter, $(NH_4)_2SO_4$ 5.5 gram/Liter $MgSO_4.7H_2O$ 0.5 g/L, KCL 0.5 g/L, NaH_2OH 4g/L, $FeSO_4.7H_2O$ 0.1 g/L and cutin 3 g/L) was prepared for the strain. The bacterial strain was cultured in nutrient broth and incubated in a shaking incubator at 37°C for 24 hours at 150 rpm. Afterward, 6% of enriched culture inoculated in 70 milliliter of growth medium in 250 milliliter of Erlenmeyer flask, and incubation was given in a shaking incubator at 150rpm at 37oC. 2 ml sample was taken and centrifuged at a speed of 10,000 rpm for 10 minutes at 4°C. The sample was then stored at -20ºC. After storage, total protein estimation and enzyme activity assays were carried out.

3.11.2 Optimization of Temperature

The optimal temperature for the enzyme assay was determined by conducting the assay at various temperatures. For this process, a 2 ml sample was taken, and it was centrifuged for 10 minutes at 4ºC at 10,000 rpm. The supernatant was collected. Enzyme activity and total protein were determined at temperatures from 30ºC, 35ºC, 40ºC, 45ºC, 50 ºC, 55 ºC and 60 ºC crude enzyme activity and specific enzyme activity were measured.

3.11.3 Optimization of pH

To optimize the pH for cutinase activity, assays were conducted at a wide range of pH levels, from 3.0 to 10.0. The supernatant required for these assays was obtained by centrifuging the samples at 10,000 rpm at 4 ºC for 10 minutes. After obtaining supernatant, the crude enzyme activity and specific enzyme activity were estimated, and enzyme assays were evaluated.

3.11.4 Optimization of Time of Incubation

To optimize the incubation time for the enzyme assay, the assay was conducted using different time intervals, specifically at 24 hours, 48 hours, 72 hours, and 96 hours. The samples were centrifuged for 10 minutes at 10,000 rpm and 4ºC for each time interval. After centrifugation, the supernatant was collected from each sample for further analysis. Protein estimation and enzyme assay were monitored, and crude enzyme activity and specific enzyme activity were calculated.

3.12 Optimization of Physiochemical Parameters for Cutinase Enzyme Assay

The submerged fermentation method was used to optimize the growth conditions of the bacterial strain Bacillus subtilis, for achieving maximum cutinase production. This method involves growing the bacteria in a liquid nutrient medium under controlled conditions to enhance enzyme yield.

3.12.1 Preparation of Inoculum

The bacterial strain was freshly cultured in a nutrient broth containing 0.4% Cutin. This culture was then placed in a shaking incubator, at 150rpm at 37oC for 24 hours.

3.12.2 Effect OF Temperature on Production of Cutinase

The bacterial strain was grown at temperatures from; 30˚C to 80˚C. Samples were centrifuged at 10,000rpm for 10 minutes at a temperature 4 ºC, and the supernatant was collected every 24, 48, 72, and 96 hours. Protein estimation and enzyme assay were monitored, and crude enzyme activity and specific enzyme activity were calculated.

3.12.3 Effect of pH on Production of Cutinase

The bacterial strain was grown at pH ranging from 3 to 10. The samples were centrifuged at 10,000 rpm at a temperature of 4ºC for 10 minutes, and the supernatant was collected at regular intervals every 30 minutes. Following that, protein estimation and enzyme assays were conducted. Through these processes, both the crude and specific enzyme activities were measured and analyzed.

3.12.4 Effect of Incubation time on Cutinase production

The bacterial strain was grown at multiple time durations; 24, 48, 72, and 96-hour intervals. The samples were subjected to centrifugation at a speed of 10,000 rpm at a temperature of 4ºC for 10 minutes. After that, the supernatant was collected at 24, 48,

72, and 96-hour intervals. Following that, protein estimation and enzyme assays were performed, and crude and specific enzyme activity were measured.

3.12.5 Plackett-Burman Design for optimizing nutritional factors for maximum Cutinase Production

To optimize the media for production using the Plackett-Burman design, various chemicals were used in specific concentrations. This approach involved selecting the interactions between different chemicals and determining the key factors in the media. The Plackett-Burman design is particularly useful for identifying which components have the most significant impact on production, thereby allowing for a more focused and efficient optimization process. The experiment was designed by using Design Expert 7 software (Stat-Ease Inc.). A total of 11 components were optimized by Placket Burman design which included glucose, $(NH_4)_2SO_4$, peptone, $NaNO_3$, K_2HPO_4, Yeast Extract, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$, Cutin and NaH_2OH. The effects of these factors were determined at 2 levels. The fermentation was conducted at 37°C and 150 rpm. The crude cutinase assay and specific activity were assayed after 24 hours for 72 hours. The results were recorded to determine the specific activity of cutinase. Significant factors were found by evaluating the results.

3.12.6 Central Composite Design Experiment

To extend the optimization process, further analysis was conducted by applying a Central Composite Design (CCD) to the significant factors identified from the Plackett-Burman design. The CCD method allowed for a detailed assessment of the interactions between these factors, providing a deeper understanding of their collective impact on enzyme productivity. This comprehensive approach facilitated a more targeted and effective optimization of the conditions for maximal enzyme output. Three factors from CDC were derived and the influence was recorded in 20 runs. These factors were yeast extract, $NaNO_3$, and K_2HPO_4.

3.13 Cutinase Production in Bulk under Optimum Culture Conditions

500 ml production media was prepared. The composition of production media; Glucose 3g/L, peptone 5g/L, Yeast extract 3g/L, $NaNO_3$ 2g/L, K_2HPO_4 2.5 gram/Liter, $(NH_4)_2SO_4$ 5.5 gram/Liter $MgSO_4.7H_2O$ 0.5 g/L, KCL 0.5 g/L, NaH_2OH 4g/L, $FeSO_4.7H_2O$ 0.1 g/L and cutin 3 g/L. The media was inoculated with 1% of 24 hours

freshly cultured bacterial strain. The inoculated bulk media was incubated for 48 hours at a temperature of 37°C. After this incubation period, the samples were centrifuged, and the supernatant was collected, resulting in the extraction of crude enzyme. After that procedures for protein estimation and enzyme assays were carried out on these collected samples.

3.14 Purification Experiment for Cutinase

For the cutinase purification from the bacterial strain *Bacillus subtilis* CS7, precipitation through ammonium sulfate and gel permeation chromatography (Sephadex G-100) was performed.

3.14.1 Ammonium Sulphate Precipitation

The production media was subjected to centrifugation for 1 minute at 10,000 rpm to get cell-free mixture. About 493ml of cell-free supernatant was extracted. To precipitate the protein from the samples, ammonium sulphate was used. The precise amount of ammonium sulphate needed for this process was determined using an online calculator provided by EnCor Biotechnology Inc. This calculator helps in accurately calculating the appropriate quantity of ammonium sulphate based on the specific requirements of the protein precipitation process. For the precipitation, ammonium sulphate was added slowly into cell free supernatant flask. The Cell-Free Supernatant (CFS) is stirred using a magnetic stirrer at a temperature of 4˚C. Once the ammonium sulphate is fully dissolved in the CFS, the solution is allowed to stand for 24 hours at 4˚C to ensure proper protein precipitation. After the 24-hour period, the solution is subjected to centrifugation for 10 minutes at a speed of 10,000 rpm. This centrifugation process separates the mixture into two components: the supernatant (CFS) and the pellet. The pellet, which contains the precipitated proteins, is then dissolved in a phosphate buffer that has a pH of 8.0. Cutinase assay and Lowry's method were used to measure the supernatant and cell pellet's enzyme activity and total protein.
The supernatant previously obtained was treated with varying concentrations of ammonium sulfate, ranging from 30% to 80%. At a specific level of ammonium sulfate concentration, a substantial amount of protein was precipitated, which led to a high yield of Cutinase activity. This process effectively separated the enzyme, optimizing its extraction and enhancing the overall yield of active Cutinase.

3.14.2 Crude Enzyme Dialysis

To remove the ammonium sulfate from the enzyme solution, the dialysis technique was used. In this technique, the ammonium sulfate-containing crude enzyme was first dissolved in an 8.0 pH phosphate buffer. This solution was then subjected to dialysis to efficiently separate and remove ammonium sulfate, leaving behind the purified enzyme in the phosphate buffer. The enzyme solution is placed into a dialysis tube, which is then immersed in the same type of phosphate buffer. This process is kept at a temperature of 4˚C for a duration of 24 hours. Throughout this period, the buffer solution is constantly stirred using a magnetic stirrer. This stirring action enables the removal of ammonium sulfate from the enzyme through the dialysis process, ensuring effective purification of the enzyme in the buffer.

3.15 Purification of Cutinase by Column Chromatography (Sephadex G-100)

According to their sizes, the separation of molecules is the fundamental basis of gel permeation chromatography (Determann, 2012).

The Sephadex gel was prepared by mixing 0.03g sodium-azide (antibacterial), 2.5g Sephadex G-100, and 0.03g fluconazole (antifungal) in 150 mL Phisphate buffer pH 8.0 and incubating at 35°C for 24 hours. After incubation, the gel swelled completely; sodium-azide prevents bacterial growth while fluconazole inhibits fungal growth. The glass column was thoroughly cleaned using distilled water and thoroughly checked for any leakage. To remove any gases, both the gel and buffer solutions were subjected to sonication for 15 minutes. The column was loaded with gel, with extreme care to avoid the formation of bubbles, The column was filled to a height of 27 cm, containing 21 cm of gel, with the remaining space, and the rest was filled with Phosphate buffer at pH 8.00. At that point, the gel was left undisturbed at room temperature for 24 hours, with the resulting gel immovably pressed in the column. The column flow rate was held at 0.33 mL/min throughout the procedure.

After those 3 milliliters of partially purified enzyme were added to the Sephadex column. To maintain a constant flow of samples in the column, Phosphate buffer supply was used regularly. During this procedure, around 30 separate fractions were collected. Each of these fractions was individually tested for protein content by measuring their absorbance at 280 nm using ultraviolet light. Both protein concentration and enzyme

activity were measured for all these fractions. Those fractions that exhibited a high level of cutinase activity were carefully stored at 4°C for further analysis in future studies.

3.16 Characterization of Purified Cutinase Enzyme

The method described by Samoylova was used to characterize purified enzymes (7). The relative activity of cutinase was determined by: Residual Activity (%) = (Activity (U/mg) of crude enzyme / Activity (U/mg) of the purified enzyme) × 100

3.16.1 Effect of Temperature on Purified Cutinase Activity

To detect the impact of temperature on enzyme activity, temperatures ranging from 30°C to 60°C were chosen to identify the optimum condition. The reaction mixture was incubated for 60 minutes at these specified temperatures. The results were recorded by measuring relative activity (%).

3.16.2 Effect of Temperature on Stability of Purified Cutinase Enzyme

To evaluate the stability of the enzyme at different temperatures, ranging from 30°C to 80°C, the reaction mixture was incubated for a duration of 240 minutes at the specified temperatures. Samples were collected at 30-minute intervals. The results were measured by calculating the percentage of relative activity.

3.16.3 Effect of pH on Purified Cutinase activity

The reaction mixture was incubated for 1 hour at a wide range of pH 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 to establish optimum temperature. The results were expressed in terms of relative activity (%).

3.16.4 Effect of pH on stability of Cutinase Enzyme

The reaction solution was incubated at a different range of temperatures; 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. This process continued for 240 minutes, with samples taken every 30 minutes. The results were expressed in terms of relative activity (%).

3.16.5 Effect of Surfactant

To evaluate the activity of the purified enzyme, it was incubated with different surfactants including Tween 20, Tween 80, SDS, CTAB, and Triton X-100. These surfactants were used at concentrations of 1% and 5% for a duration of 1 hour. The activity of enzyme was recorded by measuring relative activity (%).

3.16.6 Effect of Metals

To determine the activity of purified enzyme, the enzyme mixture was provided incubation with various metal ions such as $CaCl_2$, $MgSO_4$, $CuSO_4$, $ZnSO_4$, $HgSO_4$, $FeSO_4$, $NiSO_4$, $PB(NO_3)_2$ at different concentrations of 10mM and 10Mm for 1 hour. This procedure involved exposing the enzyme to different metallic elements to detect how these metals ions influence the activity of enzymes. This procedure is necessary for understanding the behavior and stability of enzymes in the presence of several metal ions. The results were expressed in terms of relative activity (%).

3.16.7 Effect of Organic Solvent

To determine the activity of the purified enzyme, the enzyme mixture was incubated at various organic solvents. These solvents included ethanol, methanol, acetonitrile, acetone, ethyl acetate, N-Hexane, propanol, butanol, DMSO, and glycerol. The incubation period lasted for 2 hours, during which samples were taken at 30-minute intervals for analysis. The enzyme activity observed in these samples was then calculated and expressed as a percentage, known as the relative activity (%).

3.16.8 Determination of Kinetic parameters

Cutinase from bacterial strain was analyzed at 40˚C and pH 8.0, in a reaction mixture in which cutinase was present in different concentration (0.5–35 mg/ml). The resulting data were plotted to the Lineweaver-Burk software to record the Vmax and Km of kinetic constant.

Results:

4.1 Collection of Samples

The compost soil sample was collected from NARC Islamabad. Temperature was determined as 40°C and pH was recorded 7.0 respectively. The sample physical features are described in table 4.1.

4.2 Isolation of Bacterial strain

Total 26 bacterial strains were isolated from compost soil on a nutrient agar plate. Colony forming U/ml were calculated as $5x10^{-6}$ for compost soil. These isolates were further screened for PET degradation (Satti et al., 2017).

4.3 Qualitative screening of bacterial strain for Cutinase production

To examine bacterial strain CS7 for its capacity to produce cutinase, the bacterium was cultured on PCL agar. Because of the PCL depolymerase activity of cutinase, cutinolytic bacteria formed a clear zone around the colonies on PCL agar plate. This method is very effective for obtaining information about many bacteria's cutinolytic activity. A clear zone around the colony on the agar plate appears as extracellular enzymes diffuse through the medium and break down the substrate This method is widely used because of its cost-effectiveness and rapid screening capabilities (Fig 4.1).

4.4 Morphological and biochemical Characterization of Bacterial Strain CS7

4.4.1 Growth of Bacterial strain

Bacterial strain CS7 was grown on nutrient agar and nutrient broth. Rapid growth of strain was achieved at 37°C within 24 hrs.

4.4.2 Morphological examination

Colonial characteristics were used to examine the cellular morphology of bacterial strain. The colony morphology of bacterial strain CS7 on a nutrient agar plate showed large, opaque, and flat colonies with irregular edges. The colonies were mucoid in texture, and the strain was identified as rod-shaped and Gram-positive. (Fig. 4.2A). According to microscopic findings the strain was gram negative and rod-shaped (Fig. 4.2B).

4.4.3 Physiological and biochemical characteristics of Bacterial strain CS7

Both biochemical and morphological properties of the bacterial strain CS7 are presented in Table 4.2. Bacterial strain CS7 was negative for catalase, oxidase, and protease, and glucose fermentation and positive for amylase as well as protease. Morphological (large, opaque, flat colonies with irregular margins, mucoid), microscopic (Gram-positive, rod in shape).

4.4.4 Molecular Identification

Bacterial strain CS7 demonstrated the nearest alignment to the genus *Bacillus* based on 16S RNA gene sequencing. A phylogenetic tree was constructed by using the neighbor-joining method which showed 100% similarity of strain CS7 to *Bacillus subtilis* (Fig. 4.3).

Table 4.1: Physical features of soil sample.

1	Temperature	37°C
2	pH	7.0
3	Texture	Grey moisture soil

Fig. 4.1: Cutinolytic activity of *Bacillus subtilis* CS7 on PCL medium. PCL degradation by Bacillus subtilis is indicated by clear zone around colony.

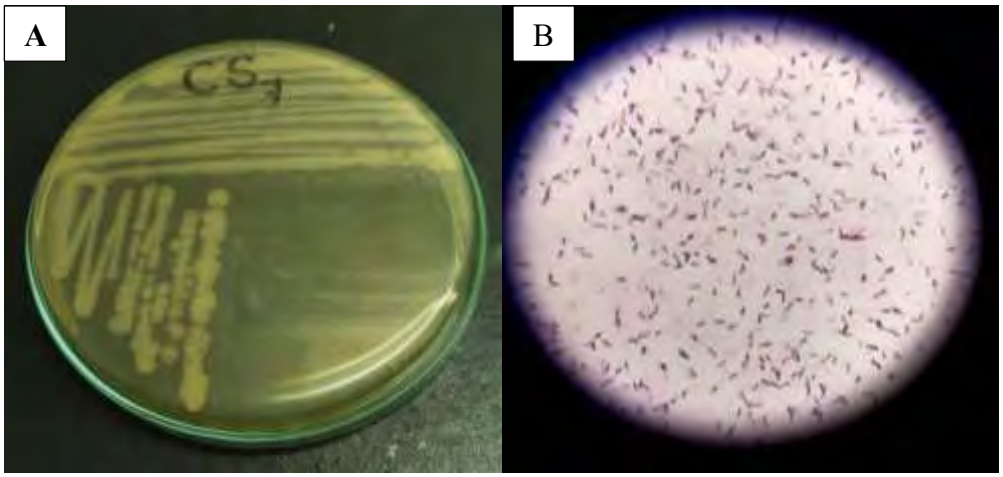

Fig. 4.2: Morphological and microscopic observation of *Bacillus subtilis* CS7 (A) Colony morphology (B) Gram identification showed Gram positive rod shaped.

Table 4.2: Physiological and biochemical characteristics of bacterial strain *Bacillus subtilis* CS7:

Tests	Results
Colony Morphology	Large, opaque, flat colonies with irregular margins
Shape	Rod shaped.
Temperature (ºC)	37 ºC – 40 ºC
pH range	8.0 – 8.5
Gram's Stain	Gram-positive, rod shaped.
Oxidase	Negative
Catalase	Negative
Triple Sugar Iron	Glucose fermenter
Amylase	Positive
Urease	Negative
Protease	Positive

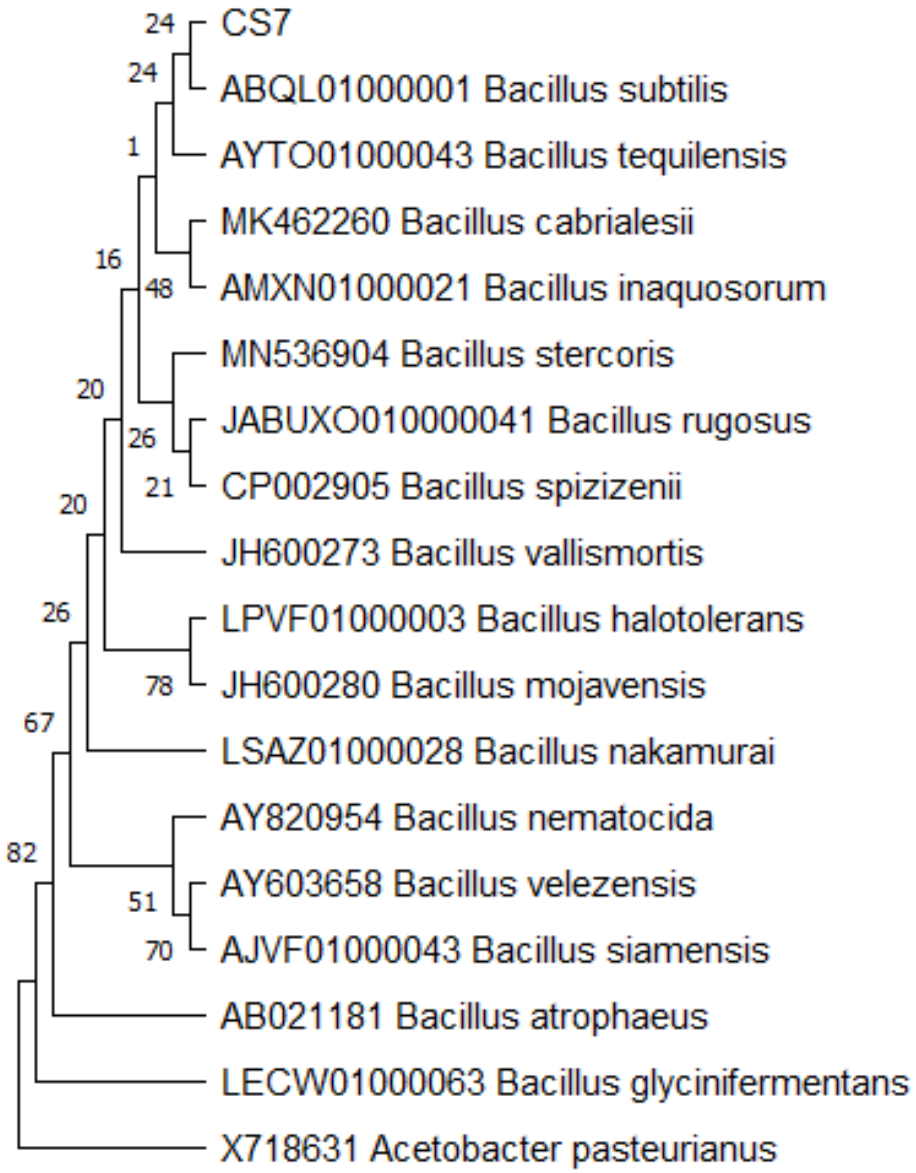

Figure 4.3: Phylogenetic tree based on 16S RNA gene sequencing, bacterial strain CS7 showed 100% similarity to *Bacillus subtilis*. *Acetobacter pasteurianus* was used for out group.

4.5 Biodegradation of PET by *Bacillus subtilis* CS7

The degradation experiment carried out with the selected *Bacillus subtilis* CS7, strain CS7 from enrichment process resulted in degradation of PET pieces (50 mg) These PET pieces were used as the sole carbon source in the medium.

After 28-day incubation period with *Bacillus subtilis* CS7, a reduction of approximately 2.8% in the weight of PET pieces was observed. The determination and measuring of weight reduction have become an easy and quick method to measure the degradation by bacterial strains.

4.5.1 Cutinase activity assay and total Protein Estimation

The samples were taken after every 48 h from the degradation experiment which showed a gradual increase in cutinase activity and protein content, the decrease was observed at the end of the experiment for *Bacillus subtilis* CS7. Highest cutinase activity 100.895 U/mL, and extracellular protein content 0.17175 mg/mL resulted after 28 days of degradation (Fig 4.4).

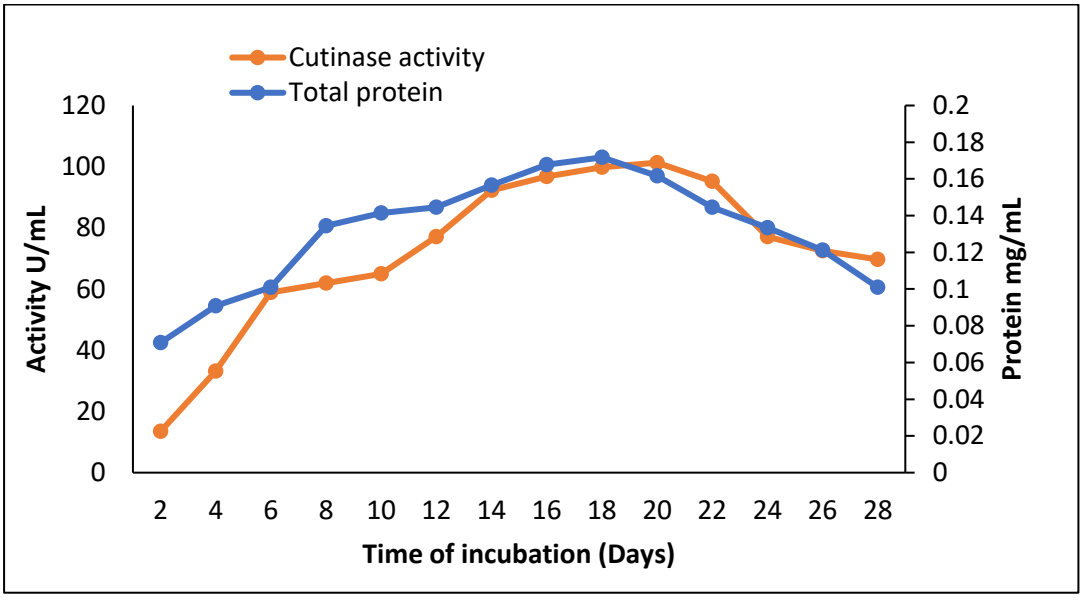

Fig.4.4: Cutinase enzyme activity and total protein in cell free supernatant by *Bacillus subtilis* CS7 during biodegradation of PET piece as a carbon source in MSM media.

4.6 Degradation Analysis

4.6.1 Determination of dry weight of residual PET

Measuring and determining the reduction in weight has become an easy and rapid approach to measure the degradation capabilities of targeted bacterial strains. The degradation of PET has been shown as the growth of bacterial strain in Minimal Salt

Medium (MSM), where PET was the only carbon source. The bacterial strain *Bacillus subtilis* CS7 successfully colonized the surface of PET films, leading to a degradation of up to 2.8% of the initial dry weight of the PET within up to 2.8% of the initial dry weight of PET in just 28 days of incubation, whereas no weight reduction was observed in the PET films in the control flask (Fig 4.5).

4.6.2 FTIR (Fourier-transform infrared spectroscopy) analysis

Structure analysis is a crucial component in determining the structural alterations that arise during degradation and are accountable for weight loss. The FTIR pattern of PET after treatment with bacterial strain *Bacillus subtilis* CS7 revealed that the plastic PET has been changed from its control form. The IR spectrum of polyethylene terephthalate after treatment revealed that the ester bonds at the glycol end are mostly affected. The peak appeared at the position 2963 cm^{-1} for strain *Bacillus subtilis* CS7, and absorption of asymmetric, stretching vibrations of C-H group was marked as it showed significant variations in comparison with the control at the peak position 2970 cm^{-1} corresponding to the vibrations of C-H group (Fig. 4.6).

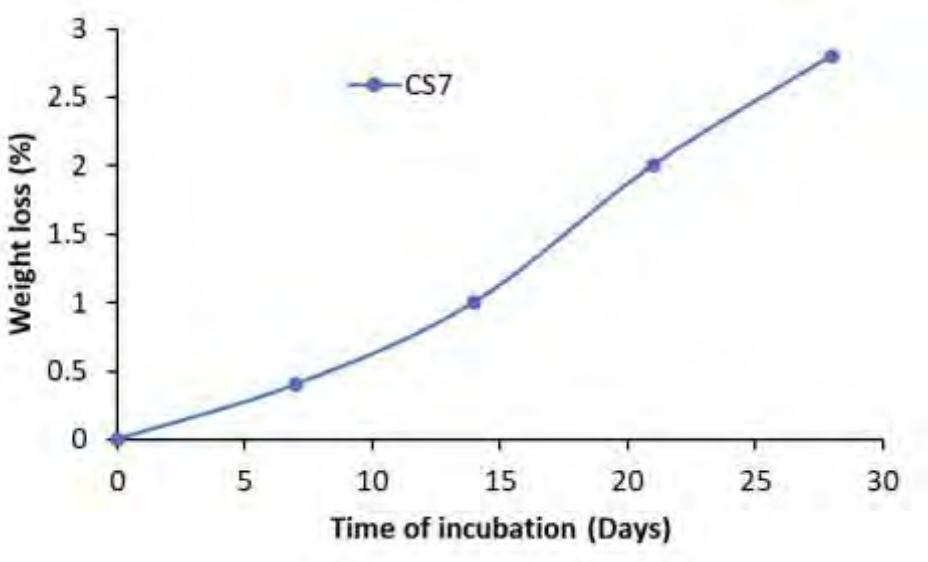

Fig. 4.5: Time course of degradation PET by *Bacillus subtilis* CS7

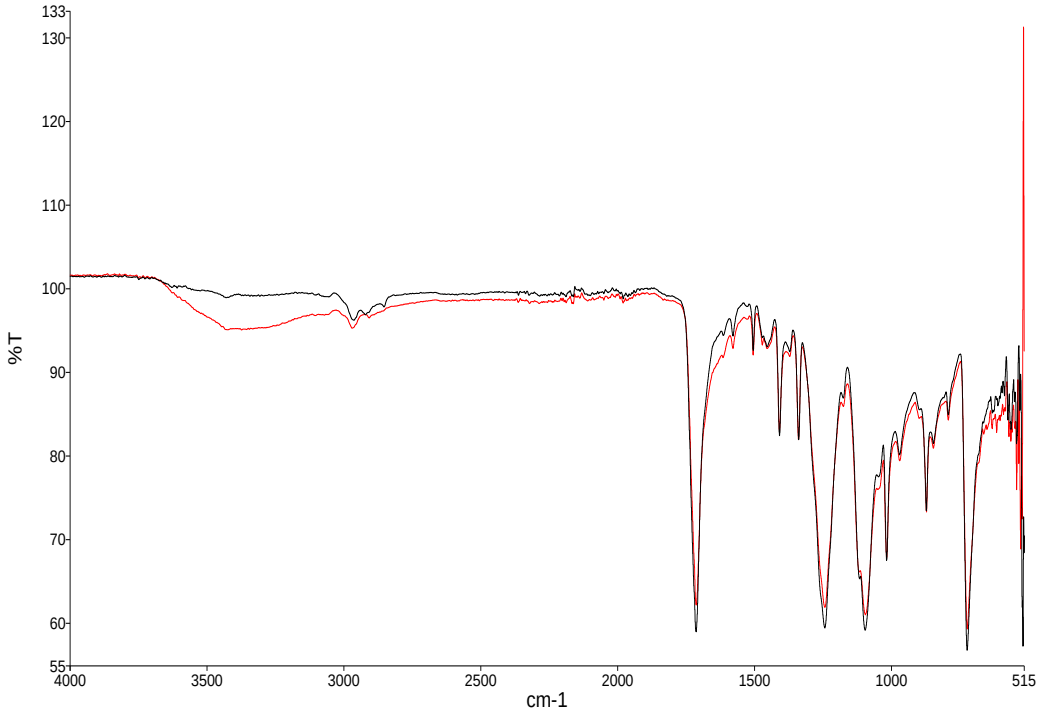

Figure 4.6 FTIR analysis of treated and untreated PET by strain *Bacillus subtilis* CS7. The black line shows untreated PET while red line shows treated PET.

4.7 Biofilm assay using Microtiter Plate Assay

The ability of *Bacillus subtilis* CS7 to form a biofilm on the surface of PET was measured using a biofilm assay in a microtiter plate. *Pseudomonas aeruginosa*, known for its biofilm-forming capacity, was used as a positive control in this evaluation. (Fig. 4.7). After incubation of 48 h, the positive control *Pseudomonas aeruginosa* and un-inoculated wells as negative control initial absorbance at 600 nm using Elisa reader were recorded 2 The initial absorbance at 600 nm was recorded using an ELISA reader after a 48-hour incubation period. For the positive control, *Pseudomonas aeruginosa* showed an absorbance of 2.17, while the un-inoculated wells, serving as the negative control, had an absorbance of 0.23. The initial optical density (OD) for *Bacillus subtilis* CS7 was observed to be 2.1. Afterward, the wells were washed twice with water and treated with 0.5% crystal violet and 30% glacial acetic acid, the positive control has shown a value of 0.922, negative control has shown a value of 0.14, and *Bacillus subtilis* CS7 had a value of 1.48.

4.7.1 Biofilm attached to the surface of PET.

In this experiment, PET pieces were recovered from the microtiter plate and were treated with 0.5% crystal violet and 30% acetic acid. The biofilm formed on the surface of PET with *Bacillus subtilis* CS7 had an absorbance (600nm) of 1.48 (Table 4.2). The results indicate that *Bacillus subtilis* CS7 could attach to the surface of PET which can be helpful in the degradation of PET (Fig 4.8).

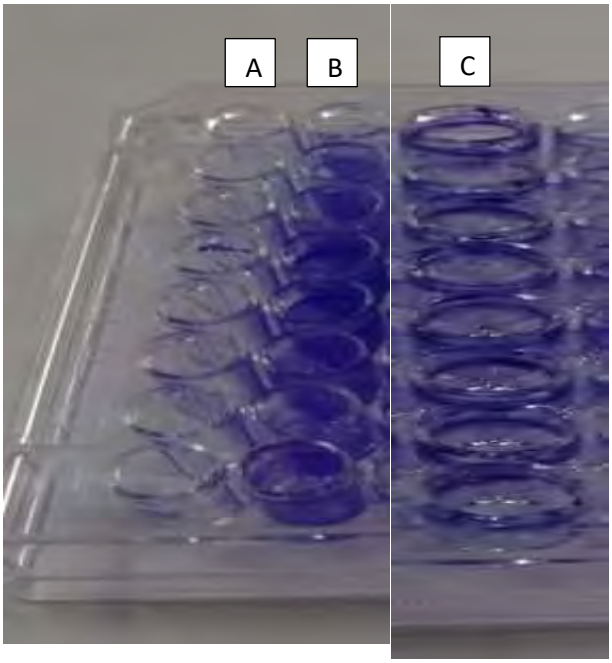

Fig 4.7: The biofilm forming ability of *Bacillus subtilis* CS7 on microtiter plate after treatment with crystal violet. (A) shows positive control of *Pseudomonas aeruginosa* (B) *Bacillus subtilis* CS7 in nutrient broth (C) *Bacillus subtilis* CS7 in nutrient broth media with PET

4.8 Evaluation of Bacterial Hydrophobicity

The BATH assay carried out for the bacterial isolate *Bacillus subtilis* CS7 clearly showed higher hydrophobicity. In the case of *Bacillus subtilis* CS7, the BATH assay revealed a significant adhesion of bacterial cells to hexadecane. This adhesion occurred even at a low hydrocarbon concentration of 0.32 mL, which resulted in a decrease of more than 15% in the turbidity (Fig. 4.8). It was confirmed that the bacterial strain is more hydrophobic in nature. As the PET is hydrophobic in nature, this hydrophobic-

hydrophobic interaction was effective in bacterial adhesion to the surface of PET piece and ultimately facilitated the degradation of PET films. The shift of hydrophobic cells from the aqueous phase to the organic phase resulted caused a decrease in the turbidity (optical density at 400 nm) of the bacterial suspension. The affinity of bacterial cells for the hydrocarbon increases with their hydrophobicity, leading to the movement of cells from the aqueous suspension to the organic phase. This transfer results in a subsequent decrease in the turbidity of the culture.

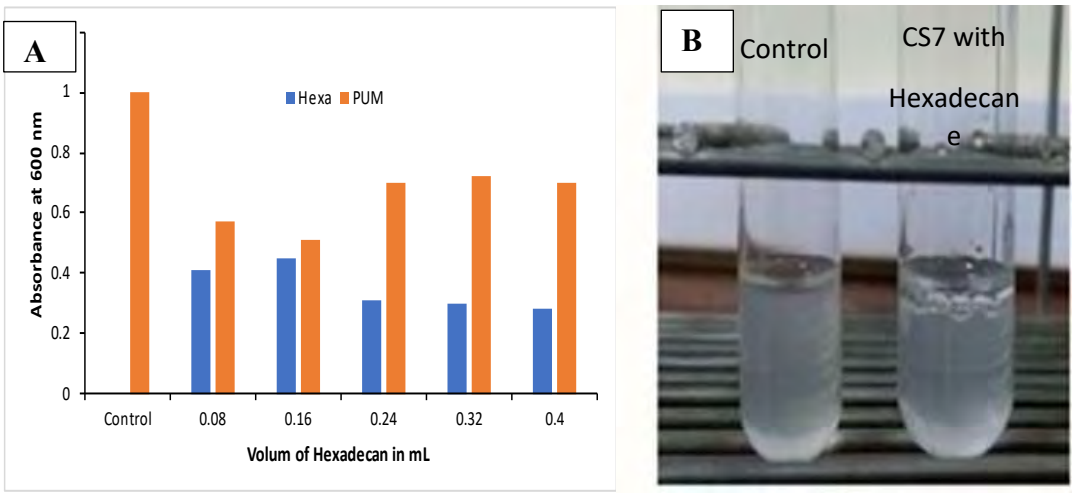

Fig. 4.8: Hydrophobicity of *Bacillus subtilis* CS7 resulted by the bacterial cells adhesion to hexadecane hydrocarbon. (A) Phase separation of aqueous phase and organic phase after the addition of Hexadecane in comparison with control. (B) Turbidity, or the optical density at 400 nm, of the bacterial solution decreases when hydrophobic cells move from the aqueous phase to the hexadecane.

4.9. Standardization of enzyme assay conditions

The enzyme assay parameters were optimized after cutinase synthesis was confirmed by the bacterial strain *Bacillus subtilis* CS7. Furthermore, the optimal assay condition was used to assess the assay at temperature 40°C and pH 8.0, the assay was carried out with an enzyme concentration of 200 µL and a substrate concentration was 20 µL, while incubation period 30 minutes.

4.10 Optimized Culture conditions for production of Cutinase

4.10.1 Effect of temperature

Production of cutinase from *Bacillus subtilis* CS7 was observed by incubating reaction mixture at different temperatures; 25 ˚C, 30 ˚C, 35 ˚C, 40 ˚C and 45˚C. Afterwards samples were taken every 24 hrs for a period of 72 hrs for protein estimation and enzyme assay. Maximum specific activity 88.56563 U/mg was achieved at 35 ˚C after 48 hrs (Fig. 4.9).

4.10.2 Effect of pH

The impact of pH on the production of cutinase by the bacterial strain *Bacillus subtilis* CS7 was analyzed. To determine the optimal pH for maximum enzyme activity, the reaction mixture was incubated at a wide range of pH; 3.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10. Samples were taken after every 24 hrs for a period of 72 hrs for protein estimation and Enzyme assay. Maximum specific activity 1.21753U/mg was achieved at pH 9.0 after 48 hrs, above and below pH 9.0 specific activity was observed decreased (Fig. 4.10).

4.10.3 Effect of incubation time

The production of cutinase from the *Bacillus subtilis* CS7 was examined. Optimized incubation time was observed by incubating the reaction mixture for 72 hrs. Samples were taken after every 24 hrs for a period of 72 hrs for enzyme assay and protein estimation. Maximum specific activity 0.706564 U/mg was achieved after 48 hrs, above and below 48 hrs specific activity was observed decreased (Fig. 4.11).

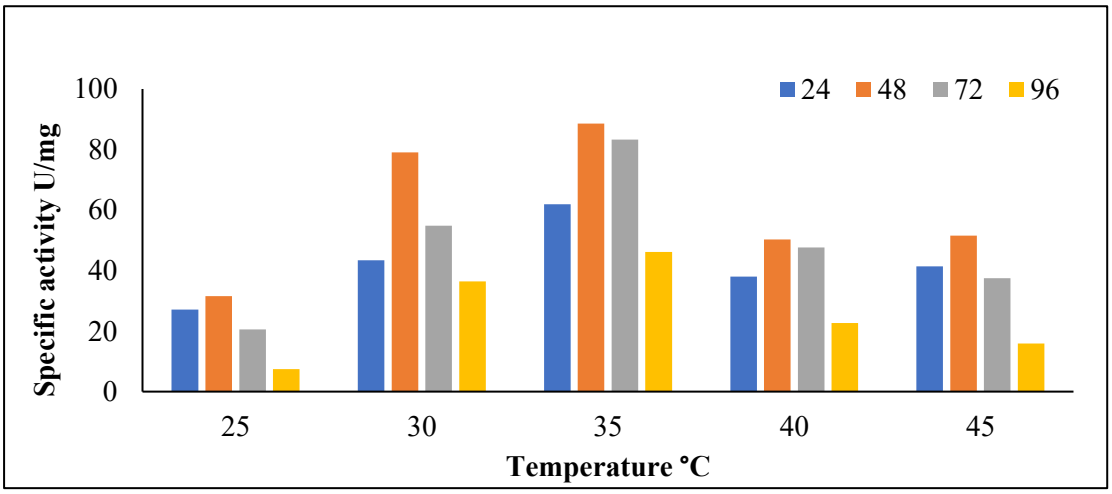

Fig 4.9: Effect of temperature on production of cutinase from *Bacillus subtilis* CS7 within 24–72 hrs of incubation.

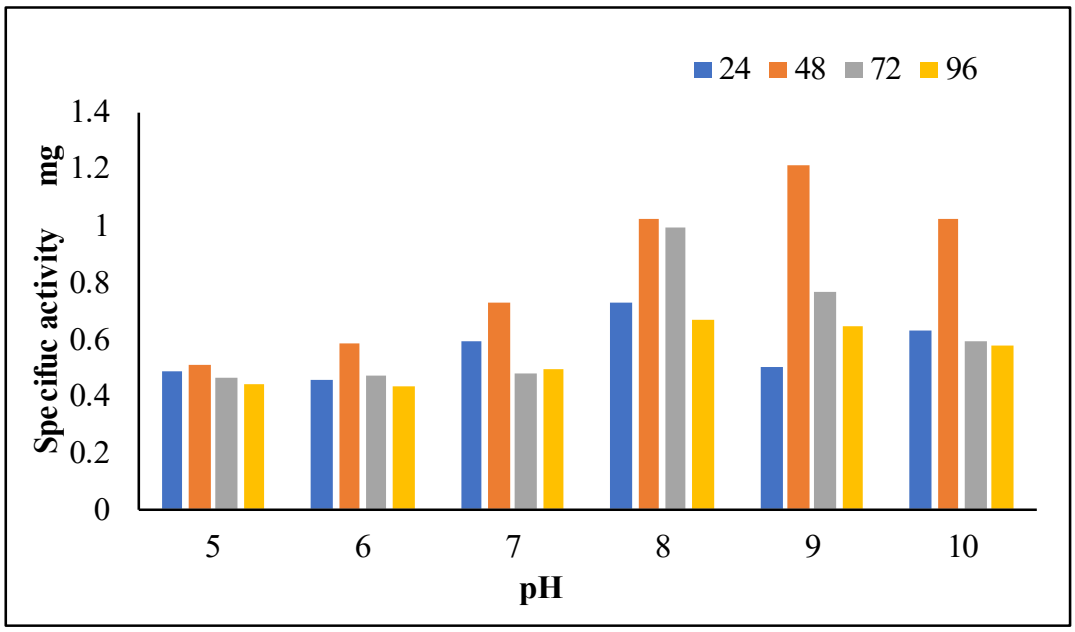

Fig 4.10: Effect of different pH levels on production of cutinase from *Bacillus subtilis* CS7 within 24–72 hrs of incubation.

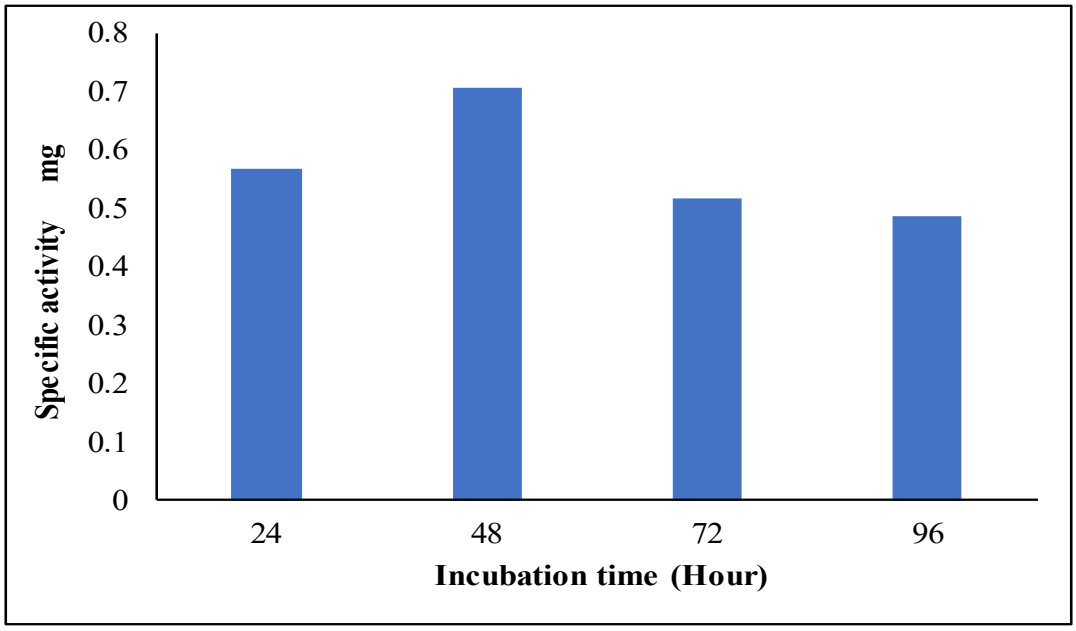

Fig 4.11: Effect of incubation time on production of cutinase from *Bacillus subtilis* CS7 from 24–72 hrs.

4.11 Optimization of Nutritional Factor for Cutinase production by Plackett-Burman Design

The Plackett-Burman design was used to optimize various nutritional factors for achieving maximum production of cutinase. Twenty experimental runs were used to analyse eleven components and run number 9 had the highest cutinase activity (18.00126 U/mg), whereas run number 7 had the lowest activity (9.72275 U/mg) (Fig 4.12). Every experiment was conducted in triplicate under the guidelines set by the software. The design was mathematically modelled using the first-order polynomial model (Eq. 1). $Y = \beta 0 + \sum \beta i\, Xi$ (Eq.1)

Where Y is the predicted response (specific activity of enzyme), $\beta 0$ is the intercept of the model and βi is the linear coefficient and Xi is the level of the independent variable. The above-mentioned factors that affect the fermentation process have also been specified in coded value by the Plackett-Burman design. as shown in table (4.3). An additional Pareto chart defines the contribution of each component to the production of Cutinase (Fig. 4.13). Three of the eleven factors—yeast extract, $NaNO_3$, and K_2HPO_4—were found to have a significant impact on the synthesis of cutinase. and evident by the values of "Prob > F" of each factor mentioned in table 4.4.

The model equation for specific activity of cutinase (U/mg) (R) could be written as: Specific activity (R)= (48.50) +(-9.78B) +(20.32D) +(11.15F) +(4.47G) +(3.27K).

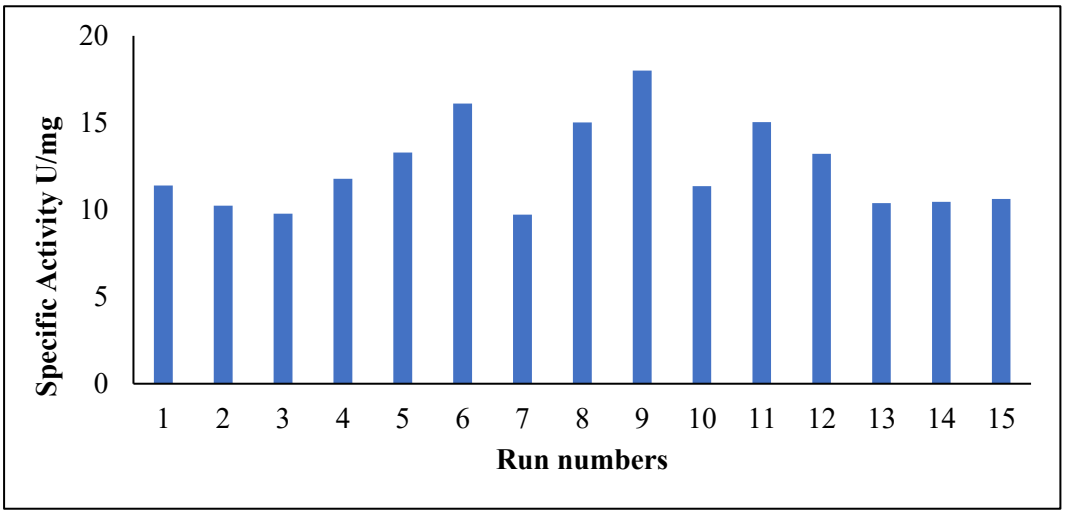

Fig 4.12: Experiment design of Plackett-Burman and observed response of bacterial strain *Bacillus subtilis* CS7 for cutinase production.

Table 4.3: Placket Burman design of factors with specific enzyme activity (U/mg) as response.

Run	Factor A Glucose %	Factor B Peptone %	Factor C Yeast Extract %	Factor D Sodium nitrate %	Factor E $((NH_4)_2SO_4)$ %	Factor F Cutin %	Factor G K_2HPO_4 %	Factor H $MgSO_4.7H_2O$ %	Factor I KCL %	Factor J $FeSO_4\,7\,H_2O$ %	Factor K NaH_2PO_4 %	Response Specific activity (u/mg)
1	0.40	0.60	0.1	0.15	0.35	0.40	0.15	0.08	0.08	0.01	0.45	11.3866
2	0.25	0.50	0.22	0.20	0.55	0.25	0.25	0.06	0.06	0.01	0.40	10.2969
3	0.10	0.40	0.10	0.15	0.35	0.10	0.15	0.03	0.03	0.01	0.35	9.77732
4	0.40	0.60	0.35	0.15	0.35	0.10	0.35	0.03	0.08	0.02	0.35	11.789
5	0.40	0.60	0.10	0.25	0.75	0.40	0.15	0.03	0.03	0.02	0.35	13.2969
6	0.40	0.40	0.35	0.25	0.75	0.10	0.15	0.03	0.08	0.01	0.45	16.1136
7	0.10	0.60	0.10	0.25	0.75	0.10	0.35	0.08	0.08	0.01	0.35	9.7227
8	0.40	0.40	0.35	0.25	0.35	0.40	0.35	0.08	0.03	0.01	0.35	15.0166
9	0.10	0.60	0.35	0.25	0.35	0.10	0.15	0.08	0.03	0.02	0.45	18.0013
10	0.10	0.60	0.35	0.15	0.75	0.40	0.35	0.03	0.03	0.01	0.45	11.3511
11	0.10	0.40	0.35	0.15	0.75	0.40	0.15	0.08	0.08	0.02	0.35	15.0436
12	0.25	0.50	0.22	0.20	0.55	0.25	0.25	0.06	0.06	0.01	0.40	13.2088
13	0.25	0.50	0.22	0.20	0.55	0.25	0.25	0.06	0.06	0.01	0.40	10.39
14	0.40	0.40	0.10	0.15	0.75	0.10	0.35	0.08	0.03	0.02	0.45	10.4621
15	0.10	0.40	0.10	0.25	0.35	0.40	0.35	0.03	0.08	0.02	0.45	10.6161

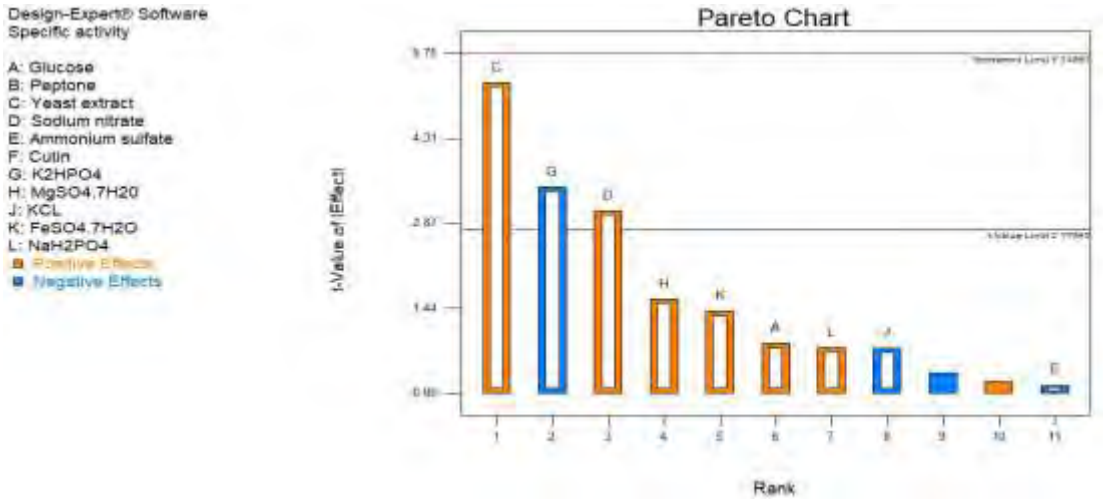

Fig 4.13: Pareto chart showing the effect of t value by important factors (Yeast extract, C; $NaNO_3$, D and K_2HPO_4, G produced by Placket Burman design with respect to the effect on the cutinase enzyme production.

Table 4.4: ANNOVA for Placket-Burman Design

Source	**Sum of Squares**	**Df**	**Mean square**	**F value**	**p-value prob>F**
Model	81.86	9	9.10	6.20	0.0472 significant
A-Glucose	1.05	1	1.05	0.72	0.4447
C-yeast extract	40.53	1	40.53	27.65	0.0063
D- Sodium nitrate	13.99	1	13.99	9.94	0.0366
E-($(NH_4)_2SO_4$)	0.030	1	0.030	0.020	0.8937
G-K_2HPO_4	17.91	1	17.91	12.22	0.0250
H-$MgSO_4.7H_2O$	3.73	1	3.73	2.54	0.1860
J-KCL	0.87	1	0.87	0.59	0.4837
K-$FeSO_4.7H_2O$	2.84	1	2.84	1.94	0.2361
L-NaH_2PO_4	0.90	1	0.90	0.61	0.4773

4.12. Optimization of significant variable using CCD

Further extension of the optimization studies was carried out, and a Central Composite Design (CCD) was applied to the critical factors identified from the Plackett-Burman design, which positively impacted the production of cutinase. The model included three factors, specified by their codes, obtained from PB and their effect was studied in 20 runs. Maximum specific activity 18.0013 U/mg was achieved at run number 9. (Table 4.5: Fig. 4.16). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was obtained to describe the specific activity of Cutinase. Specific. Activity= +109.75-6.26A+1.83B-11.05C-13.38AB-13.22AC+7.13BC10.70A2+3.27B2 -7.18C2 $Y = \beta 0 + \sum \beta i Xi\ 6\ i{=}1 + \sum \beta ii\ 6\ i{=}1\ Xi\ 2 + \sum \beta ij\ 6\ i$v

Where Y is the response (specific activity); Xi are significant independent variables; βi are linear regression coefficients; βii are quadratic regression coefficients; $\beta ij$are interactive regression coefficients while $\beta 0$ is a constant term. The acceptability of the model was evaluated using ANOVA (Table 4.6). In this circumstance among the quadratic terms and linear, only interactive term AC and BC were significant model terms, whereas A represent Yeast extract, and B represent $NaNO_3$ while C represent K_2HPO_4. Response surface plot (BC) in (Fig.4.14) shows, that increase in K_2HPO_4 and constant concentration of $NaNO_3$ potentially enhanced enzyme activity, whereas $NaNO_3$ has no effect on enzyme activity. The same response plot (AC) in (Fig. 4.15) indicates that the highest activity of cutinase was observed with an increase in both yeast extract and $NaNO_3$, indicating that these factors positively influence cutinase activity.

Table 4.5: Central Composite Design of factors with specific enzyme activity (U/mg) as response. Maximum specific activity (96.951 U/mg) was achieved at run number 14.

Run	Factor 1 Yeast Extract	Factor 2 $NaNO_3$	Factor 3 K_2HPO_4	Response 1 Specific Activity U/mg
1	0.55	0.45	0.12	18.2221
2	0.40	0.45	0.12	44.6181
3	0.48	0.25	0.10	36.779
4	0.35	0.38	0.10	59.9502
5	0.48	0.38	0.10	50.8362
6	0.48	0.38	0.10	51.1896
7	0.48	0.38	0.10	50.8598
8	0.40	0.30	0.12	71.393
9	0.60	0.38	0.10	71.1673
10	0.48	0.38	0.10	25.5266
11	0.55	0.45	0.08	57.9472
12	0.40	0.45	0.08	22.8962
13	0.48	0.38	0.10	51.9403
14	0.48	0.38	0.13	96.951
15	0.48	0.38	0.10	52.346
16	0.55	0.30	0.12	47.8488
17	0.55	0.30	0.08	35.7254
18	0.48	0.50	0.07	10.321
19	0.40	0.30	0.08	9.8682
20	0.48	0.38	0.07	76.4322

Table: 4.6: ANNOVA for Central Composite design

Source	**Sum of Squares**	**Df**	**Mean square**	**F value**	**p-value prob>F**
Model	7702.72	9	855.86	4.16	0.0183 significant
A-Yeast extract	65.21	1	65.21	0.32	0.5858
B-Sodium nitrate	315.67	1	315.67	1.53	0.2438
C-K_2HPO_4	595.26	1	595.26	2.89	0.1198
AB	5.01	1	5.01	0.024	0.8791
AC	1535.64	1	1535.64	7.46	0.0211
BC	1050.23	1	1050.23	5.10	0.0474

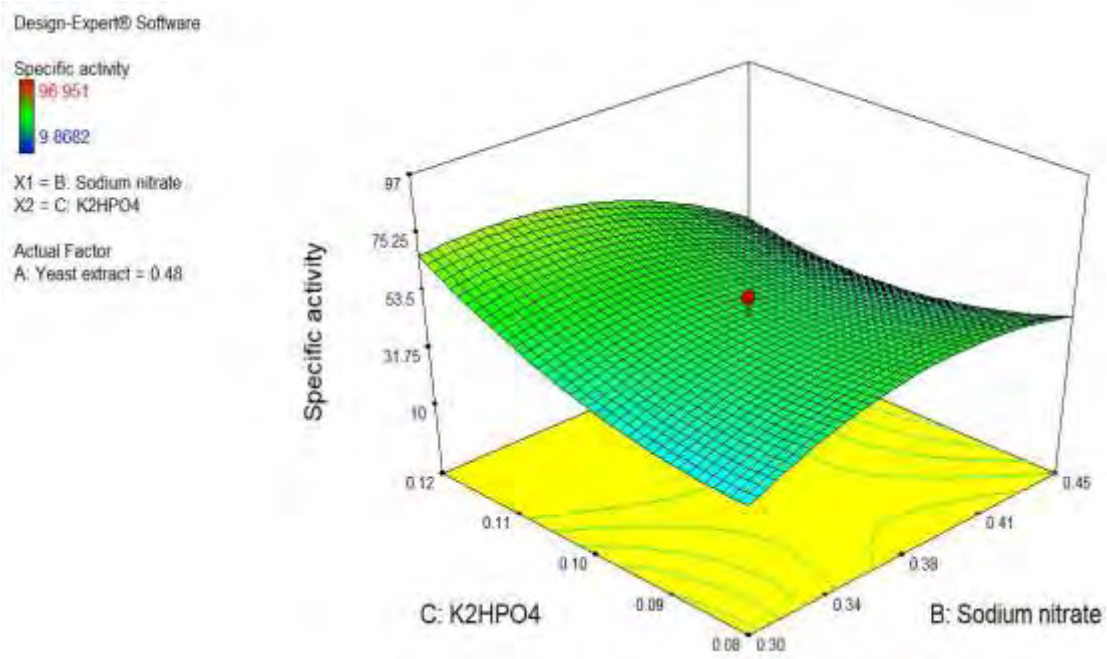

Fig 4.14: Three-dimensional response surface plot for maximum specific activity showing the interaction of the K_2HPO_4 and $NaNO_3$. (B) Design of contour between $K_2HPO_4$and $NaNO_3$ in terms of specific activity (U/mg).

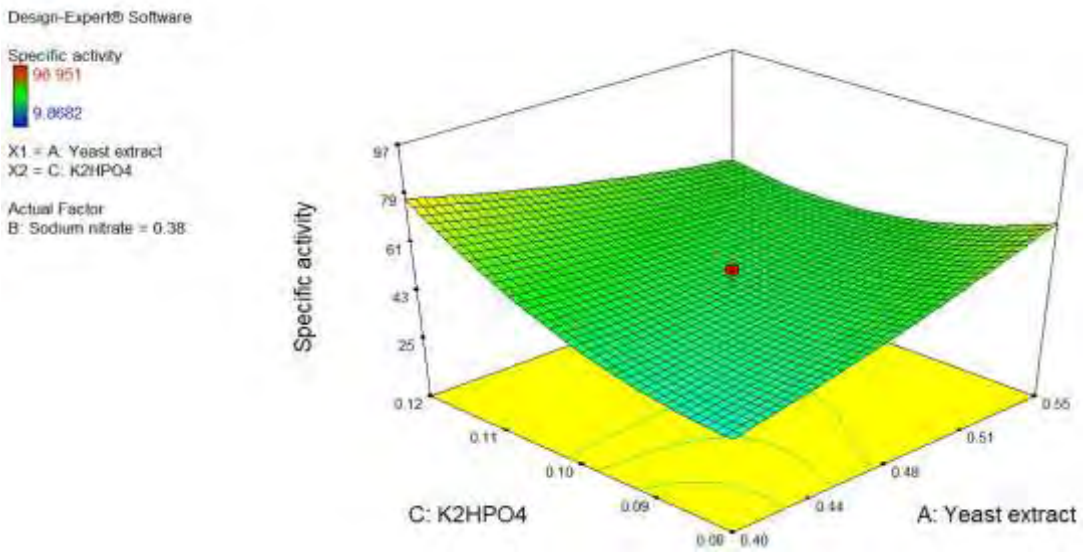

Fig 4.15: Three-dimensional response surface plot for maximum specific activity showing the interaction of the K_2HPO_4 and Yeast extract. (B) Design of contour between K_2HPO_4 and Yeast extract in terms of specific activity (U/mg).

4.13 Purification of Cutinase from *Bacillus subtilis* CS7

4.13.1 Production of cutinase at optimum condition

Cutinase production was done in shake flask at 35°C and pH 8.0 using the production media (500 ml) optimized above. Composition of production media was as follows g/L: glucose 3g, $(NH_4)_2SO_4$ 5.5g, peptone 5g, $NaNO_3$ 2g, K_2HPO_4 2.5g, Yeast Extract 3g, $MgSO_4.7H_2O$ 0.5g, KCl 0.5g, $FeSO_4.7H_2O$ 0.1g, cutin 4g and NaH_2OH 4g. The flask was placed in a shaker incubator for a duration of 48 hours, during which samples were collected at 24-hour intervals for both protein estimation and enzyme assay.

4.13.2 Precipitation with ammonium sulfate

Different concentrations of ammonium sulfate were dissolved in the cell-free supernatant to determine the optimal conditions for protein precipitation. The highest cutinase activity was found in the precipitate at a concentration of 70%. An increase in ammonium sulfate addition led to a reduction in enzyme activity, as well as concentrations below 70%. Maximum precipitate was obtained at a concentration of 70%, and enzyme specific activity was 114.598 U/mg for targeted cutinase extraction (Fig. 4.16)

4.13.3 Protein purification by column chromatography (Sephadex G-100)

Following dialysis, the semi-purified cutinase were further subjected for additional purification using a column chromatography technique with Sephadex G-100 gel resin. This process separated the proteins according to their molecular sizes. Around 30 fractions were collected. Each fraction contains 3 mL and is collected after every 10 mins. The total protein content in each fraction was analyzed at a wavelength of 280 nm, while the cutinase activity was assayed at 410 nm. Maximum activity was achieved in fraction number 17 (56.61 U/ml). These fractions showing maximum cutinase activity were combined to concentrate the cutinase for maximum activity (Fig. 4.17).

4.13.4 Total yield of purified Cutinase from *Bacillus subtilis* CS7

The total yield and purification fold for *Bacillus subtilis* CS7 cutinase that was precipitated, and gel filtered were determined. Purification fold was 6.10 percent and total yield was 38.63 percent, respectively. The purification fold and yield are given in the (Table 4.7).

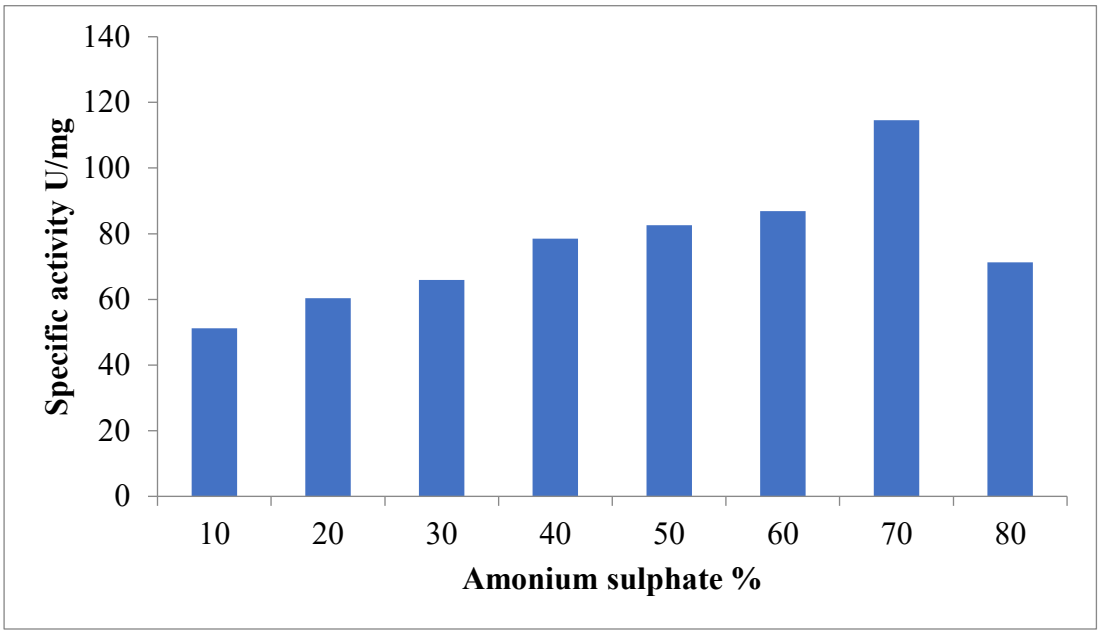

Fig. 4.16: *Bacillus subtilis* CS7 crude cutinase precipitated with ammonium sulphate and evaluation of specific activities of pellet at various percentages of ammonium sulfate precipitation.

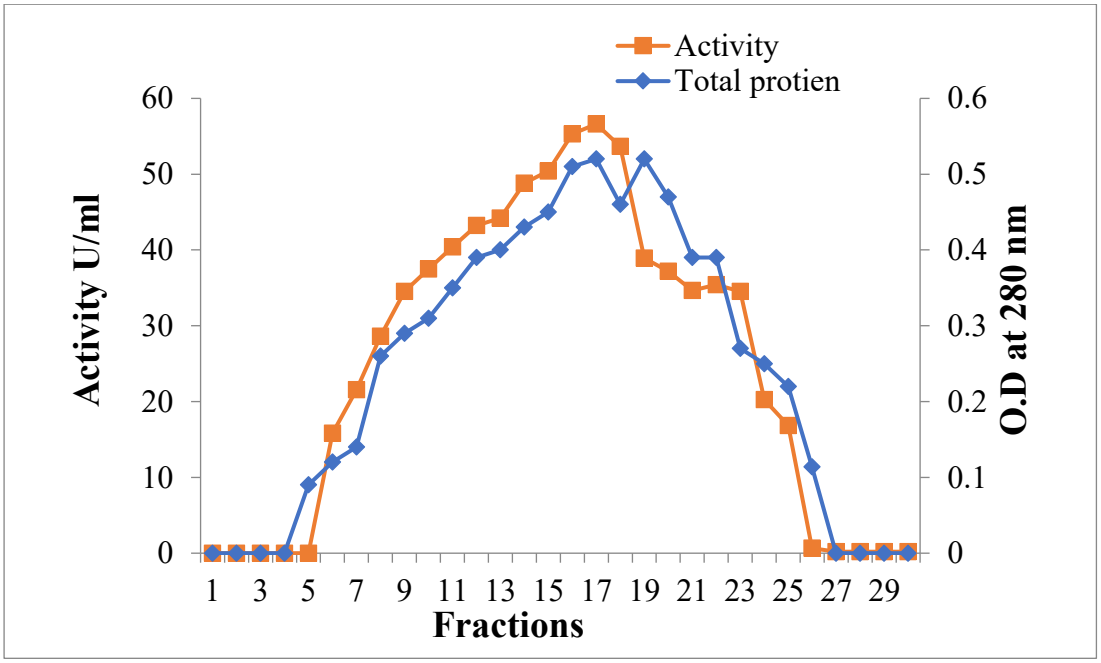

Fig 4.17: Total protein and specific activity profile of ammonium sulfate precipitated. Purification steps of cutinase from bacteria *Bacillus subtilis* CS7 through Sephadex G-100 gel column.

Table 4.7: Purification steps of cutinase from *Bacillus subtilis* CS7

Purification Steps	Total Activity U/ml	Protein mg/ml	SA U/mg	Yield %	Purification Fold
Crude Extract	352.6938	0.28	1.24219	100	1
(NH4)2SO4	170.6186	0.43	1.47203	48.3758	1.18503
Sephadex (G 100)	136.2474	0.14	7.58510	38.6305	6.1062

4.14 Characterization of purified Cutinase

The enzyme activity of purified cutinase from the bacterial strain was investigated for a variety of parameters that influence enzyme activity.

4.14.1 Effect of temperature on activity of Cutinase from bacterial strain

The effect of temperature on the activity of purified cutinase from *Bacillus subtilis* CS7 was studied by incubating the enzyme with para-nitro phenyl butyrate at various temperatures including 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C, each for a duration of 1 hour at 40°C, the enzyme preserved its peak residual activity (100%), while at 35°C, it retained more than 80% of its activity. Enzyme activity was observed to be at its lowest in the temperature range of 40°C to 80°C. (Fig 4.18).

4.14.2 Temperature stability profile Cutinase from bacterial strain

The temperature stability of purified cutinase from *Bacillus subtilis* CS7 was assessed by incubating enzymes without para-nitro phenyl butyrate at various temperatures, including 30°C, 40°C, 50°C, 60°C, 70°C, and 80°C, for a duration of 150 minutes. 100% stability was achieved at 40°C throughout the 150-minute period. At 30°C for 150 minutes, the enzyme maintained more than 70% of its stability. Temperatures between 50 and 60 °C for 150 minutes, cutinase stability was reduced by more than half (Fig 4.19).

4.14.3 Effect of pH on activity of Cutinase from bacterial strain

The effect of pH on the activity of cutinase from *Bacillus subtilis* CS7 was examined by incubating the enzyme at different pH levels, including 3, 4, 5, 6, 7, 8, 9, and 10,

while maintaining the optimal temperature of 40°C for these experiments. 100% activity was observed at pH 8.0. It was observed that the activity of enzymes reduced at pH levels above and below 8.0. (Fig 4.20).

4.14.4. pH stability profile Cutinase from bacterial strain

The effect of pH on the action of purified cutinase from *Bacillus subtilis* CS7 was examined by incubating it without para-nitro phenyl butyrate at various pH 3, 4, 5, 6, 7, 8, 9, and 10 At pH 8.0, enzyme stability was maintained at 100% for 150 min. At a pH of 7.0, more than 80% of the stability was maintained. After being exposed to pH 6.0 and below, cutinase stability was reduced significantly (Fig 4.21).

4.14.5 Effect of metals on *Bacillus subtilis* CS7 for Cutinase activity

The effect of different metal ions on *Bacillus subtilis* CS7 cutinase was investigated at concentrations of 5 mM and 10 mM. $HgSO_4$ and Pb $(NO_3)_2$ had a strong inhibitory effect at both concentrations 5mM and 10mM, while $CaCl_2$, $CuSo_4$, $NiSo_4$ and $MnSO_4$ had enhanced cutinase activity at both concentration of 5mM and 10 mM (Fig. 4.22).

4.14.6 Effect of surfactants on Cutinase activity

Cutinase from *Bacillus subtilis* CS7 was incubated with different surfactants at concentrations of 1% and 10% such as Tween 20, Tween 80, CTAB, SDS, and Triton-X100. It was observed that SDS and Triton-X 100 reduced the activity of purified enzymes at both concentrations. SDS completely inhibits the activity of enzymes at both concentrations whereas Triton X decreased 10% enzyme activity at 1% and 40% inhibition was observed at 10% concentration. Tween 80 increases approximately 46% of the cutinase activity at both concentrations (1.00% and 10.0%) (Fig 4.23).

4.14.7 Effect of organic solvent on Cutinase

The effect of different organic solvents at a final concentration of 10% on the activity of the purified enzyme was studied over a duration of 150 minutes. The effect of organic solvents on the activity of cutinase from *Bacillus subtilis* CS7 was determined by incubating it with ethanol, methanol, acetonitrile, acetone, n-hexane, ethyl acetate, propanol, DMSO, glycerol. Cutinase activity has been inhibited by acetonitrile, ethyl acetate, acetone, and propanol. Ethyle acetate decreased the activity of purified cutinase by 36% after120 min. Ethanol have been no effect on cutinase activity and retained 97% activity for 120 min. A significant increase in the activity of enzyme was observed with DMSO and retained 121% activity for 120 min (Fig 4.24).

4.15 Kinetic parameter of Cutinases from *Bacillus subtilis* CS7

Lineweaver and Burk (1934) plot was used to measure the *Km* and *Vmax* values for strain *Bacillus subtilis* CS7. The specific activity of *Bacillus subtilis* CS7 cutinase was measured after it was incubated with various substrate concentrations (1mM to 20Mm) The *Km* of strain *Bacillus subtilis* CS7 cutinase was recorded 2.87 mg/ml. Respectively, *Vmax* of strain CS7 cutinase was recorded 64.102 µmolmg-1min-1 for cutinase (Fig 4.25).

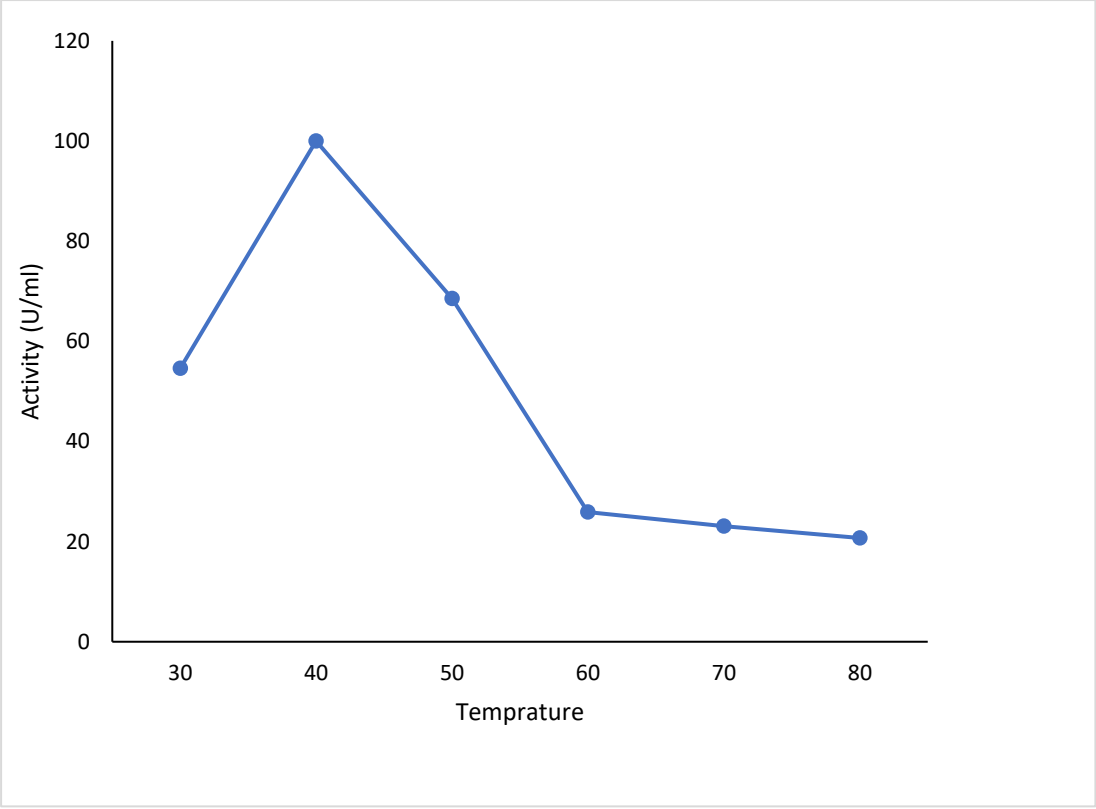

Fig 4.18: Effect of temperature on purified cutinase from *Bacillus subtilis* CS7 showed maximum activity (100 %) at 40°C.

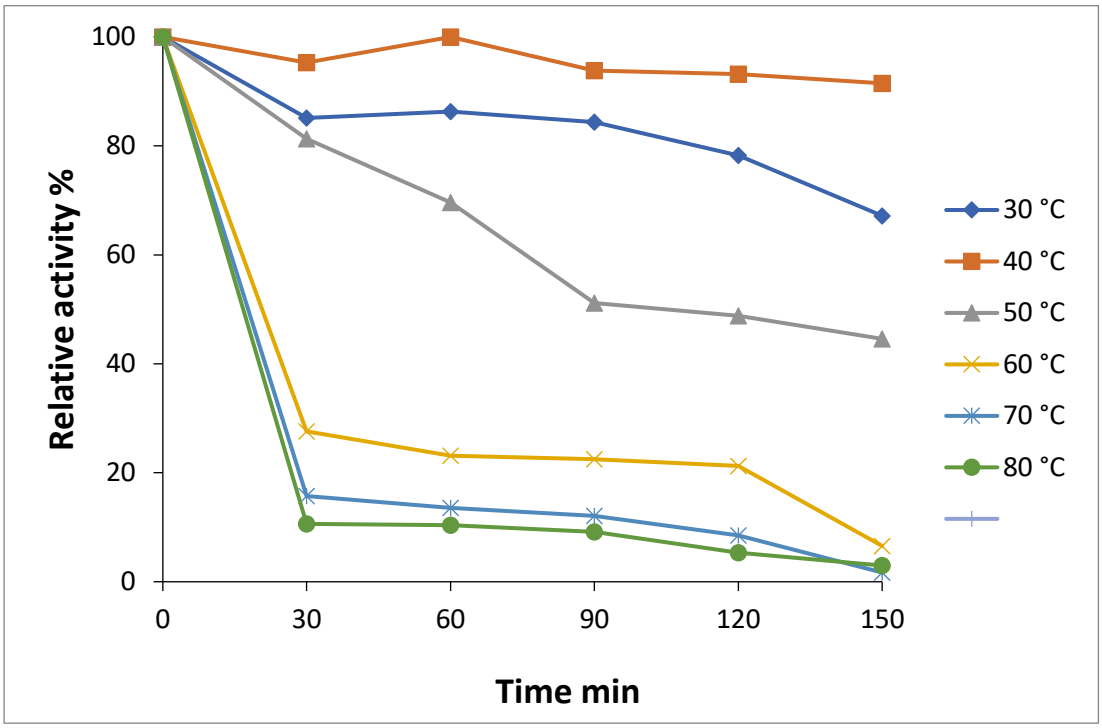

Fig 4.19: Effect of temperature on stability of purified cutinase from *Bacillus subtilis* CS7

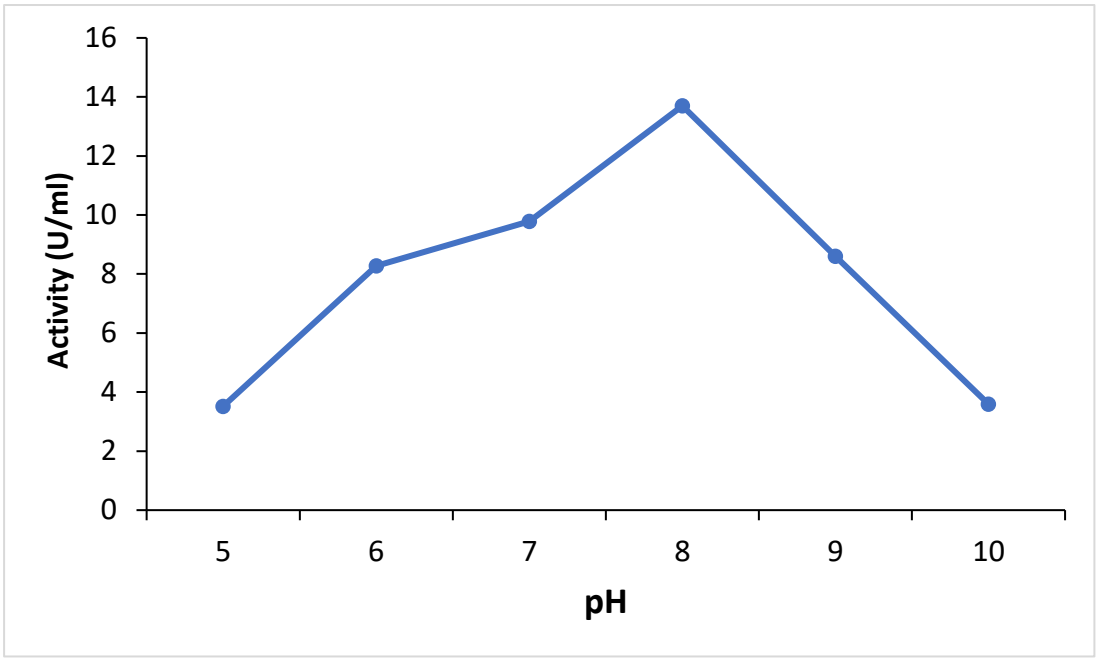

Fig 4.20: Effect of pH on purified cutinase from *Bacillus subtilis* CS7 showed maximum activity (100 %) at pH 8.0.

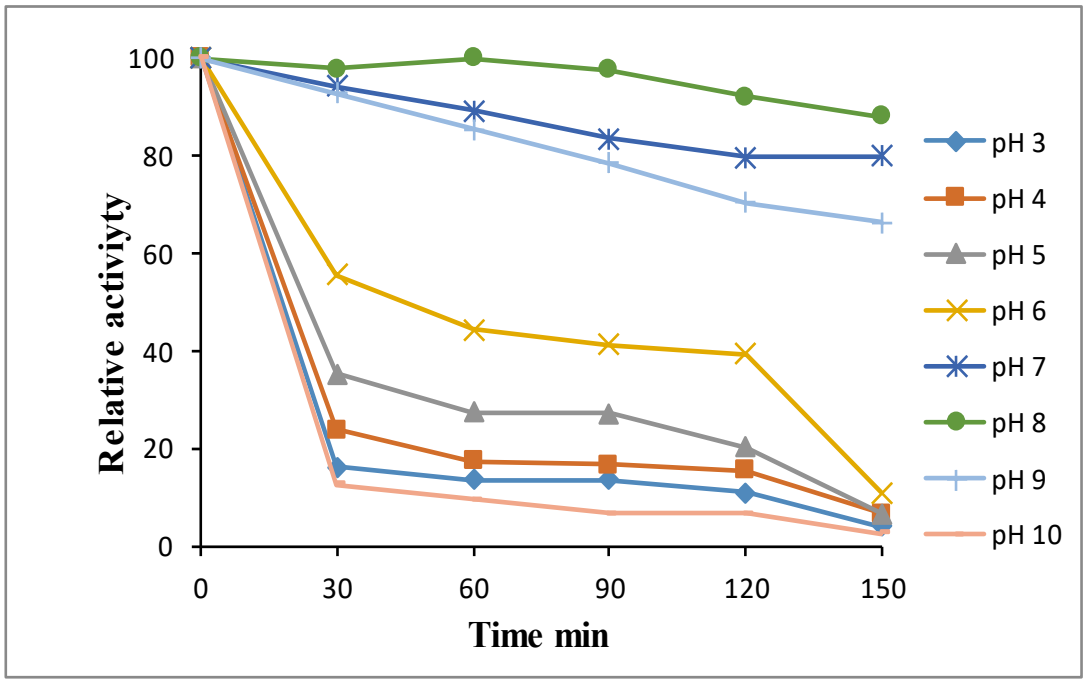

Fig 4.21: Effect of pH on stability of purified cutinase from bacterial strain *Bacillus subtilis* CS7.

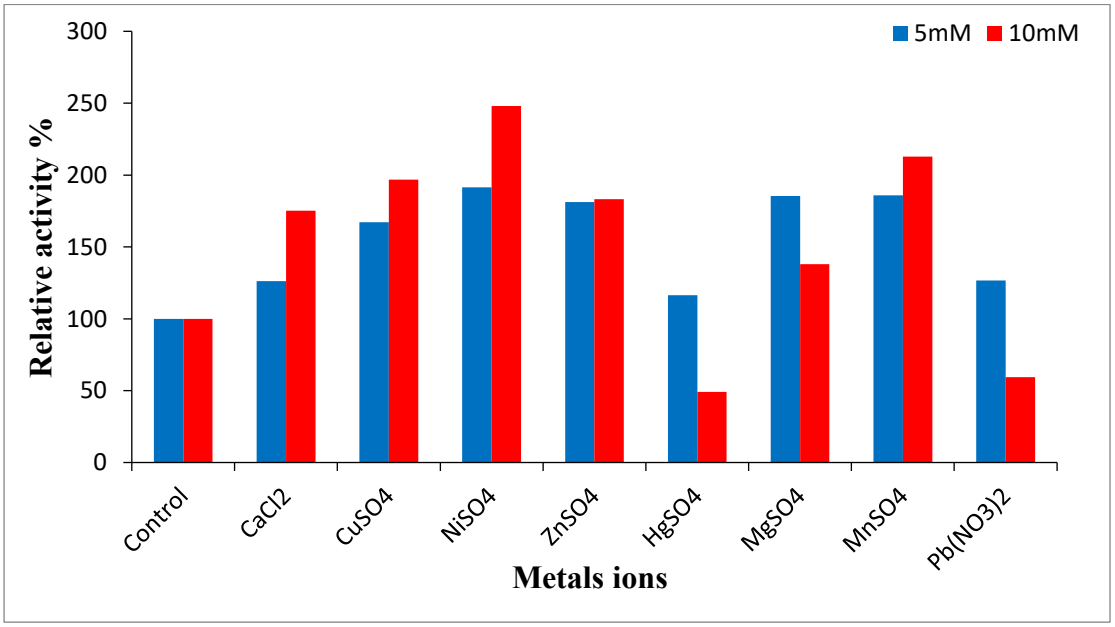

Fig 4.22: Effect of different metals ion on purified cutinase from bacterial *Bacillus subtilis* CS7.

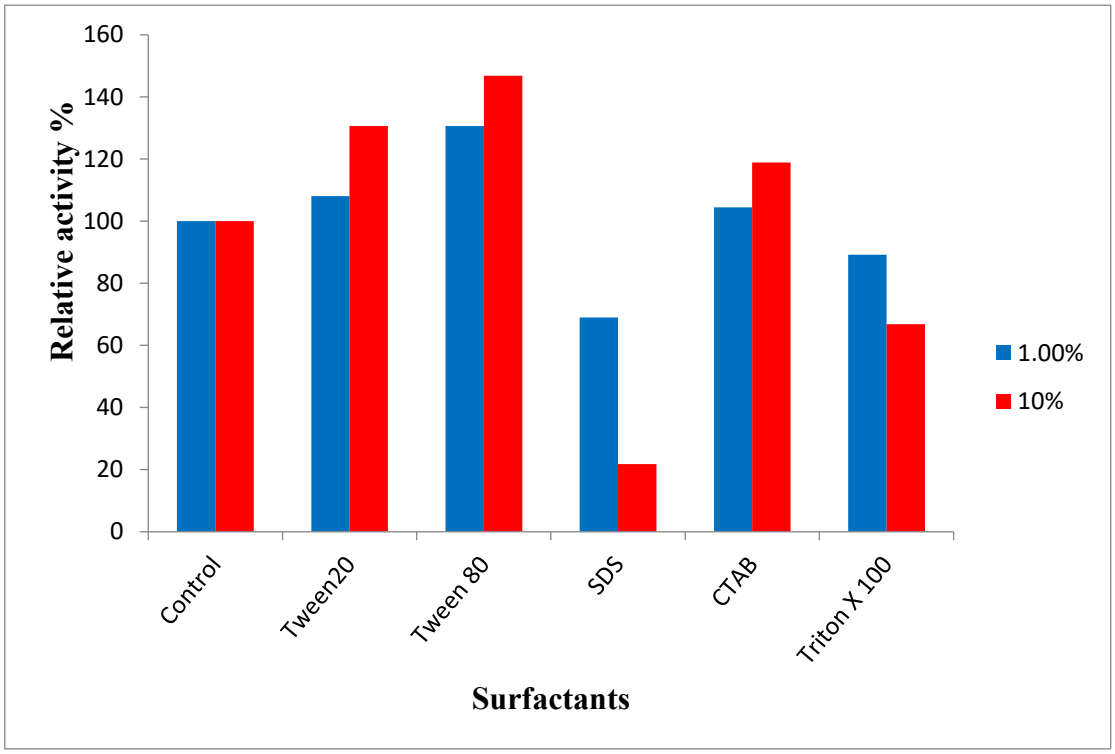

Fig 4.23: Effect of different surfactants on purified cutinase from *Bacillus subtilis* CS7.

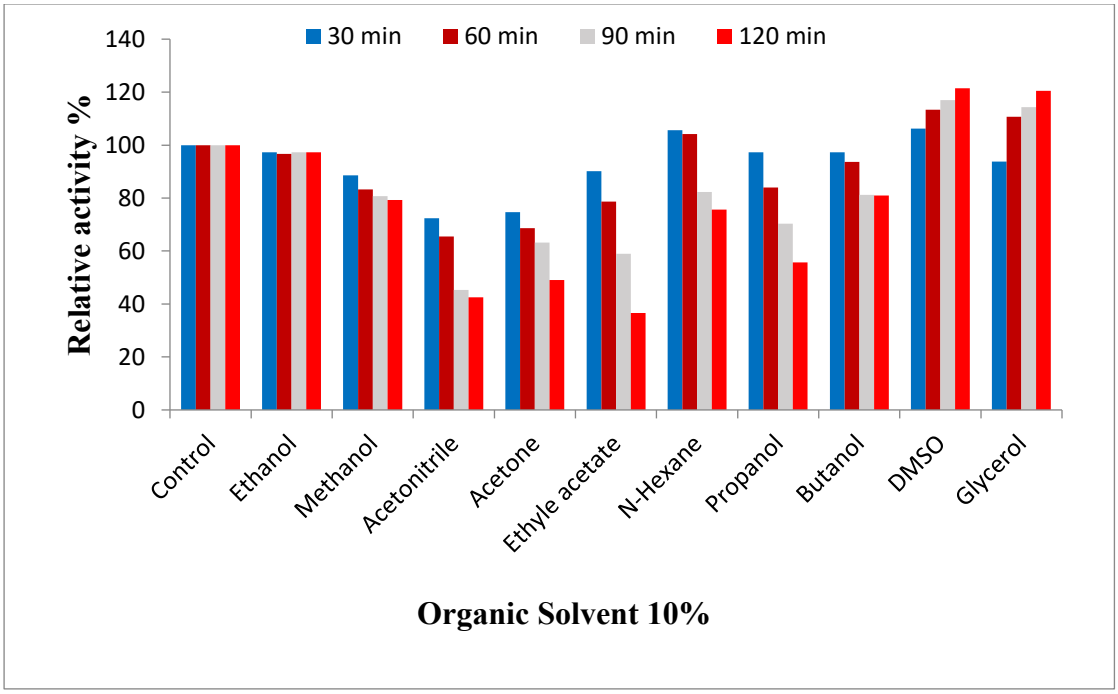

Fig 4.24: Effect of different organic solvents on purified cutinase from *Bacillus subtilis* CS7.

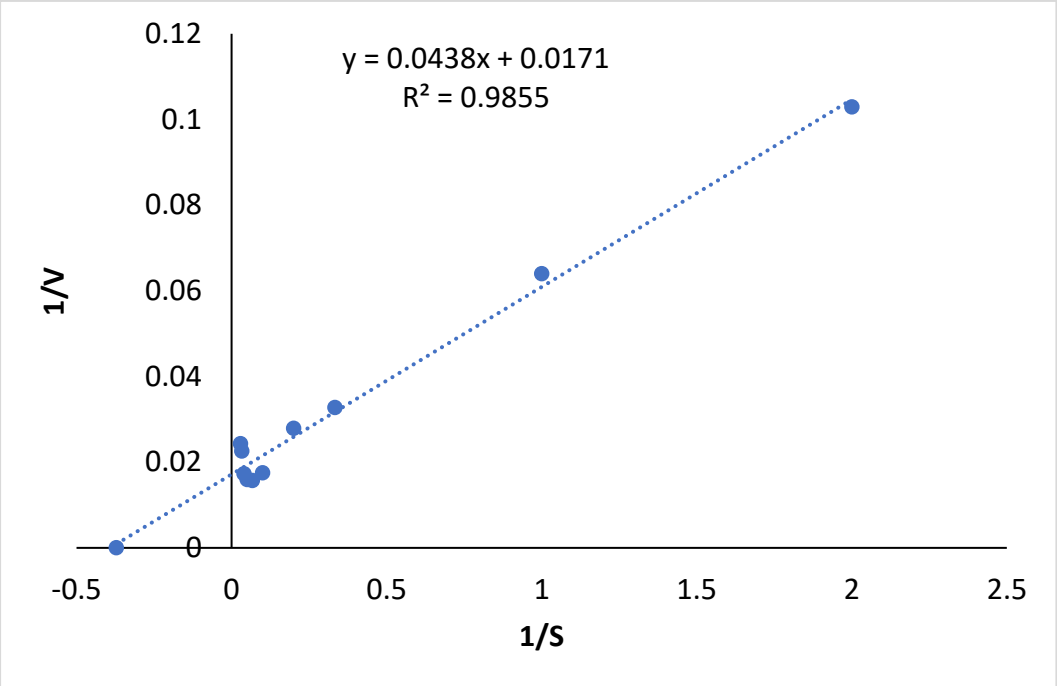

Fig 4.25: Kinetics analysis of Cutinase from *Bacillus subtilis* CS7 (*Km* and *Vmax*) value were observed using Lineweaver-Burk plot.

Discussion

Since the early 1990s, the study of synthetic polymer biodegradation has acquired significant attention due to widespread concern about the global environmental crisis. The consumption and manufacturing of biodegradable polymers have steadily increased; however, they still represent a small percentage of the overall plastic production. Evaluating the impact of plastic waste, particularly microplastics, on various ecosystem levels is crucial. This includes understanding their harmful effects on marine, freshwater, and terrestrial life (Souza Machado et al., 2018). Research has revealed alarming statistics, indicating that our oceans currently hold 5 trillion pieces of plastic, with a total weight reaching up to 0.25 million tons. Moreover, various aquatic habitats have been identified as particularly abundant in plastic debris (Cordova, 2020). This synthetic pollutant is present not only in marine sediments but also in some of the world's most remote and largest freshwater reservoirs (Pokazeev et al., 2021).

Polyethylene terephthalate (PET), one of the most prevalent, extensive, and essential synthetic polymers. The excessive use of synthetic PET has given rise to various health and environmental concerns (Mohanan et al., 2020). In addressing the issues posed by PET, the enzymatic breakdown of this synthetic polymer appears to be a feasible and viable solution. To degrade PET, enzymes like α- and β-hydrolases, including cutinases and related enzymes classified under EC3.1.1, are active. However, only a few of these enzymes have been extensively studied to date.

It is necessary not only to concentrate on the discovery of potential new bacterial strains capable of hydrolyzing PET but also to investigate the efficient and effective enzymatic mechanisms. This includes comprehensive studies on their characterization and other critical factors that contribute to the optimal production of the enzyme (Samak et al., 2020).

In this research, the strain Bacillus subtilis, which had been isolated and identified earlier and is registered under the accession number KY432689, was utilized for the purification and characterization of a PET-degrading cutinase. In this research,

microbial cultures were cultured for four weeks in minimal salt media (MSM) to isolate and enrich plastic-degrading microbes, a method detailed by (Hadar & Sivan, 2004). This four-week experiment was conducted to observe and study the degradation of PET pieces, as well as to assess the growth and survival of microbes throughout this 4-week experiment. The bacteria obtained from the selective enrichment experiment indicated an enhanced ability to degrade PET pieces and showed better survival and acclimation to PET degradation compared to bacteria found in the environment. In this study, PET served as the substrate for bacterial growth, degradation, and biofilm production. It was the sole carbon source available to the bacteria in the Minimal Salt Media (MSM).

The control results indicated that no bacteria were recovered after a specific time in the Minimal Salt Media (MSM). However, in MSM with PET as a carbon source, there was observable growth of PET-degrading bacterial strains. The PET-degrading bacterial strains altered the surface's physio-chemical characteristics and contributed to the weight reduction of the PET piece. Furthermore, these bacterial strains demonstrated the capacity to form biofilm. The assessment and measurement of weight loss proved is very simple and rapid technique for evaluating the breakdown of PET pieces by these bacterial strains. The phenomenon where microbes growing on polymers adhere to them results in an initial gain in weight. However, as the polymer structure deteriorates, there is a subsequent loss in weight. This weight reduction of the plastic pieces is proportional to the surface area of the polymer surface because of the degradation occurring at the surface (Chamas et al., 2020). In our study, Bacillus subtilis exhibited exponential growth and degradation capabilities when cultured in Minimal Salt Media (MSM) with PET as the only carbon source.

Additionally, the media facilitated the ability of the bacterial strain to induce weight loss in the PET pieces. The bacterial strain demonstrated a significant percentage (%) degradation of the initial dry weight of PET films within just 28 days of incubation. No degradation of PET pieces was exhibited by bacteria in control flask. The evaluation and determination of results, it was shown that *Bacillus subtilis* CS7 had an increased degradation rate as compared to the previous study reported by (Shah et al., 2009) and (Hadar & Sivan, 2004) for PET degradation from 3.5% to 8.4%.

The primary material used in the manufacturing of water and soft drink bottles is PET, which is designed for strong mechanical and chemical stability, as well as resistance to microbial factors. This design feature also prevents microbes, causing them to favor other surfaces that are more nutrient-rich. Microbes can degrade PET by forming biofilms on its surface. This process enables them to penetrate and effectively utilize the non-soluble substrate (Jasu & Ray, 2021). Therefore, the unexplored function of the biofilm-forming capability of bacterial strains and their capacity to degrade plastic can be estimated (Nava & Leoni, 2021). Therefore, our study focuses on the potential and ability of the bacterial strain to efficiently degrade PET and form biofilms on its surface. The assessment of biofilm formation by bacterial strain was conducted using a microtiter plate assay, a method previously employed by (Satti et al., 2017) on plastic film surfaces. In the microtiter plate assay, a biofilm-producing strain, Pseudomonas aeruginosa, served as the positive control. The analysis of absorbance at 600nm revealed values of 2.17 for the positive control and 0.1 for the negative control. On the surface of PET, the biofilm formed by bacterial strain exhibited an absorbance of 2.7 at 600nm. Following treatment with 30% acetic acid, the absorbance remained similar at 0.504. The analysis of the results indicated that bacterial strain *Bacillus subtilis* CS7 has the capability to adhere to the surface of PET and form a biofilm. This biofilm-forming potential is a key factor that could significantly contribute to the process of PET degradation.

BATH assay was conducted to determine bacterial hydrophobicity, to evaluate the bacterial ability for an affinity towards organic hydrocarbons such as hexadecane. The greater the hydrophobicity of the bacterial cells, the stronger their affinity for hydrocarbons. This affinity leads to the bacteria transitioning from a suspended state in the aqueous phase to the organic phase. As a result, there is a decrease in the turbidity of the culture (Saini, 2010). The bacterial strain demonstrated high hydrophobicity, as evidenced by its significant adherence to hexadecane even at low concentrations, amounting to 0.32ml. This adherence led to a 15% reduction in the turbidity of the culture during the logarithmic phase. Therefore, this confirms the hydrophobic nature of bacterial strain *Bacillus subtilis* CS7. since the hydrophobic nature of PET, the hydrophobic-hydrophobic interaction plays a crucial role in facilitating the adhesion of

bacteria to the PET surface. This adherence is a supportive factor in the degradation process of PET pieces. The decrease in turbidity observed in the bacterial suspension, measured at 600nm optical density, indicates the migration of hydrophobic cells from the aqueous to the organic phase. This test served as evidence of the bacterial strain capability to adhere to polymers.

Evaluating structural changes is vital for identifying how the structure is altered during weight loss caused by deterioration. The degradation of PET pieces was further confirmed by Fourier-Transform Infrared Spectroscopy (FT-IR).

The FTIR (Fourier-Transform Infrared Spectroscopy) analysis revealed that after treatment with the bacterial strain, the PET piece experienced structural changes as compared to the control. This analysis indicated that the PET piece experienced degradation at multiple positions. The study then progressed to concentrate on the optimization, production, purification, and characterization of Cutinase derived from *Bacillus subtilis* CS7. Cutinase, being an extracellular enzyme, is significantly influenced by various physicochemical and biochemical parameters such as pH, temperature, carbon source, incubation period, inorganic salts, and dissolved oxygen concentration (Altammar et al., 2022).

After a period of 48 hours at a temperature of 40°C and a pH of 8.0, the maximum production of Cutinase was observed. In the preceding analysis, similar optimal parameters for effective Cutinase production were identified (Kumari et al., 2016). To optimize the remaining parameters for effective Cutinase production, the Plackett-Burman and Central Composite Design methods were employed. These software tools have been previously demonstrated in research to effectively design medium compositions conducive to Cutinase production. This approach allows for a systematic and efficient optimization of various factors influencing enzyme production (Dutta et al., 2013, 2013; Mazzucotelli et al., 2016).

The maximum activity of cutinase, as determined by the design optimized using the Plackett-Burman method, was recorded in run number 9, showing an activity of

18.0015 U/mg and the lowest cutinase activity, which was 9.72275 U/mg, was observed in run number 7. This variation in activity levels across different runs highlights the effectiveness of the Plackett-Burman design in identifying optimal conditions for enzyme production. A positive value indicates that the factor has a greater effect on a particular activity when present in high concentrations. Conversely, a negative value suggests that the factor's impact diminishes at lower concentrations. a positive factor indicates that the specific activity of a substance, such as an enzyme, increases with higher concentrations of that factor. On the other hand, a negative value suggests that the factor has a lesser impact on the specific activity at lower concentrations This differentiation helps in understanding how various factors influence the enzyme's activity and efficiency under different conditions. The influence of factors identified in the Plackett-Burman design on cutinase production was further elaborated by Pareto chart. This chart highlighted that three specific factors – yeast extract, NaNO3 (Sodium Nitrate), and K_2HPO_4 had a significant effect on the production of cutinase. The significance of these factors was indicated by their respective "Prob > F" values, which statistically demonstrate the possibility of each factor influencing cutinase production. This analysis is crucial for understanding which components most effectively lead the enzyme's production process.

While quantitative analysis is essential for obtaining high-quality, pure enzymes, industrial applications often do not prioritize purity due to economic reasons. Despite this, the removal of unwanted proteins is still necessary. This is crucial not only for maintaining the efficiency and specificity of the enzyme but also for ensuring the safety and stability of industrial processes where these enzymes are utilized. The cutinase produced by bacillus subtilis was effectively precipitated using a 70% ammonium sulfate concentration. Following this precipitation step, the enzyme was further purified using column chromatography, specifically employing Sephadex G-100, Bacillus subtilis was purified, which increased the specific activity from 1.242 U/mg to 7.585 U/mg with a purification yield of 38.6305 % and purification fold of 6.1062. This method of chromatography is often used for the separation of desired enzymes from other proteins and impurities, thereby enhancing the enzyme's purity and quality for use in various applications.

The purified cutinase obtained from Bacillus subtilis experienced characterization over a range of temperatures, from 30°C to 60°C. The results indicated that the optimal temperature for enzyme's activity is 40°C, and it functions most effectively at a pH of 8.0. At higher pH and temperatures the rate of reaction started to slow down.The purified cutinase extract from *Bacillus subtilis* CS7 was subjected to tests involving various metals, surfactants, and organic solvents to investigate its stability and reactivity under different conditions. The isolated Cutinase enzyme from Bacillus subtilis demonstrated complete stability when maintained at 40°C for 120 minutes. Reports indicate that enzymes from Thermobifida fusca , T. vulgaris , and Bacillus subtilis strains exhibit thermostability within a temperature range of 20 to 60°C and a pH range of 8.0 to 11.0 (Adıgüzel & Tunçer, 2017; Fett et al., 2000; Gomez Del Pulgar & Saadeddin, 2014). These parameters are closely like the results obtained in our research, indicating a comparable level of thermostability in the Cutinase enzyme from *Bacillus subtilis* CS7.

The production of Cutinase by *Bacillus subtilis* CS7 was positively affected by the presence of various metals. These included $CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$, $ZnSO_4$, $NiSO_4$, and $MnSO_4$, as noted in observed and recorded findings. The activity of Cutinase was inhibited by $HgSO_4$ and Pb $(NO3)_2$ at both low (5mM) and high (10mM) concentrations. This inhibition is attributed to the interaction between the tryptophan amino acid and the Cutinase binding site by (Sulaiman, 2014). The enzyme exhibited stability due to the presence of metals that act as co-factors, particularly with $MnSO_4$ and $NiSO_4$. The relative activity was 212.938 and 247.978, respectively, under these conditions respectively (Araújo et al., 2007) and (Speranza et al., 2011).

Cutinase from *Bacillus subtilis* CS7 showed remarkable stability in the presence of most surfactants. Specifically, when incubated with Tween 20, Tween 80, and CTAB, Triton X 100 the enzyme maintained its maximum activity. Enzyme activity was inhibited by both SDS and Triton X 100 at concentrations of 1 and 10 percent. These findings are consistent with the results reported in the studies conducted by, (Chen et al., 2010) and (Adıgüzel & Tunçer, 2017) aligning with our own research results.

Maximum activity by Cutinase was observed when incubated for 120 minutes in a variety of organic solvents, including ethanol, methanol, ethyl acetate, DMSO, glycerol, butanol, and n-Hexane. However, its activity was inhibited in the presence of butanol, acetonitrile, DMSO, acetone, and propanol. Similar results were reported by (Hasnaoui et al., 2022).

Bacillus subtilis CS7 cutinase had the maximum specific activity (7.58510) and Km value (2.87 mg/ml), which is almost identical to Thermobifida fusca's Km value (Barth et al., 2016). The cutinases developed Michaelis–Menten kinetics, with the maximum affinity for pNPB, according to the findings. Chen et al. previously described similar kinetic activity for T. fusca cutinase under various conditions.

This research is significant because identifying all the optimal conditions greatly effects the production of the cutinase enzyme from Bacillus subtilis. Once optimized and purified on a large scale, this enzyme has the potential for diverse applications across various fields. The confirmation of PET-hydrolyzing ability in the cutinase produced by *Bacillus subtilis* CS7, along with the identification of key factors in enzymes from PET-degrading strains, can be used for the development of innovative processes for PET waste recovery. Cutinase can be utilized to degrade plastics, including synthetic polyethylene terephthalate (PET), into water-soluble products.

Future prospective

- Over expression of cutinase by transformation of cutinase coding gene in some more appropriate host like E. coli.
- Search for the detection of more potent PET degrading microorganisms to solve various side effect issues.
- In addition to assessing their enzymatic potential, cutinases could also be examined for their efficacy in recycling amorphous PET.
- To speed up PET biodegradation, it is essential to assess high-quality, well-organized degradation processes based on microorganisms that degrade PET.
- Emphasizing research on PET degradation and hydrolysis is essential, as it can lead to the discovery of novel biocatalysts.

Conclusion

1. A total of 7 out of 26 bacterial isolates exhibited good growth after 28 days of incubation while utilizing PET as a sole carbon source.
2. Bacterial strain CS7 degraded PET piece with 2.8% reduction in weight loss after 28 days of incubation.
3. Bacterial strain CS7 was able to effectively colonize on the surface of PET in the form of biofilm formation.
4. Maximum production of cutinase by bacterial strain CS7 was achieved at temperature 35°C and pH 8.0.
5. Maximum production of cutinase by bacterial strain CS7 through Placket Burman design was achieved at run number 9.
6. Enhance production of cutinase by CS7 through central composite design was achieved at rum number 14.
7. Cutinase was purified using column chromatography after being produced in bulk at optimal conditions.
8. The purified cutinase was stable at temperature 40 ºC and pH 8.0.
9. Purified cutinase was found stable in the presence of various metal ions, surfactants, and organic solvent.

References

Adıgüzel, A. O., & Tunçer, M. (2017). Purification and characterization of cutinase from Bacillus sp. KY0701 isolated from plastic wastes. *Preparative Biochemistry and Biotechnology*, *47*(9), 925–933.

Ahmad, H., Chhipi-Shrestha, G., Hewage, K., & Sadiq, R. (2022). A Comprehensive Review on Construction Applications and Life Cycle Sustainability of Natural Fiber Biocomposites. *Sustainability*, *14*(23), Article 23. https://doi.org/10.3390/su142315905

Ahmaditabatabaei, S., Kyazze, G., Iqbal, H. M., & Keshavarz, T. (2021). Fungal enzymes as catalytic tools for polyethylene terephthalate (PET) degradation. *Journal of Fungi*, *7*(11), 931.

AlMaadeed, M. A. A., Ponnamma, D., & El-Samak, A. A. (2020). Chapter 1 - Polymers to improve the world and lifestyle: Physical, mechanical, and chemical needs. In M. A. A. AlMaadeed, D. Ponnamma, & M. A. Carignano (Eds.), *Polymer Science and Innovative Applications* (pp. 1–19). Elsevier. https://doi.org/10.1016/B978-0-12-816808-0.00001-9

Altammar, K. A., Ling, J. G., Al-Bajalan, H. M., Chin, I.-S., Mackeen, M. M., Mahadi, N. M., Murad, A. M. A., & Bakar, F. D. A. (2022). Characterization of AnCUT3, a plastic-degrading paucimannose cutinase from Aspergillus niger expressed in Pichia pastoris. *International Journal of Biological Macromolecules*, *222*, 2353–2367.

Alvarez, A., Saez, J. M., Davila Costa, J. S., Colin, V. L., Fuentes, M. S., Cuozzo, S. A., Benimeli, C. S., Polti, M. A., & Amoroso, M. J. (2017). Actinobacteria: Current research and perspectives for bioremediation of pesticides and heavy metals. *Chemosphere*, *166*, 41–62. https://doi.org/10.1016/j.chemosphere.2016.09.070

Araújo, R., Silva, C., O'Neill, A., Micaelo, N., Guebitz, G., Soares, C. M., Casal, M., & Cavaco-Paulo, A. (2007). Tailoring cutinase activity towards polyethylene terephthalate and polyamide 6, 6 fibers. *Journal of Biotechnology*, *128*(4), 849–857.

Arribas Arias, H. (2021). *Microorganismos de la plastisfera y la biodegradación del plástico por la microbiota digestiva de diferentes insectos*. https://gredos.usal.es/handle/10366/147486

Ashby, M. F., & Johnson, K. (2013). *Materials and design: The art and science of material selection in product design*. Butterworth-Heinemann. https://books.google.com/books?hl=en&lr=&id=lyDEACMTtUEC&oi=fnd&pg=PP2&dq=22.%09Ashby,+M.+F.,+%26+Johnson,+K.+(2013).+Materials+and+design:+the+art+and+science+of+material+selection+in+product+design.+Butterworth-Heinemann&ots=MUq4_8lR0u&sig=mswDkOokUmVaHI49YulQaEDDpEQ

Auta, H. S., Emenike, C. U., Jayanthi, B., & Fauziah, S. H. (2018). Growth kinetics and biodeterioration of polypropylene microplastics by Bacillus sp. And Rhodococcus

sp. Isolated from mangrove sediment. *Marine Pollution Bulletin*, *127*, 15–21. https://doi.org/10.1016/j.marpolbul.2017.11.036

Avendaño Toledo, C. A., & Castro Velazco, A. M. (2020a). *Determinación del pretratamiento más efectivo sobre el polietileno tereftalato para el aumento en la eficiencia del proceso de degradación realizado por hongos y bacterias autóctonas de lixiviado de relleno sanitario*. http://repository.unilibre.edu.co/handle/10901/18618

Avendaño Toledo, C. A., & Castro Velazco, A. M. (2020b). *Determinación del pretratamiento más efectivo sobre el polietileno tereftalato para el aumento en la eficiencia del proceso de degradación realizado por hongos y bacterias autóctonas de lixiviado de relleno sanitario*. http://repository.unilibre.edu.co/handle/10901/18618

Bano, K., Kuddus, M., R Zaheer, M., Zia, Q., F Khan, M., Md Ashraf, G., Gupta, A., & Aliev, G. (2017). Microbial enzymatic degradation of biodegradable plastics. *Current Pharmaceutical Biotechnology*, *18*(5), 429–440.

Barboza, L. G. A., Lopes, C., Oliveira, P., Bessa, F., Otero, V., Henriques, B., Raimundo, J., Caetano, M., Vale, C., & Guilhermino, L. (2020). Microplastics in wild fish from North East Atlantic Ocean and its potential for causing neurotoxic effects, lipid oxidative damage, and human health risks associated with ingestion exposure. *Science of the Total Environment*, *717*, 134625.

Benyathiar, P., Kumar, P., Carpenter, G., Brace, J., & Mishra, D. K. (2022). Polyethylene Terephthalate (PET) Bottle-to-Bottle Recycling for the Beverage Industry: A Review. *Polymers*, *14*(12), Article 12. https://doi.org/10.3390/polym14122366

Berselli, A., Ramos, M. J., & Menziani, M. C. (2021). Novel Pet-Degrading Enzymes: Structure-Function from a Computational Perspective. *ChemBioChem*, *22*(12), 2032–2050.

Berselli, A., Ramos, M. J., & Menziani, M. C. (2021). Novel Pet-Degrading Enzymes: Structure-Function from a Computational Perspective. *ChemBioChem*, *22*(12), 2032–2050.

Bharadwaj, A., Yadav, D., & Varshney, S. (2015). Non-biodegradable waste–its impact & safe disposal. *Int. J. Adv. Technol. Eng. Sci*, *3*(1). https://www.researchgate.net/profile/Alok-Bharadwaj-3/publication/301283637_NON-BIODEGRADABLE_WASTE_-_ITS_IMPACT_SAFE_DISPOSAL/links/570f12e508aed4bec6fdf29c/NON-BIODEGRADABLE-WASTE-ITS-IMPACT-SAFE-DISPOSAL.pdf

Bracco, P., Brunella, V., Trossarelli, L., Coda, A., & Botto-Micca, F. (2005). Comparison of polypropylene and polyethylene terephthalate (Dacron) meshes for abdominal wall hernia repair: A chemical and morphological study. *Hernia*, *9*(1), 51–55. https://doi.org/10.1007/s10029-004-0281-y

Bubpachat, T., Sombatsompop, N., & Prapagdee, B. (2018). Isolation and role of polylactic acid-degrading bacteria on degrading enzymes productions and PLA biodegradability at mesophilic conditions. *Polymer Degradation and Stability*, *152*, 75–85.

Carpenter, E., & Wolverton, S. (2017). Plastic litter in streams: The behavioral archaeology of a pervasive environmental problem. *Applied Geography*, *84*, 93–101.

Carr, C. M., Clarke, D. J., & Dobson, A. D. W. (2020). Microbial Polyethylene Terephthalate Hydrolases: Current and Future Perspectives. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.571265

Carr, C. M., Clarke, D. J., & Dobson, A. D. W. (2020). Microbial Polyethylene Terephthalate Hydrolases: Current and Future Perspectives. *Frontiers in Microbiology*, *11*. https://www.frontiersin.org/articles/10.3389/fmicb.2020.571265

Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J. H., Abu-Omar, M., Scott, S. L., & Suh, S. (2020). Degradation Rates of Plastics in the Environment. *ACS Sustainable Chemistry & Engineering*, *8*(9), 3494–3511. https://doi.org/10.1021/acssuschemeng.9b06635

Chamas, A., Moon, H., Zheng, J., Qiu, Y., Tabassum, T., Jang, J. H., Abu-Omar, M., Scott, S. L., & Suh, S. (2020). Degradation Rates of Plastics in the Environment. *ACS Sustainable Chemistry & Engineering*, *8*(9), 3494–3511. https://doi.org/10.1021/acssuschemeng.9b06635

Chen, G.-Q., & Patel, M. K. (2012). Plastics Derived from Biological Sources: Present and Future: A Technical and Environmental Review. *Chemical Reviews*, *112*(4), 2082–2099. https://doi.org/10.1021/cr200162d

Chen, S., Su, L., Billig, S., Zimmermann, W., Chen, J., & Wu, J. (2010). Biochemical characterization of the cutinases from Thermobifida fusca. *Journal of Molecular Catalysis B: Enzymatic*, *63*(3–4), 121–127.

Chu, J., Cai, Y., Li, C., Wang, X., Liu, Q., & He, M. (2021). Dynamic flows of polyethylene terephthalate (PET) plastic in China. *Waste Management*, *124*, 273–282.

Çınar, M. E., & Kar, F. (2018). Characterization of composite produced from waste PET and marble dust. *Construction and Building Materials*, *163*, 734–741.

Cole, M., & Galloway, T. S. (2015). Ingestion of Nanoplastics and Microplastics by Pacific Oyster Larvae. *Environmental Science & Technology*, *49*(24), 14625–14632. https://doi.org/10.1021/acs.est.5b04099

Cordova, M. R. (2020). Marine plastic debris: Distribution, abundance, and impact on our seafood. In *Handbook of Research on Environmental and Human Health Impacts of Plastic Pollution* (pp. 94–121). IGI Global. https://www.igi-global.com/chapter/marine-plastic-debris/233350

Cox, K. D., Covernton, G. A., Davies, H. L., Dower, J. F., Juanes, F., & Dudas, S. E. (2019). Human Consumption of Microplastics. *Environmental Science & Technology*, *53*(12), 7068–7074. https://doi.org/10.1021/acs.est.9b01517

da Costa, A. M., de Oliveira Lopes, V. R., Vidal, L., Nicaud, J.-M., de Castro, A. M., & Coelho, M. A. Z. (2020). Poly (ethylene terephthalate)(PET) degradation by Yarrowia lipolytica: Investigations on cell growth, enzyme production and monomers consumption. *Process Biochemistry*, *95*, 81–90.

Dąbrowska, G. B., Janczak, K., & Richert, A. (2021). Combined use of Bacillus strains and Miscanthus for accelerating biodegradation of poly (lactic acid) and poly (ethylene terephthalate). *PeerJ*, *9*, e10957.

Danso, D., Chow, J., & Streit, W. R. (2019). Plastics: Environmental and biotechnological perspectives on microbial degradation. *Applied and Environmental Microbiology*, *85*(19), e01095-19.

Danso, D., Chow, J., & Streit, W. R. (2019). Plastics: Environmental and biotechnological perspectives on microbial degradation. *Applied and Environmental Microbiology*, *85*(19), e01095-19.

de Castro, A. M., Carniel, A., Nicomedes Junior, J., da Conceição Gomes, A., & Valoni, É. (2017). Screening of commercial enzymes for poly (ethylene terephthalate)(PET) hydrolysis and synergy studies on different substrate sources. *Journal of Industrial Microbiology and Biotechnology*, *44*(6), 835–844.

de Queiros Eugenio, E., Campisano, I. S. P., Dias, A. G., de Castro, A. M., Coelho, M. A. Z., & Langone, M. A. P. (2022). Novel efficient enzymatic synthesis of the key-reaction intermediate of PET depolymerization, mono (2-hydroxyethyl terephthalate)–MHET. *Journal of Biotechnology*, *358*, 102–110.

de Souza Machado, A. A., Kloas, W., Zarfl, C., Hempel, S., & Rillig, M. C. (2018). Microplastics as an emerging threat to terrestrial ecosystems. *Global Change Biology*, *24*(4), 1405–1416.

Dehghani, S., Moore, F., & Akhbarizadeh, R. (2017). Microplastic pollution in deposited urban dust, Tehran metropolis, Iran. *Environmental Science and Pollution Research*, *24*(25), 20360–20371. https://doi.org/10.1007/s11356-017-9674-1

Demirel, B. (2017). Optimisation of mould surface temperature and bottle residence time in mould for the carbonated soft drink PET containers. *Polymer Testing*, *60*, 220–228.

Demirel, B., Yaraş, A., & Elcicek, H. (2011). *Crystallization behavior of PET materials*. http://acikerisim.bartin.edu.tr/handle/11772/1592

Dhawan, R., Bisht, B. M. S., Kumar, R., Kumari, S., & Dhawan, S. K. (2019). Recycling of plastic waste into tiles with reduced flammability and improved tensile strength. *Process Safety and Environmental Protection*, *124*, 299–307.

Diggle, A., & Walker, T. R. (2022). Environmental and Economic Impacts of Mismanaged Plastics and Measures for Mitigation. *Environments*, *9*(2), Article 2. https://doi.org/10.3390/environments9020015

Dimarogona, M., Nikolaivits, E., Kanelli, M., Christakopoulos, P., Sandgren, M., & Topakas, E. (2015). Structural and functional studies of a Fusarium oxysporum cutinase with polyethylene terephthalate modification potential. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1850*(11), 2308–2317.

Dissanayake, P. D., Kim, S., Sarkar, B., Oleszczuk, P., Sang, M. K., Haque, M. N., Ahn, J. H., Bank, M. S., & Ok, Y. S. (2022). Effects of microplastics on the terrestrial environment: A critical review. *Environmental Research*, *209*, 112734. https://doi.org/10.1016/j.envres.2022.112734

Dombre, C., Marais, S., Chappey, C., Lixon-Buquet, C., & Chalier, P. (2014). The behaviour of wine aroma compounds related to structure and barrier properties of virgin, recycled and active PET membranes. *Journal of Membrane Science*, *463*, 215–225.

Durgashyam, K., Reddy, M. I., Balakrishna, A., & Satyanarayana, K. (2019). Experimental investigation on mechanical properties of PETG material processed by fused deposition modeling method. *Materials Today: Proceedings*, *18*, 2052–2059.

Dutta, K., Dasu, V. V., & Hegde, K. (2013). Development of medium and kinetic modeling for enhanced production of cutinase from Pseudomonas cepacia NRRL B-2320. *Advances in Microbiology*, *2013*. https://www.scirp.org/html/2-2270221_38157.htm

Egmond, M. R., & de Vlieg, J. (2000). Fusarium solani pisi cutinase. *Biochimie*, *82*(11), 1015–1021.

Encarnação, T., Pais, A. A., Campos, M. G., & Burrows, H. D. (2019). Endocrine disrupting chemicals: Impact on human health, wildlife and the environment. *Science Progress*, *102*(1), 3–42.

Enzymes revolutionize the bioproduction of value-added compounds: From enzyme discovery to special applications. (2020). *Biotechnology Advances*, *40*, 107520. https://doi.org/10.1016/j.biotechadv.2020.107520

Fan, P., Yu, H., Xi, B., & Tan, W. (2022). A review on the occurrence and influence of biodegradable microplastics in soil ecosystems: Are biodegradable plastics substitute or threat? *Environment International*, *163*, 107244. https://doi.org/10.1016/j.envint.2022.107244

Fecker, T., Galaz-Davison, P., Engelberger, F., Narui, Y., Sotomayor, M., Parra, L. P., & Ramírez-Sarmiento, C. A. (2018). Active site flexibility as a hallmark for efficient PET degradation by I. sakaiensis PETase. *Biophysical Journal*, *114*(6), 1302–1312.

Ferronato, N., & Torretta, V. (2019). Waste Mismanagement in Developing Countries: A Review of Global Issues. *International Journal of Environmental Research and Public Health*, *16*(6), 1060. https://doi.org/10.3390/ijerph16061060

Fett, W. F., Wijey, C., Moreau, R. A., & Osman, S. F. (2000). Production of cutinolytic esterase by filamentous bacteria. *Letters in Applied Microbiology*, *31*(1), 25–29.

Foschi, E., D'Addato, F., & Bonoli, A. (2021). Plastic waste management: A comprehensive analysis of the current status to set up an after-use plastic strategy in Emilia-Romagna Region (Italy). *Environmental Science and Pollution Research International*, *28*(19), 24328–24341. https://doi.org/10.1007/s11356-020-08155-y

Furukawa, M., Kawakami, N., Tomizawa, A., & Miyamoto, K. (2019). Efficient degradation of poly (ethylene terephthalate) with Thermobifida fusca cutinase exhibiting improved catalytic activity generated using mutagenesis and additive-based approaches. *Scientific Reports*, *9*(1), 16038.

Gajendiran, A., Krishnamoorthy, S., & Abraham, J. (2016). Microbial degradation of low-density polyethylene (LDPE) by Aspergillus clavatus strain JASK1 isolated from landfill soil. *3 Biotech*, *6*(1), 52. https://doi.org/10.1007/s13205-016-0394-x

Gao, R., Pan, H., & Lian, J. (2021). Recent advances in the discovery, characterization, and engineering of poly (ethylene terephthalate)(PET) hydrolases. *Enzyme and Microbial Technology*, *150*, 109868.

Gao, R., Pan, H., & Lian, J. (2021). Recent advances in the discovery, characterization, and engineering of poly (ethylene terephthalate)(PET) hydrolases. *Enzyme and Microbial Technology*, *150*, 109868.

Geyer, B., Lorenz, G., & Kandelbauer, A. (2016). Recycling of poly (ethylene terephthalate)–A review focusing on chemical methods. *Express Polymer Letters*, *10*(7), 559–586.

Geyer, B., Lorenz, G., & Kandelbauer, A. (2016). Recycling of poly (ethylene terephthalate)–A review focusing on chemical methods. *Express Polymer Letters*, *10*(7), 559–586.

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, *3*(7), e1700782. https://doi.org/10.1126/sciadv.1700782

Geyer, R., Jambeck, J. R., & Law, K. L. (2017). Production, use, and fate of all plastics ever made. *Science Advances*, *3*(7), e1700782. https://doi.org/10.1126/sciadv.1700782

Gilan, I., & Sivan, A. (2013). Effect of proteases on biofilm formation of the plastic-degrading actinomycete Rhodococcus ruber C208. *FEMS Microbiology Letters*, *342*(1), 18–23. https://doi.org/10.1111/1574-6968.12114

Giridhar, G., Manepalli, R., & Apparao, G. (2017). Size-exclusion chromatography. In *Thermal and Rheological Measurement Techniques for Nanomaterials Characterization* (pp. 51–65). Elsevier. https://www.sciencedirect.com/science/article/pii/B9780323461399000037

Gomez Del Pulgar, E. M., & Saadeddin, A. (2014). The cellulolytic system of *Thermobifida fusca*. *Critical Reviews in Microbiology*, *40*(3), 236–247. https://doi.org/10.3109/1040841X.2013.776512

Gorrasi, G., Bugatti, V., Milone, C., Mastronardo, E., Piperopoulos, E., Iemmo, L., & Di Bartolomeo, A. (2018). Effect of temperature and morphology on the electrical properties of PET/conductive nanofillers composites. *Composites Part B: Engineering*, *135*, 149–154.

Gregory, M. R. (2009). Environmental implications of plastic debris in marine settings—Entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2013–2025. https://doi.org/10.1098/rstb.2008.0265

Gricajeva, A., Nadda, A. K., & Gudiukaite, R. (2022). Insights into polyester plastic biodegradation by carboxyl ester hydrolases. *Journal of Chemical Technology & Biotechnology*, *97*(2), 359–380. https://doi.org/10.1002/jctb.6745

Hadar, Y., & Sivan, A. (2004). Colonization, biofilm formation and biodegradation of polyethylene by a strain of Rhodococcus ruber. *Applied Microbiology and Biotechnology*, *65*, 97–104.

Hahladakis, J. N., Velis, C. A., Weber, R., Iacovidou, E., & Purnell, P. (2018). An overview of chemical additives present in plastics: Migration, release, fate and environmental impact during their use, disposal and recycling. *Journal of Hazardous Materials*, *344*, 179–199. https://doi.org/10.1016/j.jhazmat.2017.10.014

Hasnaoui, I., Dab, A., Mechri, S., Abouloifa, H., Saalaoui, E., Jaouadi, B., Noiriel, A., Asehraou, A., & Abousalham, A. (2022). Purification, Biochemical and Kinetic Characterization of a Novel Alkaline sn-1, 3-Regioselective Triacylglycerol Lipase from Penicillium crustosum Thom Strain P22 Isolated from Moroccan Olive Mill Wastewater. *International Journal of Molecular Sciences*, *23*(19), 11920.

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

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

Ho, B. T., Roberts, T. K., & Lucas, S. (2018). An overview on biodegradation of polystyrene and modified polystyrene: The microbial approach. *Critical Reviews in Biotechnology*, *38*(2), 308–320. https://doi.org/10.1080/07388551.2017.1355293

Hodson, M. E., Duffus-Hodson, C. A., Clark, A., Prendergast-Miller, M. T., & Thorpe, K. L. (2017). Plastic Bag Derived-Microplastics as a Vector for Metal Exposure in Terrestrial Invertebrates. *Environmental Science & Technology*, *51*(8), 4714–4721. https://doi.org/10.1021/acs.est.7b00635

Hossain, R., Islam, M. T., Ghose, A., & Sahajwalla, V. (2022). Full circle: Challenges and prospects for plastic waste management in Australia to achieve circular economy. *Journal of Cleaner Production*, *368*, 133127. https://doi.org/10.1016/j.jclepro.2022.133127

Hurley, R., Woodward, J., & Rothwell, J. J. (2018). Microplastic contamination of river beds significantly reduced by catchment-wide flooding. *Nature Geoscience*, *11*(4), 251–257.

Hussain, S., Mubeen, I., Ullah, N., Shah, S. S. U. D., Khan, B. A., Zahoor, M., Ullah, R., Khan, F. A., & Sultan, M. A. (2022). Modern Diagnostic Imaging Technique Applications and Risk Factors in the Medical Field: A Review. *BioMed Research International*, *2022*, 5164970. https://doi.org/10.1155/2022/5164970

Hwang, J.-H., Sadmani, A., Lee, S.-J., Kim, K.-T., & Lee, W. H. (2020). Microalgae: An Eco-friendly Tool for the Treatment of Wastewaters for Environmental Safety. In R. N. Bharagava & G. Saxena (Eds.), *Bioremediation of Industrial Waste for Environmental Safety: Volume II: Biological Agents and Methods for Industrial Waste Management* (pp. 283–304). Springer. https://doi.org/10.1007/978-981-13-3426-9_12

Jachimowicz, P., Nosek, D., & Cydzik-Kwiatkowska, A. (2022). Chemical and microbiological changes on the surface of microplastic after long term exposition to different concentrations of ammonium in the environment. *Science of The Total Environment*, *830*, 154784.

Jadaun, J. S., Bansal, S., Sonthalia, A., Rai, A. K., & Singh, S. P. (2022). Biodegradation of plastics for sustainable environment. *Bioresource Technology*, *347*, 126697.

Janczak, K., Hrynkiewicz, K., Znajewska, Z., & Dąbrowska, G. (2018). Use of rhizosphere microorganisms in the biodegradation of PLA and PET polymers in compost soil. *International Biodeterioration & Biodegradation*, *130*, 65–75.

Jasu, A., & Ray, R. R. (2021). Biofilm mediated strategies to mitigate heavy metal pollution: A critical review in metal bioremediation. *Biocatalysis and Agricultural Biotechnology*, *37*, 102183.

Ji, L. N. (2013). Study on preparation process and properties of polyethylene terephthalate (PET). *Applied Mechanics and Materials*, *312*, 406–410.

Joo, S., Cho, I. J., Seo, H., Son, H. F., Sagong, H.-Y., Shin, T. J., Choi, S. Y., Lee, S. Y., & Kim, K.-J. (2018). Structural insight into molecular mechanism of poly (ethylene terephthalate) degradation. *Nature Communications*, *9*(1), 382.

Juers, D. H., Matthews, B. W., & Huber, R. E. (2012). LacZ β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. *Protein Science*, *21*(12), 1792–1807. https://doi.org/10.1002/pro.2165

Kawai, F. (2021). *The Current State of Research on PET Hydrolyzing Enzymes Available for Biorecycling. Catalysts 2021, 11, 206*. s Note: MDPI stays neutral with regard to jurisdictional claims in published …. https://search.proquest.com/openview/37b451d155e3bda81f8bb92fe668c26e/1?pq-origsite=gscholar&cbl=2032420

Kawai, F., Kawabata, T., & 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. https://doi.org/10.1007/s00253-019-09717-y

Kawai, F., Kawabata, T., & Oda, M. (2020). Current State and Perspectives Related to the Polyethylene Terephthalate Hydrolases Available for Biorecycling. *ACS Sustainable Chemistry & Engineering*, *8*(24), 8894–8908. https://doi.org/10.1021/acssuschemeng.0c01638

Khairul Anuar, N. F. S., Huyop, F., Ur-Rehman, G., Abdullah, F., Normi, Y. M., Sabullah, M. K., & Abdul Wahab, R. (2022). An overview into polyethylene terephthalate (PET) hydrolases and efforts in tailoring enzymes for improved plastic degradation. *International Journal of Molecular Sciences*, *23*(20), 12644.

Khoaele, K. K., Gbadeyan, O. J., Chunilall, V., & Sithole, B. (2023). The Devastation of Waste Plastic on the Environment and Remediation Processes: A Critical Review. *Sustainability*, *15*(6), Article 6. https://doi.org/10.3390/su15065233

Khoironi, A., Anggoro, S., & Sudarno, -. (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.

Kibria, Md. G., Masuk, N. I., Safayet, R., Nguyen, H. Q., & Mourshed, M. (2023). Plastic Waste: Challenges and Opportunities to Mitigate Pollution and Effective Management. *International Journal of Environmental Research*, *17*(1), 20. https://doi.org/10.1007/s41742-023-00507-z

Kim, S. A., Kim, L., Kim, T. H., & An, Y.-J. (2022). Assessing the size-dependent effects of microplastics on zebrafish larvae through fish lateral line system and gut damage. *Marine Pollution Bulletin*, *185*, 114279.

Koczoń, P., Bartyzel, B., Iuliano, A., Klensporf-Pawlik, D., Kowalska, D., Majewska, E., Tarnowska, K., Zieniuk, B., & Gruczyńska-Sękowska, E. (2022). Chemical

Structures, Properties, and Applications of Selected Crude Oil-Based and Bio-Based Polymers. *Polymers*, *14*(24). https://doi.org/10.3390/polym14245551

Koshti, R., Mehta, L., & Samarth, N. (2018). Biological Recycling of Polyethylene Terephthalate: A Mini-Review. *Journal of Polymers and the Environment*, *26*(8), 3520–3529. https://doi.org/10.1007/s10924-018-1214-7

Koshti, R., Mehta, L., & Samarth, N. (2018). Biological Recycling of Polyethylene Terephthalate: A Mini-Review. *Journal of Polymers and the Environment*, *26*(8), 3520–3529. https://doi.org/10.1007/s10924-018-1214-7

Kumar, A., Samadder, S. R., Kumar, N., & Singh, C. (2018). Estimation of the generation rate of different types of plastic wastes and possible revenue recovery from informal recycling. *Waste Management*, *79*, 781–790.

Kumari, V., Kumar, V., Chauhan, R., Asif, M., & Bhalla, T. C. (2016). Optimization of medium parameters by response surface methodology (RSM) for enhanced production of cutinase from Aspergillus sp. RL2Ct. *3 Biotech*, *6*, 1–10.

Landon-Lane, M. (2018). Corporate social responsibility in marine plastic debris governance. *Marine Pollution Bulletin*, *127*, 310–319.

Lebreton, L. C. M., van der Zwet, J., Damsteeg, J.-W., Slat, B., Andrady, A., & Reisser, J. (2017). River plastic emissions to the world's oceans. *Nature Communications*, *8*(1), Article 1. https://doi.org/10.1038/ncomms15611

Lepoittevin, B., Devalckenaere, M., Pantoustier, N., Alexandre, M., Kubies, D., Calberg, C., Jérôme, R., & Dubois, P. (2002). Poly (ε-caprolactone)/clay nanocomposites prepared by melt intercalation: Mechanical, thermal and rheological properties. *Polymer*, *43*(14), 4017–4023.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265–275.

Lozano, Y. M., Aguilar-Trigueros, C. A., Onandia, G., Maaß, S., Zhao, T., & Rillig, M. C. (2021). Effects of microplastics and drought on soil ecosystem functions and multifunctionality. *Journal of Applied Ecology*, *58*(5), 988–996. https://doi.org/10.1111/1365-2664.13839

Lucas, N., Bienaime, C., Belloy, C., Queneudec, M., Silvestre, F., & Nava-Saucedo, J.-E. (2008). Polymer biodegradation: Mechanisms and estimation techniques – A review. *Chemosphere*, *73*(4), 429–442. https://doi.org/10.1016/j.chemosphere.2008.06.064

Lucas, N., Bienaime, C., Belloy, C., Queneudec, M., Silvestre, F., & Nava-Saucedo, J.-E. (2008). Polymer biodegradation: Mechanisms and estimation techniques – A review. *Chemosphere*, *73*(4), 429–442. https://doi.org/10.1016/j.chemosphere.2008.06.064

Ma, M. M., Wang, L. Y., & Zhu, H. Y. (2012). Enzymatic degradation of polyester-nanoparticles by lipases and adsorption of lipases on the polyester-nanoparticles. *Advanced Materials Research*, *418*, 2302–2307.

Macko, S. A. (2019). Increasing challenges to the future marine environment. In *Cooperation and Engagement in the Asia-Pacific Region* (pp. 161–176). Brill Nijhoff. https://brill.com/downloadpdf/book/edcoll/9789004412026/BP000014.pdf

Magnan, A. K., Schipper, E. L. F., Burkett, M., Bharwani, S., Burton, I., Eriksen, S., Gemenne, F., Schaar, J., & Ziervogel, G. (2016). Addressing the risk of maladaptation to climate change. *Wiley Interdisciplinary Reviews: Climate Change*, *7*(5), 646–665.

Marten, E., Müller, R.-J., & Deckwer, W.-D. (2005). Studies on the enzymatic hydrolysis of polyesters. II. Aliphatic–aromatic copolyesters. *Polymer Degradation and Stability*, *88*(3), 371–381.

Martínez, A., & Maicas, S. (2021). Cutinases: Characteristics and Insights in Industrial Production. *Catalysts*, *11*(10), Article 10. https://doi.org/10.3390/catal11101194

Masud, M. H., Akram, W., Ahmed, A., Ananno, A. A., Mourshed, M., Hasan, M., & Joardder, M. U. H. (2019). Towards the effective E-waste management in Bangladesh: A review. *Environmental Science and Pollution Research*, *26*(2), 1250–1276.

Maurya, A., Bhattacharya, A., & Khare, S. K. (2020). Enzymatic remediation of polyethylene terephthalate (PET)–based polymers for effective management of plastic wastes: An overview. *Frontiers in Bioengineering and Biotechnology*, *8*, 602325.

Mazhandu, Z. S., Muzenda, E., Mamvura, T. A., Belaid, M., & Nhubu, T. (2020). Integrated and Consolidated Review of Plastic Waste Management and Bio-Based Biodegradable Plastics: Challenges and Opportunities. *Sustainability*, *12*(20), Article 20. https://doi.org/10.3390/su12208360

Mazzucotelli, C. A., Moreira, M. D. R., & Ansorena, M. R. (2016). Statistical optimization of medium components and physicochemical parameters to simultaneously enhance bacterial growth and esterase production by *Bacillus thuringiensis*. *Canadian Journal of Microbiology*, *62*(1), 24–34. https://doi.org/10.1139/cjm-2015-0463

McKeen, L. W. (2018). *The effect of sterilization on plastics and elastomers*. William Andrew.

Methane fermentation process as anaerobic digestion of biomass: Transformations, stages and microorganisms | African Journal of Biotechnology. (n.d.). Retrieved January 24, 2024, from https://www.ajol.info/index.php/ajb/article/view/101067

Mohanan, N., Montazer, Z., Sharma, P. K., & Levin, D. B. (2020). Microbial and enzymatic degradation of synthetic plastics. *Frontiers in Microbiology*, *11*, 580709.

Mohsin, M. A., Abdulrehman, T., & Haik, Y. (2017). Reactive extrusion of polyethylene terephthalate waste and investigation of its thermal and mechanical properties after treatment. *International Journal of Chemical Engineering*, *2017*. https://www.hindawi.com/journals/ijce/2017/5361251/abs/

Moog, D., Schmitt, J., Senger, J., Zarzycki, J., Rexer, K.-H., Linne, U., Erb, T., & Maier, U. G. (2019). Using a marine microalga as a chassis for polyethylene terephthalate (PET) degradation. *Microbial Cell Factories*, *18*(1), 171. https://doi.org/10.1186/s12934-019-1220-z

Moreman, J., Lee, O., Trznadel, M., David, A., Kudoh, T., & Tyler, C. R. (2017). Acute Toxicity, Teratogenic, and Estrogenic Effects of Bisphenol A and Its Alternative Replacements Bisphenol S, Bisphenol F, and Bisphenol AF in Zebrafish Embryo-Larvae. *Environmental Science & Technology*, *51*(21), 12796–12805. https://doi.org/10.1021/acs.est.7b03283

Morris, B. A. (2022). *The science and technology of flexible packaging: Multilayer films from resin and process to end use*. William Andrew.

Müller, R.-J., Kleeberg, I., & Deckwer, W.-D. (2001). Biodegradation of polyesters containing aromatic constituents. *Journal of Biotechnology*, *86*(2), 87–95. https://doi.org/10.1016/S0168-1656(00)00407-7

Nava, V., & Leoni, B. (2021). A critical review of interactions between microplastics, microalgae and aquatic ecosystem function. *Water Research*, *188*, 116476.

Nikolaivits, E., Kanelli, M., Dimarogona, M., & Topakas, E. (2018). A middle-aged enzyme still in its prime: Recent advances in the field of cutinases. *Catalysts*, *8*(12), 612.

Ogwueleka, T. C., & B p, N. (2021). Activities of informal recycling sector in North-Central, Nigeria. *Energy Nexus*, *1*, 100003. https://doi.org/10.1016/j.nexus.2021.100003

Panowicz, R., Konarzewski, M., Durejko, T., Szala, M., Łazińska, M., Czerwińska, M., & Prasuła, P. (2021). Properties of Polyethylene Terephthalate (PET) after Thermo-Oxidative Aging. *Materials*, *14*(14), 3833. https://doi.org/10.3390/ma14143833

Pokazeev, K., Sovga, E., Chaplina, T., Pokazeev, K., Sovga, E., & Chaplina, T. (2021). Main natural and anthropogenic sources of pollution of the Black Sea, its shelf zones and small water reservoirs. *Pollution in the Black Sea: Observations about the Ocean's Pollution*, 97–141.

Puglisi, E., Romaniello, F., Galletti, S., Boccaleri, E., Frache, A., & Cocconcelli, P. S. (2019). Selective bacterial colonization processes on polyethylene waste samples in an abandoned landfill site. *Scientific Reports*, *9*(1), Article 1. https://doi.org/10.1038/s41598-019-50740-w

Pujic, P., Valour, F., Lebeaux, D., & Rodriguez-Nava, V. (2024). Chapter 38—Nocardia and Actinomyces. In Y.-W. Tang, M. Y. Hindiyeh, D. Liu, A. Sails, P. Spearman, & J.-R. Zhang (Eds.), *Molecular Medical Microbiology (Third Edition)* (pp. 775–809). Academic Press. https://doi.org/10.1016/B978-0-12-818619-0.00132-5

Qi, Y., Ossowicki, A., Yang, X., Huerta Lwanga, E., Dini-Andreote, F., Geissen, V., & Garbeva, P. (2020). Effects of plastic mulch film residues on wheat rhizosphere and soil properties. *Journal of Hazardous Materials*, *387*, 121711. https://doi.org/10.1016/j.jhazmat.2019.121711

Ragaert, K., Delva, L., & Van Geem, K. (2017). Mechanical and chemical recycling of solid plastic waste. *Waste Management*, *69*, 24–58.

Ramakrishna, M., Girigoswami, A., Chakraborty, S., & Girigoswami, K. (2021). Bisphenol A—An overview on its effect on health and environment. *Biointerface Res Appl Chem*, *12*(1), 105–119.

Rhodes, C. J. (2018). Plastic pollution and potential solutions. *Science Progress*, *101*(3), 207–260.

Rhodes, C. J. (2018). Plastic pollution and potential solutions. *Science Progress*, *101*(3), 207–260.

Ribitsch, D., Heumann, S., Trotscha, E., Herrero Acero, E., Greimel, K., Leber, R., Birner-Gruenberger, R., Deller, S., Eiteljoerg, I., Remler, P., Weber, T., Siegert, P., Maurer, K.-H., Donelli, I., Freddi, G., Schwab, H., & Guebitz, G. M. (2011). Hydrolysis of polyethyleneterephthalate by p-nitrobenzylesterase from Bacillus subtilis. *Biotechnology Progress*, *27*(4), 951–960. https://doi.org/10.1002/btpr.610

Ronkvist, Å. M., Xie, W., Lu, W., & Gross, R. A. (2009). Cutinase-Catalyzed Hydrolysis of Poly(ethylene terephthalate). *Macromolecules*, *42*(14), 5128–5138. https://doi.org/10.1021/ma9005318

Ronkvist, Å. M., Xie, W., Lu, W., & Gross, R. A. (2009). Cutinase-Catalyzed Hydrolysis of Poly(ethylene terephthalate). *Macromolecules*, *42*(14), 5128–5138. https://doi.org/10.1021/ma9005318

Saini, G. (2010). *Bacterial hydrophobicity: Assessment techniques, applications and extension to colloids*. Oregon State University. https://search.proquest.com/openview/5f3961d76e0931031a7cbb9be6382ac1/1?pq-origsite=gscholar&cbl=18750

Saini, G. (2010). *Bacterial hydrophobicity: Assessment techniques, applications and extension to colloids*. Oregon State University. https://search.proquest.com/openview/5f3961d76e0931031a7cbb9be6382ac1/1?pq-origsite=gscholar&cbl=18750

Salam, M. D., Varma, A., Prashar, R., & Choudhary, D. (2021). Review on efficacy of microbial degradation of polyethylene terephthalate and bio-upcycling as a part of plastic waste management. *Appl. Ecol. Environ. Sci*, *9*, 695–703.

Salvador, M., Abdulmutalib, U., Gonzalez, J., Kim, J., Smith, A. A., Faulon, J.-L., Wei, R., Zimmermann, W., & Jimenez, J. I. (2019). Microbial genes for a circular and sustainable bio-PET economy. *Genes*, *10*(5), 373.

Samak, N. A., Jia, Y., Sharshar, M. M., Mu, T., Yang, M., Peh, S., & Xing, J. (2020). Recent advances in biocatalysts engineering for polyethylene terephthalate plastic waste green recycling. *Environment International*, *145*, 106144.

Samoylova, Y. V., Sorokina, K. N., Romanenko, M. V., & Parmon, V. N. (2018). Cloning, expression and characterization of the esterase estUT1 from Ureibacillus thermosphaericus which belongs to a new lipase family XVIII. *Extremophiles*, *22*(2), 271–285. https://doi.org/10.1007/s00792-018-0996-9

Sánchez, C. (2020). Fungal potential for the degradation of petroleum-based polymers: An overview of macro- and microplastics biodegradation. *Biotechnology Advances*, *40*, 107501. https://doi.org/10.1016/j.biotechadv.2019.107501

Sarioğlu, E., & Kaynak, H. K. (2017). PET bottle recycling for sustainable textiles. *Polyester-Production, Characterization and Innovative Applications*, 5–20.

Sarmah, P., & Rout, J. (2018). *Algal colonization on polythene carry bags in a domestic solid waste dumping site of Silchar town in Assam*. *48*.

Sarmah, P., & Rout, J. (2018). *Algal colonization on polythene carry bags in a domestic solid waste dumping site of Silchar town in Assam*. *48*.

Satti, S. M., Shah, A. A., Auras, R., & Marsh, T. L. (2017). Isolation and characterization of bacteria capable of degrading poly (lactic acid) at ambient temperature. *Polymer Degradation and Stability*, *144*, 392–400.

Satti, S. M., Shah, A. A., Auras, R., & Marsh, T. L. (2017). Isolation and characterization of bacteria capable of degrading poly (lactic acid) at ambient temperature. *Polymer Degradation and Stability*, *144*, 392–400.

Scalenghe, R. (2018). Resource or waste? A perspective of plastics degradation in soil with a focus on end-of-life options. *Heliyon*, *4*(12), e00941. https://doi.org/10.1016/j.heliyon.2018.e00941

Shah, A. A., Hasan, F., Hameed, A., & Ahmed, S. (2008). Biological degradation of plastics: A comprehensive review. *Biotechnology Advances*, *26*(3), 246–265.

Shah, A. A., Hasan, F., Hameed, A., & Akhter, J. I. (2009). Isolation of Fusarium sp. AF4 from sewage sludge, with the ability to adhere the surface of polyethylene. *Afr. J. Microbiol. Res*, *3*(10), 658–663.

Sharma, S., & Chatterjee, S. (2017). Microplastic pollution, a threat to marine ecosystem and human health: A short review. *Environmental Science and Pollution Research*, *24*, 21530–21547.

Sharma, S., & Chatterjee, S. (2017). Microplastic pollution, a threat to marine ecosystem and human health: A short review. *Environmental Science and Pollution Research*, *24*, 21530–21547.

Siddiqua, A., Hahladakis, J. N., & Al-Attiya, W. A. K. A. (2022). An overview of the environmental pollution and health effects associated with waste landfilling and open dumping. *Environmental Science and Pollution Research*, *29*(39), 58514–58536. https://doi.org/10.1007/s11356-022-21578-z

Singh, M. K., Mohanty, A. K., & Misra, M. (2023). Upcycling of waste polyolefins in natural fiber and sustainable filler-based biocomposites: A study on recent developments and future perspectives. *Composites Part B: Engineering*, *263*, 110852. https://doi.org/10.1016/j.compositesb.2023.110852

Siracusa, V. (2012). Food Packaging Permeability Behaviour: A Report. *International Journal of Polymer Science*, *2012*, e302029. https://doi.org/10.1155/2012/302029

Song, X., Xu, Y., Li, G., Zhang, Y., Huang, T., & Hu, Z. (2011). Isolation, characterization of Rhodococcus sp. P14 capable of degrading high-molecular-weight polycyclic aromatic hydrocarbons and aliphatic hydrocarbons. *Marine Pollution Bulletin*, *62*(10), 2122–2128.

Speranza, P., de Oliveira Carvalho, P., & Macedo, G. A. (2011). Effects of different solid state fermentation substrate on biochemical properties of cutinase from Fusarium sp. *Journal of Molecular Catalysis B: Enzymatic*, *72*(3–4), 181–186.

Šudomová, L., Doležalová Weissmannová, H., Steinmetz, Z., Řezáčová, V., & Kučerík, J. (2023). A differential scanning calorimetry (DSC) approach for assessing the quality of polyethylene terephthalate (PET) waste for physical recycling: A proof-of-concept study. *Journal of Thermal Analysis and Calorimetry*, *148*(20), 10843–10855. https://doi.org/10.1007/s10973-023-12430-8

Šujica, M. Ž., & Smole, M. S. (2003). Structure-mechanical properties relationship of poly (ethylene terephthalate) fibers. *Journal of Applied Polymer Science*, *89*(12), 3383–3389.

Sulaiman, S. (2014). *Structure, stability and activity of metagenome-derived LC-cutinase with polyethylene terephthalate (PET) degrading ability*. https://ir.library.osaka-u.ac.jp/repo/ouka/all/50519/27073_Dissertation.pdf

Taghavi, N., Udugama, I. A., Zhuang, W.-Q., & Baroutian, S. (2021). Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness. *Biotechnology Advances*, *49*, 107731.

Tamoor, M., Samak, N. A., Jia, Y., Mushtaq, M. U., Sher, H., Bibi, M., & Xing, J. (2021). Potential use of microbial enzymes for the conversion of plastic waste into value-added products: A viable solution. *Frontiers in Microbiology*, *12*, 777727.

Taniguchi, I., Yoshida, S., Hiraga, K., Miyamoto, K., Kimura, Y., & Oda, K. (2019). Biodegradation of PET: Current Status and Application Aspects. *ACS Catalysis*, *9*(5), 4089–4105. https://doi.org/10.1021/acscatal.8b05171

Team, R. C. (2019). Available online: Https://www. *R-Project. Org/(Accessed on 8 April 2020)*.

Temporiti, M. E. E., Nicola, L., Nielsen, E., & Tosi, S. (2022). Fungal enzymes involved in plastics biodegradation. *Microorganisms*, *10*(6), 1180.

Tokiwa, Y., Calabia, B. P., Ugwu, C. U., & Aiba, S. (2009). Biodegradability of plastics. *International Journal of Molecular Sciences*, *10*(9), 3722–3742.

Tournier, V., Duquesne, S., Guillamot, F., Cramail, H., Taton, D., Marty, A., & André, I. (2023). Enzymes' power for plastics degradation. *Chemical Reviews*, *123*(9), 5612–5701.

Tymiński, \Lukasz P., Znajewska, Z., & Dąbrowska, G. B. (2019). Characteristics and functions of hydrophobins and their use in manifold industries. *Postępy Mikrobiologii-Advancements of Microbiology*, *57*(4), 374–384.

Usman, M. A., Momohjimoh, I., & Usman, A. O. (2020). Mechanical, physical and biodegradability performances of treated and untreated groundnut shell powder recycled polypropylene composites. *Materials Research Express*, *7*(3), 035302. https://doi.org/10.1088/2053-1591/ab750e

Wang, E., Miao, C., & Chen, X. (2022). Circular economy and the changing geography of international trade in plastic waste. *International Journal of Environmental Research and Public Health*, *19*(22), 15020.

Wang, X., Lu, D., Jönsson, L. J., & Hong, F. (2008). Preparation of a PET-Hydrolyzing Lipase from *Aspergillus oryzae* by the Addition of Bis(2-hydroxyethyl) Terephthalate to the Culture Medium and Enzymatic Modification of PET Fabrics. *Engineering in Life Sciences*, *8*(3), 268–276. https://doi.org/10.1002/elsc.200700058

Waterborg, J. H. (2009). The Lowry Method for Protein Quantitation. In J. M. Walker (Ed.), *The Protein Protocols Handbook* (pp. 7–10). Humana Press. https://doi.org/10.1007/978-1-59745-198-7_2

Wojnowska-Bary\la, I., Bernat, K., & Zaborowska, M. (2022). Plastic waste degradation in landfill conditions: The problem with microplastics, and their direct and indirect environmental effects. *International Journal of Environmental Research and Public Health*, *19*(20), 13223.

Yang, S., Xu, H., Yan, Q., Liu, Y., Zhou, P., & Jiang, Z. (2013). A low molecular mass cutinase of Thielavia terrestris efficiently hydrolyzes poly (esters). *Journal of Industrial Microbiology and Biotechnology*, *40*(2), 217–226.

Yeung, C. W., Teo, J. Y., Loh, X. J., & Lim, J. Y. (2021). Polyolefins and polystyrene as chemical resources for a sustainable future: Challenges, advances, and prospects. *ACS Materials Letters*, *3*(12), 1660–1676.

Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., & Oda, K. (2016). A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, *351*(6278), 1196–1199. https://doi.org/10.1126/science.aad6359

Zekriardehani, S., Jabarin, S. A., Gidley, D. R., & Coleman, M. R. (2017). Effect of Chain Dynamics, Crystallinity, and Free Volume on the Barrier Properties of Poly(ethylene terephthalate) Biaxially Oriented Films. *Macromolecules*, *50*(7), 2845–2855. https://doi.org/10.1021/acs.macromol.7b00198

Zhang, J., Gao, D., Li, Q., Zhao, Y., Li, L., Lin, H., Bi, Q., & Zhao, Y. (2020). Biodegradation of polyethylene microplastic particles by the fungus Aspergillus flavus from the guts of wax moth Galleria mellonella. *Science of The Total Environment*, *704*, 135931. https://doi.org/10.1016/j.scitotenv.2019.135931

Production and Characterization of Cutinase from Polyethylene Terephthalate Degrading Bacterial Strain Isolated from Compost Soil

ORIGINALITY REPORT

17%	9%	12%	5%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Jiarong Qiu, Yuxin Chen, Liangqing Zhang, Jinzhi Wu, Xianhai Zeng, Xinguo Shi, Lemian Liu, Jianfeng Chen. "A comprehensive review on enzymatic biodegradation of polyethylene terephthalate", Environmental Research, 2023 Publication	1%
2	Submitted to Higher Education Commission Pakistan Student Paper	1%
3	www.mdpi.com Internet Source	1%
4	Ayodeji Amobonye, Prashant Bhagwat, Suren Singh, Santhosh Pillai. "Plastic biodegradation: frontline microbes and their enzymes", Science of The Total Environment, 2020 Publication	1%
5	Sadia Mehmood Satti, Amina Mumtaz Abbasi, Salahuddin, Qurrat ul Ain Rana et al.	1%