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POLYHYDROXYALKANOATE PRODUCTION FROM RAISIN WASTE USING RECOMBINANT *Escherichia coli*

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2025

TAAHHÜTNAME

Bu tezin Manisa Celal Bayar Üniversitesi Lisansüstü Eğitim Enstitüsü Biyomühendislik Ana Bilim Dalında akademik ve etik kurallara uygun olarak yazıldığını ve kullanılan tüm literatür bilgilerinin referans gösterilerek tezde yer aldığını, tamamen kendi çalışmam olduğunu, her alıntıya kaynak gösterdiğimi, tezin yazımında akademik ve etik kurallara aykırı herhangi bir yapay zeka ve program kullanmadığımı beyan ederim.

ALEYNA FİLİZFİDANOĞLU

ÖZET

Yüksek Lisans

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1900'lerin başından itibaren petrol türevi polimerlerin hızlı yayılımı, pek çok alanda kullanım imkânı sağlamış ancak ciddi çevre sorunlarına yol açmıştır. Bu sorunlara çözüm olarak biyolojik kaynaklardan üretilen ve biyoplastik olarak adlandırılan çevre dostu alternatifler geliştirilmiştir. Biyoplastikler, biyolojik olarak parçalanabilirlikleri, çevre dostu yapıları ve biyouyumlulukları ile öne çıkmaktadır. Polihidroksialkanoat (PHA) ailesinin en bilinen üyesi olan poli-3-hidroksibutirat (P3HB), çeşitli mikroorganizmalar tarafından üretilebilmekte ve ambalaj, tıp, ilaç, tarım, otomotiv endüstrisi, hijyen ürünleri ve çevre teknolojileri gibi alanlarda geniş bir uygulama alanına sahiptir.

Bu tez çalışması, kuru üzüm atığını karbon kaynağı olarak kullanarak rekombinant *Escherichia coli* Rosetta pQEAL1124 suşu ile P3HB üretmeyi amaçlamaktadır. Çalışma, üretim sürecini etkileyen fizikokimyasal parametrelerin optimizasyonunu ve farklı ekstraksiyon yöntemlerinin karşılaştırılmasını kapsamaktadır. P3HB üretiminin optimizasyonu için Yanıt Yüzey Yöntemi (RSM) uygulanmış ve maksimum üretim için en uygun koşullar 7,03 g/L kuru üzüm atığı, 2,46 g/L tripton ve 194 rpm olarak belirlenmiştir. Ayrıca, konvansiyonel yöntemle maksimum %47 verimle P3HB elde edilirken, yeşil ekstraksiyon yöntemi kapsamında 1,3-dioksolan kullanılarak %33 verimle P3HB üretimi gerçekleştirilmiştir.

Anahtar Kelimeler: Biyoplastikler, biyouyumluluk, Poli-3-hidroksibutirat, *Escherichia coli*, kuru üzüm atığı

2025, 65 sayfa

ABSTRACT

M.Sc. Thesis

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Since the early 1900s, the rapid proliferation of petroleum-derived polymers has enabled their use in a wide range of applications; however, this widespread utilization has also led to severe environmental problems. As a solution, environmentally friendly alternatives known as bioplastics, produced from biological resources, have been developed. Bioplastics are distinguished by their biodegradability, eco-friendly nature, and biocompatibility. Among them, poly(3-hydroxybutyrate) (P3HB), a member of the polyhydroxyalkanoate (PHA) family, is one of the most widely studied bioplastics. It can be synthesized by various microorganisms and has broad applications in packaging, medicine, pharmaceuticals, agriculture, the automotive industry, hygiene products, and environmental technologies.

This thesis aims to produce P3HB using raisin waste as a carbon source with recombinant *Escherichia coli* Rosetta pQEAL1124. The study includes the optimization of physicochemical parameters affecting the production process and a comparison of conventional and green extraction methods. Response Surface Methodology (RSM) was applied for the optimization of P3HB production, and the optimum conditions providing maximum production were determined as 7.03 g/L raisin waste, 2.46 g/L tryptone, and 194 rpm. Furthermore, the conventional extraction method yielded a maximum of 47% efficiency, while the green extraction method using 1,3-dioxolane resulted in a maximum of P3HB with 33% efficiency.

Keywords: Bioplastics, biocompatibility, Poly-3-hydroxybutyrate, *Escherichia coli*, raisin waste.

2025, 65 pages

ÖNSÖZ VE TEŞEKKÜR

Bu tez, Literatür Araştırması, Giriş, Kullanılan Gereçler ve Yöntem, Bulgular ve Tartışma, Sonuç ve Öneriler ile Başvurular olmak üzere altı ana bölümden oluşmaktadır. Çalışma kapsamında, *Escherichia coli* Rosetta pQEAL1124 suşu kullanılarak kuru üzüm atığından poly(-3-hydroxybutyrate) üretiminin gerçekleştirilmesi amaçlanmıştır.

Yüksek lisans eğitim ve tez sürecim boyunca bilgi ve tecrübesiyle benim yoluma ışık tutan, karşılaştığım her sorunda sabırla ve özveriyle yardımcı olan ve moral ve motivasyonumu kaybettiğimi düşündüğüm zamanlarda desteğini hiçbir zaman esirgemeyen değerli danışman hocam Doç. Dr. İrem DENİZ CAN'a en içten teşekkürlerimi sunuyorum. Bilime yaklaşımı ve etik değerleriyle birlikte yapıcı eleştirileri sayesinde yalnızca tez çalışmamı bitirmekle kalmayıp aynı zamanda araştırma yapma yetkinliğimi de geliştirme fırsatı buldum. Bu uzun süreçte göstermiş olduğu anlayış ve sabır benim için oldukça kıymetlidir. Kendisine teşekkür eder, bana kazandırmış olduğu değerleri bundan sonraki mesleki hayatım boyunca devam ettirmek için çabalayacağımı belirtmekten onur duyarım.

Her koşulda, maddi ve manevi desteklerini daima yanımda hissettiren, tüm eğitim hayatımda başarılı olacağıma inanan pek değerli annem Neriman FİLİZFİDANOĞLU'na ve babam Ahmet FİLİZFİDANOĞLU'na sonsuz teşekkürlerimi sunarım.

Destekleriyle her zaman yanımda olan, laboratuvar ortamını aile gibi hissettiren ve bana yardımcı olan başta Tuğbay Sıla ALTAÇ'a ve Özcan GEZEN'e teşekkürü borç bilirim.

Bu tez çalışması, Türkiye Bilimsel ve Teknolojik Araştırma Kurumu (TÜBİTAK) tarafından danışman hocam Sayın Doç. Dr. İrem Deniz CAN'ın yürütücülüğünü yaptığı, 124M105 numaralı "Biyoteknolojik Yaklaşımlar Kullanarak Polihidroksialkanoat Üretimi ve Kemik Doku Mühendisliğinde Kullanım Potansiyelinin Araştırılması (PHA4Bone)" proje kapsamında desteklenmiştir. 124M105 numaralı proje kapsamında yüksek lisans bursiyeri olduğum TÜBİTAK'a beni ve çalışmamı destekledikleri için teşekkürlerimi sunarım.

Aleyna FİLİZFİDANOĞLU
Manisa, 2025

LIST of ABBREVIATIONS

CCD	Central Composite Design
CO_2	Carbon Dioxide
CP	Casein Polymers
CR	Cellulose Regenerates
CSTR	Continuously-stirred Tank Reactor
C. necator	*Cupriavidus necator*
DMC	Dimethyl Carbonate
DNS	3,5-Dinitrosalicylic Acid
E. coli	*Escherichia coli*
EU	European Union
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
H_2SO_4	Sulphuric Acid
HDPE	High Density Polyethylene
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-β-D-Thiogalactopyranoside
LB	Luria Bertani
LDPE	Low Density Polyethylene
MCL	Medium-chain length
NMR	Nuclear Magnetic Resonance
OD	Optical Density
P3HB	Poly (-3-horoxivalerate)
P3HH	Poly (-3-hydroxyhexanoate)
P3HO	Poly (-3-hydroxyoctanoate)
P3HP	Poly (-3-hydroxybutyrate)
P3HV	Poly (-3-hydroxyhexanoate)
P4HB	Poly (-4-hydroxybutyrate)
P5HV	Poly (-5-hydroxyvalerate)
PA	Polyamide
PBAT	Polybutyrateadipateterephthalate

PBS	Polybuthylene Succinate And Copolymers
PCL	Polycaprolactone
PE	Polyethylene
PET	Poly (ethyleneterephtalate)
PHA	Polyhydroxyalkanoate
PLA	Poly (lactic acid)
PP	Polypropylene
RSM	Response Surface Methodology
RW	Raisin Waste
SCL	Short-Chain Length
SDS	Sodium Dodecyl Sulfate

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SECTION ONE

INTRODUCTION

1.1. Literature Research

1.1.1. From conventional plastics to bioplastics

Since the early 1900s, petroleum-derived polymers have rapidly spread on a global scale and directly affected the world economy due to their wide range of applications (Vieyra et al., 2022). Thanks to their high thermal and mechanical durability, suitability for chemical modification, effective barrier properties and low production costs, these materials have contributed to the steady growth of the polymer industry and have become indispensable in industry (La Mantia et al., 2020; Tong et al., 2022). According to PlasticsEurope data, the production volume of plastics increased rapidly from 2 million tons in 1950 to 359 million tons in 2018 (PlasticsEurope, 2019). This situation has brought the sustainability issue of plastics to the agenda rapidly (Wang et al., 2021). In response to the increase in plastics, the ecosystem is facing problems such as a decrease in water resources, a decrease in soil fertility, a decrease in the number and species of living things and ultimately poses serious threats to human health. Various countries are making efforts to minimize the damage caused by plastics.

Following the 2015 Paris Agreement, the European Union (EU) set its own roadmap and published the European Green Deal in December 2019. The EU's Green Deal includes a very serious and comprehensive transformation for both EU members and countries that cooperate and trade with the EU. The European Union plans to reduce Europe's carbon emissions by 55% by 2030 compared to 1990 levels. The EU has announced the “Fit for 55” package, a development plan in line with this goal, with the ultimate goal of becoming the world's first carbon neutral continent by 2050. The main objective of the European Green Deal is to reduce greenhouse gases that cause global warming. Reducing these gases is extremely important as harmful gases damage the ozone layer. Therefore, reducing the use of conventional plastics is one of the main steps towards becoming a pioneer of green energy and sustainable living (The European Green Deal, 2019). Although large European countries such as Germany and the Netherlands have achieved a great success rate of 80% in the recovery of plastic waste, this rate still corresponds to 28% in Europe in general (European Union, 2013).

In European countries, 76% of waste plastics are incinerated for disposal. Although all standards are met to minimize environmental damage, the amount of carbon dioxide (CO_2) given to nature is quite high (European Commission, 2013). Thus, despite the measures taken for the use and disposal of conventional petroleum-derived plastics, alternative ways have been resorted due to undeniable damage to the environment. One of the comprehensive approaches to tackle plastic pollution is the use of plastics derived from biological sources. Unlike conventional plastics, bioplastics are environmentally friendly, biodegradable and biocompatible (Sudhakar et al., 2024).

Bioplastics are also defined as plastics that have biological origin, biodegradability, and both properties. Thus, not all bioplastics are biodegradable, and not all biodegradable plastics are produced from renewable resources. Figure 1. indicates examples of non-biodegradable and biodegradable bioplastics. Biodegradable plastics can be produced from a variety of sources, including petroleum-based plastics that have been made biodegradable. While bio-based plastics are produced from renewable resources, biodegradable plastics can be converted into natural components by the action of microorganisms. A bioplastic may or may not be biodegradable; therefore, although these two terms are related, they are not interchangeable (Cinar et al, 2020).

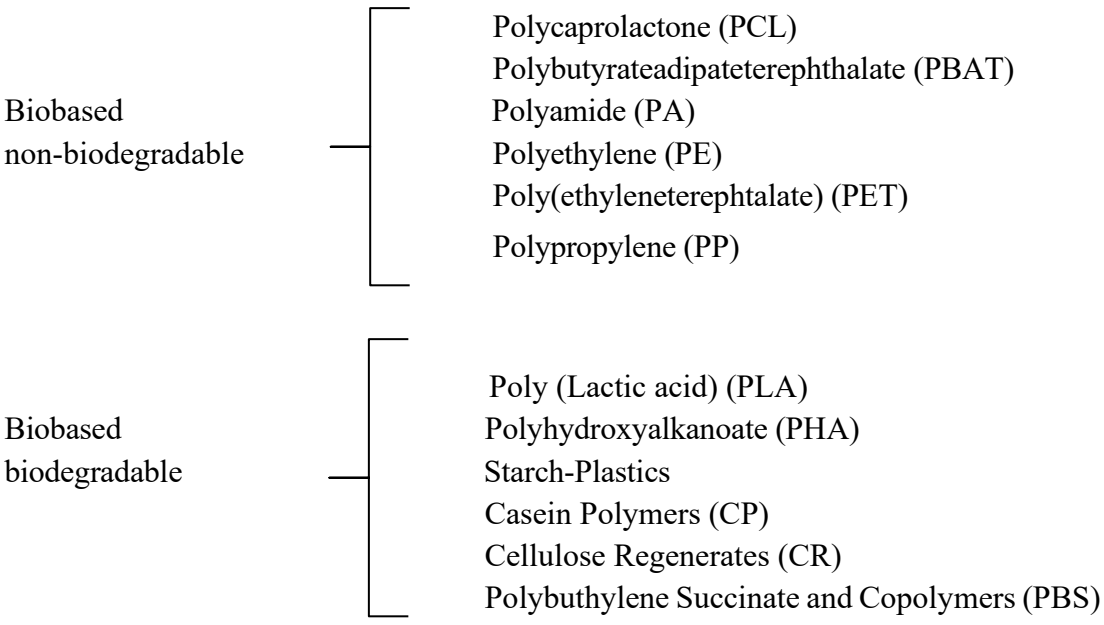


Figure 1. Non- biodegradable and biodegradable bioplastics.

In view of the rapid spread of global plastic production, new products and application areas are expected to increase. It is inevitable that biobased and degradable bioplastics will gain importance in terms of sustainability as the areas of use of plastics expand to a wide area with increasing technology. According to data from European Bioplastics (European Union, 2013), bioplastics production capacity was 2.47 million tons in 2024 (Figure 2). According to these data, the use of biobased non-biodegradable bioplastics in 2024 was 43.7%, while biobased degradable plastics are 56.3%. In 2029, this amount is targeted to reach 5.73 million tons and 66% of biobased-degradable bioplastics is desired.

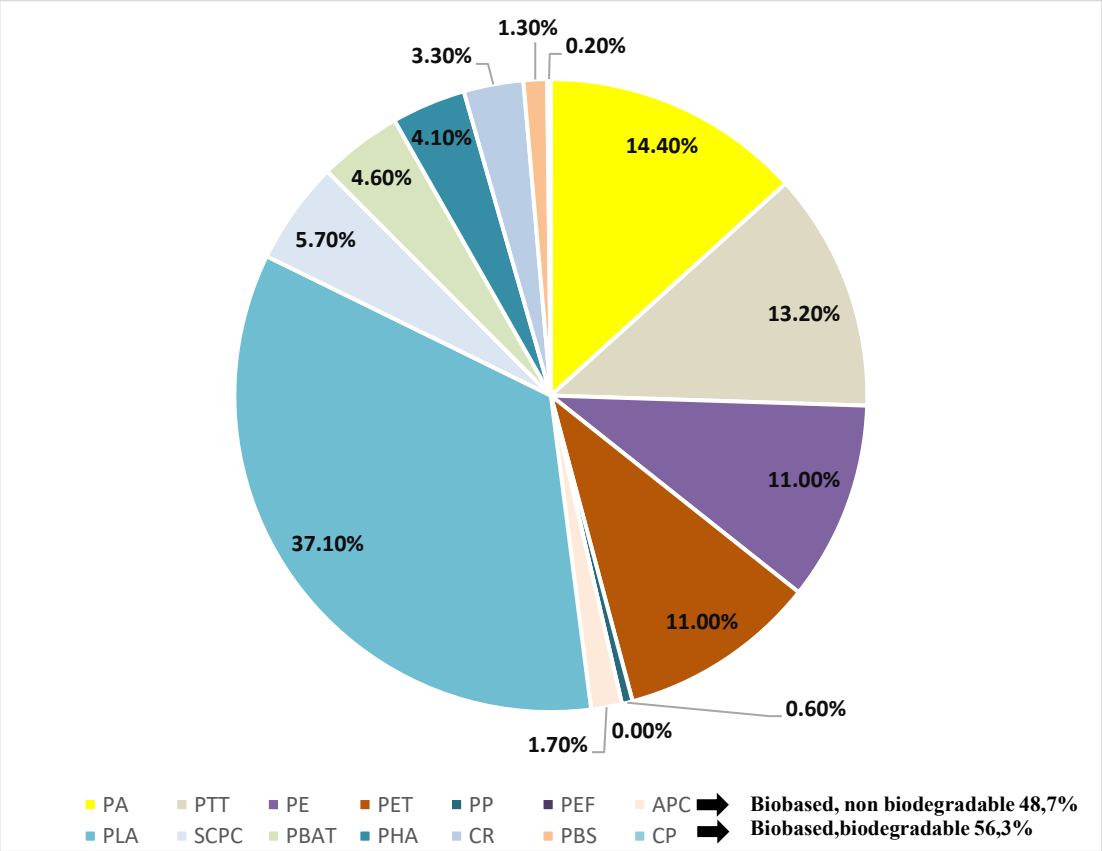


Figure 2. Global production capacities of bioplastics in 2024.

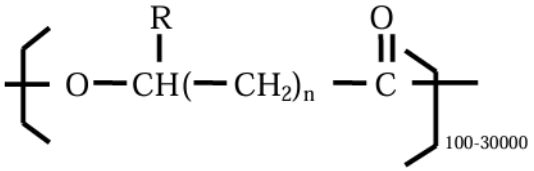
1.1.2. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are group of microbial polyesters that are biobased and biodegradable, sustainable and fully recyclable, especially in bioplastics production (Chanprateep, 2010; Reddy et al., 2003). Willem Bijerinck, a Dutch microbiologist, first identified PHAs in microorganisms in 1988. In 1925,

French microbiologist Lemoigne discovered PHAs in *Bacillus megaterium* (Marchessault, 2009).

PHAs can be produced from many Gram-positive and Gram-negative bacteria, either wild-type or genetically modified. Various microorganisms synthesize PHAs, especially in carbon-abundant environments, and store nearly all of their dry weight in granules in their cytoplasm (Madison and Huisman, 1999). PHA accumulated in the cytoplasm has been observed to be more than carbon storage over time. It has been determined that it helps microorganisms to survive in nutrient lack, provide energy, and adapt to stress conditions such as osmotic pressure, temperature and pH (Chen, 2009). In general, PHAs are classified according to the number of carbons in their units (monomer size) (Figure 3);

- PHAs containing 5 carbons; short-chain length (SCL).
- PHAs containing 6-14 carbons; medium-chain length (MCL).



$n = 1$	R= hydrogen	Poly (-3- hydroxypropionate) (P3HP)
	methyl	Poly (-3-hydroxybutyrate) (P3HB)
	ethyl	Poly (-3-horoxivalerate) (P3HV)
	propyl	Poly (-3-hydroxyhexanoate) (P3HH)
	pentyl	Poly (-3-hydroxyoctanoate) (P3HO)
$n = 2$	R= hydrogen	Poly (-4-hydroxybutyrate) (P4HB)
$n = 3$	R= hydrogen	Poly (-5-hydroxyvalerate) (P5HV)

Figure 3. General structure of PHAs (Ensari, 2012).

Polyhydroxybutyrate (P3HB), the most studied and well-received PHA type, belongs to this group and is a homopolymer. Many bacteria, such as *Bacillus megaterium, Cupriavidus necator,* and others, can produce scl-PHAs (Favaro et

al., 2019). One of the advantages that monomer differences provide to PHAs is the carbon source association of PHA synthase (polymerase) enzymes that recognize monomers. This allows different microorganisms to synthesize PHA to combine with diverse carbon sources to produce polymers in various variations (Purwadi, 2006).

1.1.3. Poly (-3-hydroxybutyrate)

Poly-3-hydroxybutyrate (P3HB) is one of the most preferred PHAs due to its similarity to polypropylene (PP). P3HB can be shaped, extruded or molded into fibers or films. P3HB has other advantages that make it a preferred alternative to traditional petrochemical-based polymers (Chen and Wang, 2002). Today, P3HB is produced using many different techniques. The most preferred of these techniques is the production by microorganisms as a secondary metabolite. P3HB is usually produced in response to dietary deficiencies such as nitrogen or phosphorus, or in response to the limitation of abundant carbon sources such as glucose or other sugars. Enzymatic reactions are part of a complex mechanism that produces P3HB. As the first step in microbial P3HB synthesis, the enzyme 3-ketothiolase condenses two acetyl-CoA moieties into acetoacetyl-CoA, which is converted to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase. (R)-3-hydroxybutyryl-CoA is polymerized to P3HB by P3HBsynthase. All genes involved in P3HB synthesis are contained within a structure known as the P3HB biosynthesis operon (Zarei et al., 2013; Wróbel et al., 2020).

1.1.3.1. Structure and properties of P3HB

P3HB is a-short-chained PHA, with a molecular weight ranging between 50 kDa-1000 kDa and is obtained by methyl bonding instead of radical group (Madison and Huisman, 1999). Thanks to the attached functional groups, the polymer has some physical and mechanical properties such as hydrophobicity, thermoplasticity, melting temperature, high crystallinity or brittleness (Philip et al. 2007; Grigore at el., 2019). The thermal properties of P3HB and derivatives of

semi-crystalline materials are usually divided according to three temperature values.

- glass transition temperature (Tg) for amorphous phases,
- melting temperature (Tm) for crystalline phases,
- decomposition temperature (Td) where the material degenerates (Kansiz et al., 2007).

In Table 1, a comparison of the mechanical properties of P3HB and petroleum-based plastics with high commercial production capacity is given (McAdam et al.,2020). When the mechanical properties are examined, melting temperature, tensile strength and degree of crystallinity are quite similar to PP. One of the most important properties in determining the mechanical properties of a polymer material is crystallinity. The reason is that crystallinity gives information about the molecular structure of the material (Shrivastava, 2018; Kong and Hay, 2002). In biopolymers, as the degree of crystallinity increases, hard and strong but at the same time more brittle material is obtained. According to the studies, the degree of crystallinity of P3HB is between 60%-80% (Kansiz et al., 2007; Vroman et al., 2009; Sharma et al., 1995; Jendrossek et al., 2002; Santos et al., 2017). For this reason, P3HB processing is considered one of the most challenging steps in the industrialization of P3HB. In order to expand the processing area of the produced P3HB, to improve the polymer or to increase its processability, it can be blended with derivatives of the polymer. For instance, while P3HB is brittle and hard, the addition of P3HV monomers provides a low melting temperature and this can improve/change the physical properties of the final material (Philip et al. 2007). As another example, P4HB is a strong but easy to process polymer. Its use in combination with P3HB, especially due to its high tensile strength, expands its range of applications (McAdam et al.,2020; Philip et al., 2007).

Table 1. Mechanical properties of P3HB, polypropylene (PP), polyethylene terephthalate (PET), low density polyethylene (LDPE), high density polyethylene (HDPE).

Mechanical Property	P3HB	PP	PET	LDPE	HDPE
Crystallinity (%)	50-60	42-58	7.97	25-50	60-80
Melting Temperature (°C)	170	160-169	260	115	135
Tensile strength (MPa)	20-40	31-45	62	30	30-40
Tensile modulus (GPa)	3-3.5	1.95	9.35	0.26-0.5	0.18

1.1.3.2. Production of P3HB

P3HBs are microbially synthesized biopoliesters. They are produced by wild-type or genetically modified microorganisms including bacteria and archaea (Chen, 2009). In addition, there are studies involving the production of biopolymers from genetically modified plants (transgenic plants) (Dobrogojski et al., 2018; Bohmert et al., 2000; Nawrath et al., 1994). Several bacterial, such as *Alcaligenes, Pseudomonas and Cupriavidus,* have been identified as wild-type P3HB producers and the synthesized P3HB is used as carbon reserves in the microorganism to fulfill energy needs (Sayyed et al., 2021; Zhang et al., 2022).

Gram-negative and gram-positive bacterial species have the ability to synthesize P3HB and accumulate it as granules in their cytoplasm. However, the production of P3HB is classified into two groups according to microorganism' ability to produce P3HB and variability of the culture conditions. In the first group, microorganisms are exposed to various stress conditions such as limiting essential nutrients (nitrogen, phosphorus and sulfur) in the presence of a carbon source. P3HB production by *Alcaligenes eutrophus, Protomonas oleovorans* and *Protomonas extorquens* can be classified in this group. The second group can produce P3HB during growth without the need for any restrictions. P3HB production by *Azetobacter vinelandi, Alcaligenes latus* and recombinant *Escherichia coli* belong to this group (Khanna and Srivastava, 2005).

Especially analytical methods have been used in the determination of P3HB in recent years. Devices such as Gas chromatograph (GC), Nuclear magnetic resonance

(NMR), High performance liquid chromatography (HPLC), Fourier transform infrared spectroscopy (FTIR) provide ethical and rapid P3HB analysis (Kanzariya et al., 2024; Kanabenia et al., 2024; Khang et al., 2021; Duvigneau et al., 2021). In addition, it is also possible to observe the granules accumulated in the microorganism microscopically. Staining is required to observe P3HB from microscopic processes. Generally, Sudan Black B stain is used in light microscopy observations (Porras et al., 2017), while Nile Blue stain is used in fluorescence microscopy (Mesquita et al., 2015). Electron microscopes are also very useful for detailed structural images. Microscope images of P3HB producing microorganisms are given in Figure 4.

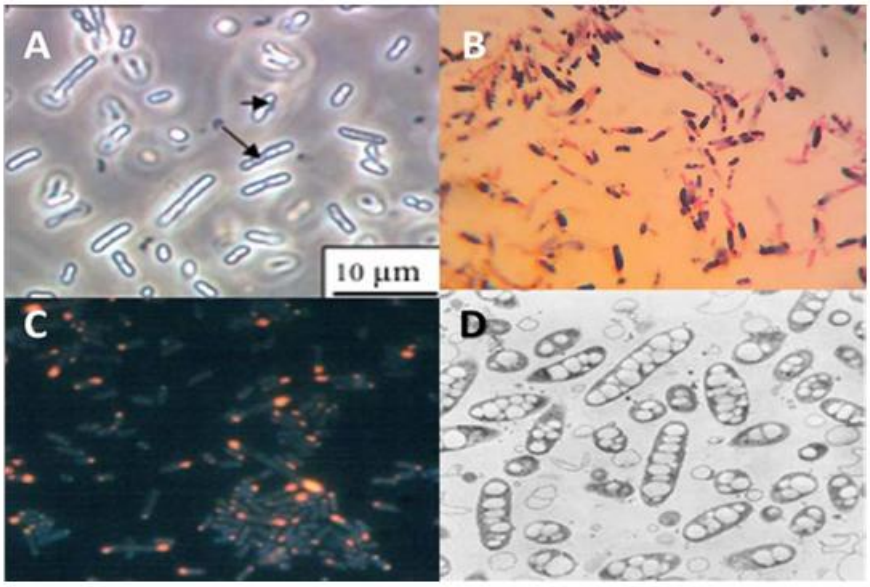


Figure 4. Microscopic images of some microorganisms producing P3HB. **A**) Phase-contrast microscopy visualization of P3HB granules in *C. necator* (Chee et al. 2010). **B**) Visualization of P3HB granules in recombinant *E. coli* by light microscopy (Ensari, 2012). **C**) Fluorescence microscopy visualization of P3HB granules in *recombinant E. coli* (Sato et al. 2007). **D**) TEM image of P3HB granules in *Pseudomonas resinovorans* (Solaiman et al. 2003).

1.1.3.3. Wild-type microorganisms for the production of P3HB

The first initiative that produce commercialized PHAs and its derivatives by microorganisms is Imperial Chemical Industries Ltd (ICI) which was built in the 1970s (Philip et al., 2007). Initially, ICI focused on *Methylophilus methylotrophus* and its

strains, conducting research on wild-type bacterial species with PHA production potential (Bourque et al., 1995). These bacteria, which use methanol as a carbon source for polymer production, have a very low yield in terms of the polymer produced per cell. The second preferred microorganism was *Azotobacter* species as a result of their well-characterized structure (Lasemi et al., 2013). During polymer production, these species produced polysaccharides as a by-product, resulting in lower product yields. Thirdly, *Ralstonia eutropha*, which can synthesize P3HB using fructose as carbon source, was evaluated. This microorganism, which stands out for its capacity to accumulate P3HB with the restriction of essential nutrients such as nitrogen, was successfully produced in 200,000 liter continuously-stirred tank reactor (CSTR) (Sandhya et al., 2013). Wild-type P3HB producing microorganisms commonly used in industrial P3HB production are given in Table 2.

Table 2. Wild-type P3HB producing organisms commonly used in industrial P3HB production.

Microorganism	**Carbon Source**	**P3HB Productivity (g/L/h)**	**References**
Alcaligenes latus	Fructose and glucose	0.12	Gomez et al., (1996)
Alcaligenes latus	Waste of malt	0.45	Yu et al., (1998)
Azotobacter beijerinckii	Glucose	0.09	Lasemi et al., (2013)
Cupriavidus necator	Carbon dioxide	0.23	Sonnleitner et al., (1979)
Pseudomonas multivorans	Glycerol	0.10	Zhu et al., (2010)
Cupriavidus necator	Potato starch	1.47	Haas et al., (2008)
Cupriavidus necator	Waste glycerol	4.20	Cavalheiro et al., (2009)
Methylobacterium extorquens	Methanol	0.60	Bourque et al., (1995)

Pseudomonas aeruginosa	Cane molasses, fructose, glucose, glycerol, sucrose	0.01	Tripathi et al., (2012)
Methylobacterium extorquens	Methanol	0.98	Mokhtari-Hosseini et al., (2009)
Hydrogenophaga pseudoflava	Hydrolyzed whey and valerate	0.05	Koller et al., (2007)
Cupriavidus necator	Molasses	0.12	Beaulieu et al., (1995)
Alcaligenes latus	Sucrose	3.97	Yamane et al., (1996)
Azotobacter vinelandii	Molasses	1.40	Page, (1996)

1.1.3.4. Recombinant microorganisms

Escherichia coli is one of the most widely used bacteria for P3HB production among recombinant microorganisms. One of the main reasons why *E. coli* is frequently preferred in this field is that it does not naturally possess genes encoding the P3HB depolymerase enzyme (Chen, 2009). PHA depolymerase enzyme causes the polymer produced (PHA) to break down inside the cell. *E. coli* lacking this enzyme allows the polymer to accumulate and stockpile inside the cell (cytoplasm). This provides an important advantage in terms of increasing the final product yield. Moreover, *E. coli* shows very rapid growth under optimal conditions. The increase in its metabolic activity, especially at high temperatures, allows the biomass yield and thus PHA accumulation to reach high levels in a short time (Kim et al.,2021; Yang et al., 2022; Jung et al., 2019). In addition, the cell wall of *E. coli* is surrounded by a thin layer of peptidoglycan and an outer membrane. This structure allows the cells to be easily broken down by physical and chemical methods (Lee, 1996). This feature allows both shortening the processing time and working with various solvents in the extraction and purification stages, which are an important part of the production process. Thus, a more cost-effective and environmentally friendly process design becomes possible (Chen and Jiang, 2018; Madkour et al., 2013).

rec.*E. coli* can work with high specificity in the production of targeted P3HB monomers and production efficiency can be further increased by various genetic engineering approaches. *E. coli* has the capacity to efficiently metabolize various substrates as carbon sources. These carbon sources are usually available from renewable and sustainable resources such as agricultural wastes or industrial residues (Kourmentza et al., 2017). This reduces the cost of raw materials used in PHA production and increases environmental sustainability. In particular, the ability of rec. *E. coli* to produce polymers in high yields using such low-cost carbon source provides a great advantage in terms of economic production on an industrial scale (Obruca et al., 2014). When all these features are evaluated, the use of rec. *E. coli* for PHA production draws attention with its high production efficiency, low process costs, easy cell lysis and ability to utilize various carbon sources. For these reasons, *E. coli* is one of the most preferred and studied model organisms in industrial biotechnology applications for bioplastic production (Kourmentza et al., 2017).

In literature, there are several studies that show the important potential of rec. *E. coli* usage for the production of PHA and its derivatives. The metabolically engineered *E. coli* was reported to be able to produce enantiomerically pure (R)-3-hydroxybutyric acid (R3HB) of 7.70 g/L using glucose as carbon source with a yield of 49.5% (g product/g dry cell weight, DCW) (Lee et al., 2003). Similarly, the effect of *Ralstonia eutropha* on P3HB production in recombinant *E. coli* expressing P3HB biosynthesis genes was investigated, and it was reported that 5.31 g P3HB/L and 9.24 g DCW/L were obtained after 48 hours of culture (Park et al., 2019). In a different approach, *Alcaligenes latus* was used as the source of PHAs biosynthesis genes. Rec. *E. coli* using glucose and oleic acid produced 2.88 g/L P3HB with 78% yield (Choi and Lee, 1999). Moreover another study, using molasses, rec. *E. coli* constructed from *Ralstonia eutropha* biosynthesis genes produced 1g/L P3HB with a yield of 80% (Liu et al., 1998).

1.1.3.5. Raw material for the production of P3HB

In order to produce P3HB from bacteria, it is desirable to produce the most efficiently and in high quantities, and the carbon source selected should be low-cost, easily accessible and the growth of the bacteria should be rapid. In addition to the

microorganism selection for P3HB production, bioconversion parameters, such as carbon source and amount, nitrogen source and amount, agitation rate, temperature, pH, etc., are very essential. It is a rule of thumb that in order to produce P3HB from microorganisms, the first parameter to be examined is the choice of carbon source (Sudesh and Abe, 2010). Animal and vegetable oil, glucose, molasses, starch, whey, waste food products, waste fruit and vegetable peels are used in P3HB production (Reddy et al., 2003). The choice of a specific carbon source in these processes is important, not only in terms of influencing the overall cost of P3HB production, but also in terms of influencing various important parameters such as cell growth, product efficiency, molecular mass of P3HB, carbon efficiency and polymer composition (Favaro et al., 2019). Table 3 shows the amount of P3HB (polymer concentration) produced by wild-type and recombinant microorganisms used in the studies according to carbon sources.

Table 3. Different micoorganisms and carbon sources used to produce P3HB.

Microorganism	**Carbon source**	**P3HB (g/L)**	**References**
Wild- type strains			
Paraburkholderia sacchari	Xylose	16.40	Oliveira-Filho et al., (2020)
Haloferax mediterranei	Macroalgal biomass	2.20	Ghosh et al., (2019)
Cupriavidus necator	Seed oil	11.77	Yousuf, (2018)
Cupriavidus necator	Palm oil	109.50	Zainab-L and Sudesh, (2019)
Pseudomonas putida	Glucose and nonanoic acid	31.40	Davis et al., (2015)
Cupriavidus necator	Molasses	0.77	Sen et al., (2019)
Chelatococcus daeguensis	Glycerol	17.40	Cui et al., (2015)
Cupriavidus necator	Sugarcane vinnase	11.70	Dalsasso et al., (2019)

Ralstonia eutropha	Paddy straw	7.21	Sandhya et al., (2013)
Cupriavidus necator	Beer brewery wastewater and maltose	3.00	Amini et al., (2020)
Bacillus megaterium	Sucrose	41.60	Kanjanachumpol et al., (2013)
Halomonas sp.	Glucose and corn powder	71.30	Ren et al., (2018)
Pseudomonas mendocina	Biodiesel liquid waste	2.60	Chanasit et al., (2016)
Azotobacter vinelandii	Glucose	30.30	Subramanian et al., (2020)
Cobetia amphilecti	Avocado seeds	2.10	Gnaim et al., (2023)
	Recombinant strains		
Pseudomonas oleovorans NRRL B-14682	Crude glycerol	1.14	Ashby et al., (2011)
Bacillus safensis EBT1	Sugarcane bagasse	1.20	Sakthiselvan and Madhumathi, (2018)
Cupriavidus necator CCGUG	Kitchen waste	0.38	Omar et al., (2011)
Cupriavidus necator 428	Glycerol	0.54	Das and Grover, (2018)
Cupriavidus necator H16 (DSM 428)	Waste fish oil	103.80	Loan et al., (2022)

1.1.3.6. Extraction and purification of P3HB

P3HB extraction is one of the most challenging processes in the bioplastics production flow. After the production of P3HB granules, the steps of cell harvesting, pretreatment, if necessary, extraction and purification of the polymer follow one another (Samorì et al., 2015; Jacquel et al., 2008). The most extensively studied methods for P3HB extraction are enzymatic digestion (Koller, 2020), acid/base extraction (Mannina et al., 2020), mechanical digestion (Jacquel et al., 2008), solvent extraction (Mongili et al., 2021), sodium dodecyl sulfate (SDS) sonication and gamma irradiation (Raza et al., 2018). The methods used for P3HB extraction, and their advantages and disadvantages are summarized in Table 4.

Table 4. Methods used in P3HB purification and their advantages and disadvantages.

Extraction Methods	**Advantages**	**Disadvantages**
Enzyme extraction	Moderate application conditions Good quality product Environmentally friendly	Complex process High cost
Mechanical extraction	No chemicals used Low contamination	Long time required Complex process
Solvent extraction	High purity High quality High molecular weight Low P3HB degradation	Not environmentally friendly Use of toxic solvents Use of volatile solvents High cost High temperature required
Surfactants with chemicals	Simple Not complex process Cheap Fast Environmentally friendly	Low purity High wastewater
Supercritical fluids		Dependent on process parameters

Gamma radiation	No chemicals used Low contamination	Length of the radiation time High investment costs
High pressure homogenization	No chemicals used Low contamination	Dependent on process parameters Thermal degradation
Green chemical	Enviromental friendly Sustainable process High elasticity	High cost Complex process Long time required High temperature required

Enzymatic digestion is an eco-friendly extraction method compared to solvent or chemical digestion, but it is not preferred due to polymer-specific enzyme selection, expensive and complex processes. Bead mill disruption, high pressure homogenization, ultrasonication systems are the most commonly used methods in mechanical digestion. Although those mechanical digestions are economical approach, they have low efficiency due to their long time and lack of chemical/surfactant/organic solvent.

Solvent extraction is the most preferred method because it is simple and fast. In this process, the permeability of the cell membrane is firstly increased and P3HB is dissolved in organic solvents. Surfactants such as SDS, Triton X-100, betaine and palmitoylcarnitine are also used to extract P3HB. One of the most significant advantages of SDS is that it does not disrupt P3HB granules while lysing the cell membrane (Ramsay et al., 1990; Jacquel et al., 2008). However, when used by itself, P3HB cannot be obtained in high purity, so the use of sodium hypochlorite increases efficiency and purity (Ramsay et al., 1990). P3HB production followed by treatment with a solvent such as 1,2-dichloroethane, chloroform, cyclohexanone and 1,2-propylene carbonate, can be used as an alternative solvent extraction method and P3HB is precipitated in non-solvent alcohols (Kunasundari and Sudesh, 2011; Fiorese et al., 2009). It is well known that extractions using solvents have the advantages of higher yields and pure products but have negligible polymer degradation. The most

commonly used solvents for the extraction of P3HBs are chlorophosome or sodium hypochlorite (Getachew and Woldesenbet, 2016; Kanavaki et al., 2021). In the production of P3HB from *E. coli*, sodium hypochlorite and chloroform are most commonly preferred together for cell lysis and extraction processes (Ramachander et al., 2002; Agus et al, 2010; Wang et al., 2012; Leong et al., 2014). Organic solvents or alcohols such as methanol and ethanol used for the precipitation of P3HB can be used to obtain high purity P3HB, but at the same time, they are very harmful to the environment as toxic substances due to their untreated disposal, especially on commercial scales (Kunasundari and Sudesh 2011).

While the purpose of P3HB production is to pave the way for the use of a sustainable and ecosystem-friendly alternative plastic, utilization of environmentally unfriendly chemicals in its extraction casts a shadow on the preferability of P3HB when the whole process is considered. Therefore, various alternative approaches used for P3HB recovery have focused on the use of more sustainable solvents such as dimethyl carbonate, propylene carbonate, ethyl lactate and γ-valerolactone (Pagliano et al., 2021). However, additional inexpensive, non-toxic, biodegradable and sustainable solvents are needed to expand the knowledge on P3HB extraction methods and in the biopolymer industry.

More recently, green chemistry-based purification approaches are preferred, where P3HB is dissolved in an environmentally friendly solvent and the cell membrane (and cell wall, if there is any) is disrupted to release the polymer. In order to obtain the polymer (to separate it from the solvent), evaporation, drying and precipitation processes are usually applied, preferably sometimes at high temperatures. In the study conducted by Wongmoon and Napathorn (2022), P3HB was extracted from *Cupriavidus necator* using 1,3-dioxolane and a yield between 99.3% was obtained as a result of extraction using high temperature (80°C) and continuous shaking processes. In another study, Gnaim et al. (2023) produced P3HB by *Cobetia amphilecti* using avocado seeds as carbon source and used ethyl levulinate as the green solvent. They reported that the obtained P3HB had superior properties compared to the extraction process with chloroform (a conventional method), with a recovery rate of 97.4%. In a similar article, Mongili et al. (2021) extracted P3HB from recombinant *E. coli* using

dimethyl carbonate (DMC) and ethanol with an extraction efficiency of over 67% using wet biomass.

1.1.4. Application areas of P3HB

P3HB, which stands out as an alternative to petrochemical plastics, has gained potential for use in a wide range of products, including reusable and disposable bags, plates and cutlery, packaging, coating materials, feminine hygiene products, beverage cans, and the automotive sector (Chen, 2009). Its low oxygen permeability makes it particularly advantageous for use in food packaging (Naser et al., 2021). In the medical and pharmaceutical sectors, due to its biocompatibility, it is frequently used in surgical sutures and bandages, as capsules in drug delivery systems, wound dressings and scaffolds, tablets, and implants (Pulingan et al., 2022). In agriculture and horticulture, biopolymers are used in the production of biodegradable coatings and films, without harming the soil or the environment (Oyedeji et al., 2024). P3HB has been used in wastewater treatment, biofilm support materials, and corrosion-preventive biopolymer coatings. It provides environmental protection by promoting biofilm formation, especially in submarine structures such as concrete barriers. (Chai et al., 2024).

1.1.4.1. Application of packaging and disposable products

The most common use of P3HB is in biodegradable packaging materials. Due to its low oxygen permeability, moisture barrier properties, and mechanical strength, it is widely used in the food packaging industry (Koller et al., 2005). At the same time, due to the low glass transition temperature of P3HB, it is also considered a suitable alternative for cold chain packaging (Chen and Patel, 2012). Additionally, P3HB-based films and containers are used to extend the shelf life of food products and reduce microbial contamination. They also offer environmentally friendly solutions for single-use products such as cutlery, cups, and plates (Laycock et al., 2013).

1.1.4.2. Application of medicine and biomedical

The fact that P3HB is biocompatible and does not produce toxic degradation products makes it an important biomaterial in the medical field. It is widely used in applications such as tissue engineering, biodegradable suture threads, bone-tissue scaffolds, and controlled drug delivery systems (Misra et al., 2006). The ability of

P3HB to degrade into water and carbon dioxide in a biological environment allows it to be used safely in such implants (Lezcano et al., 2022). P3HB is frequently used as a scaffold material in bone and cartilage tissue engineering studies (Misra et al., 2006). Various in vitro and in vivo studies have shown that it supports osteoblast proliferation and exhibits high compatibility with bone cells (Kai et al., 2016). Additionally, it is known that P3HB microcapsules in controlled drug delivery systems contribute to targeted therapy by enabling time-dependent drug release (Philip et al., 2007). In the other hand, P3HB-based fibers used in suture threads have been reported to accelerate the healing process and have the potential to reduce post-implant inflammation (Wu et al., 2022).

1.1.4.3. Application of agriculture

P3HB also has significant potential applications in the agricultural sector due to its environmentally friendly properties. The use of P3HB-based biodegradable materials in agriculture plays an important role in reducing environmental waste. They are particularly used in seedling bags, mulching films, biodegradable fertilizer capsules, and pesticide carrier systems (Amelia et al., 2019). P3HB can be easily broken down by microorganisms in the soil and therefore causes no damage to the physical or chemical structure of the soil (Sudesh et al., 2000). Through its controlled release property, fertilizers integrated into P3HB matrices can be delivered to plants in a more sustained and balanced release (Ntaikou et al., 2009). It has also been reported that the degradation products released during P3HB production are easily utilized by soil microorganisms, which improves soil quality and supports microbial activity (Tokiwa et al., 2004). This demonstrates that P3HB is a material that can be integrated into the environmental cycle and is compatible with nature (Anderson and Dawes, 1990). Within the scope of environmental engineering applications, P3HB-based materials are evaluated in many areas such as wastewater treatment, heavy metal adsorption, and contaminant removal with biofilms. For example, the modified form of P3HB with polyionic complexes can act as an effective adsorbent for removing heavy metals (e.g., Pb^{2+}, Cd^{2+}) from aqueous environments (Misra et al., 2006).

1.1.4.4. Applications textile and nanofiber

The applicability of P3HB in fiber production, particularly its ability to be processed using techniques such as melt-spinning and electrospinning, provides a

solution to the textile industry's search for biodegradable materials. P3HB fibers have significant potential for widespread use in medical textiles, hygiene products, and short-lived fiber-based products (Battegazzore et al., 2019). These fibers are used in a wide range of applications, including wound dressings, biodegradable clothing, medical textiles, and filtration systems (Laycock et al., 2013). Especially, the large surface area of electrospun P3HB nanofibers provides advantages in terms of cell adhesion and proliferation. P3HB fibers impregnated with antibacterial properties are also promising for medical surface coatings (Kaniuk et al., 2021). Additionally, P3HB-based fibers may replace conventional polyesters, and industrial-scale production formulations are being developed (Bugnicourt et al., 2014).

1.1.4.5. Applications in electronics and biodegradable technologies

In recent years, there has been growing interest in P3HB for environmentally degradable electronics and nanoscale devices due to its high purity and suitability for controlled surface modifications (Schramm et al., 2012). It is particularly evaluated as a biodegradable base material in microfluidic systems, biosensor substrates, and temporary electronic devices (Bozó et al., 2021). P3HB-based nanocomposites can be used in biodegradable electronic circuits and sensor bases (Uva et al., 2022). Also, P3HB has been made suitable for use in the production of flexible electronic circuit substrates by combining it with conductive polymers (Arrieta et al., 2015). Such composites offer sustainable and biocompatible solutions in implantable biosensors or environmentally sensitive medical monitoring systems (Keshavarz and Roy, 2010). The development of electronic components that leave a zero footprint in nature is particularly important in the production of short-lived environmental sensors from the perspective of sustainable technological approaches.

1.1.4.6. Applications in composite materials and the automotive industry

P3HB can be used in the production of composite materials together with various biopolymers and natural fibers. These composites, made with PLA, PCL, and cellulose reinforcements, are used in construction materials, automotive interiors, and durable consumer products (Wang et al., 2014). P3HB-based composites are most commonly used in the automotive industry. In particular, the use of these biocomposites is becoming increasingly widespread in interior panel coatings, door interior parts, dashboard panels, and decorative surfaces. The primary reasons for the

preference of such materials in the automotive industry include weight reduction and fuel consumption reduction, environmental sustainability, and ease of disposal at the end of their service life (Satyanarayana et al., 2009). P3HB-based natural fiber-reinforced composites offer a weight advantage over traditional PP-based materials due to their lower density, while also providing sufficient stiffness and impact resistance to make them competitive for automotive interior applications (Rahman et al., 2023). P3HB composites supported by lignocellulosic fillers have a high Young's modulus, tensile strength, and thermal resistance. These types of biocomposite systems are also important in terms of reducing the environmental footprint and carbon emissions (Brodin et al., 2017).

1.1.5. Global P3HB producer companies

P3HB and its derivatives, produced at pilot and industrial scales, are listed below, along with the companies and their annual production volumes. Numerous companies are working with P3HB in the hope of commercializing it and expanding its applications. P3HB, frequently used in biomedical and packaging applications, plays a critical role in its production and waste management. The companies that produce PHA and derivatives commercially are listed in Table 5.

Founded in Italy in 2007, Bio-on, with the mission of producing PHA and its derivatives, aimed to integrate with the agri-food industry using its existing technological infrastructure. Unfortunately, their investments were unsuccessful, and the company sought to produce polymers using carbon dioxide as raw material. They also utilize glycerol, sugar cane, and food waste to achieve sustainable and environmentally friendly production (Bio-on, 2025). TianAn Biopolymers, with an annual production capacity of 10,000 tons, became the world's largest P(3HB-co-3HV) producer in 2021. They produce polymers by fermenting *Cupriavidus necator* on D-glucose and propionic acid. They use P(3HB-co-3HV) to produce injection molding, films, and extrusion materials (TianAn Biopolymer, 2025).

Tianjin is the first company in China to produce 10,000 tons of PHA per year. They have developed fully biodegradable products for blown film processing. They have also developed P3HB foam pellets, which can be processed into fully

biodegradable foams for the food industry and appliance packaging. Kaneka Corporation, operating in Japan, produces P(3HB-co-3HHx). Production capacity reported in 2007 was 100 tons per year and reached 50,000 tons in 2020. It is produced through a microorganism fermentation process using vegetable oils and fatty acids as the main raw materials.

In recent years, P3HB production has reached commercial scale as technology has advanced, and sustainable production models have been adopted, particularly from waste products, replacing acid-derived carbon sources. In this context, China and the US are leading the market with annual production capacities of tens of thousands of tons. New technology companies are producing low-carbon, renewable biopolymers using diverse carbon sources such as atmospheric gases or food waste. Companies focusing on medical applications are developing P3HB-based products for surgery and tissue engineering with Food and Drug Administration (FDA) approvals.

Table 5. Companies that commercially produce PHA and its derivatives.

Country	**Company**	**PHA Type**	**Capacity (tones/year)**
UK	ICI	PHBV	300
China	Tianan	P(3HB-co-3HV)	50,000
China	Jiangsu Nan Tian	P3HB	Pilot scale
Japan	Kaneka	P(3HB-co-3HHx	50,000
Germany	Biomers	P3HB	Unknown
USA	ADM	Various	50,000
USA	Danimer	Various	272,000
Austria	Chemie Linz	P3HB	20-100
Austria	BTF	P3HB	20-100
Brazil	PHB Industrial	P3HB P(3HB-co-3HV)	50,000
Brazil	Biocycles	P3HB	100
China	TianAn Biopolymer	P(3HBV)	2,000
China	Tianjin Northern Food	P3HB	10,000

Japan	Mitsubishi	P3HB	10
USA	Yield10 Bioscience	P3HB copolymers	50,000
Italy	Bio-On	Various	10,000
China	GreenBio-DSM (TEDA Tianjin)	P(3HB-co-4HB)	10,000

SECTION TWO

MATERIALS AND METHODS

2.1. Materials

This section provides detailed explanations of the equipment, media, raw materials, and chemicals used.

2.1.1. Equipment

The equipment used in this thesis and their brand and model details are given in Table 6.

Table 6. Equipment used in this thesis.

Equipment	Model
Grinder	Lavion HC100
Magnetic stirrer	DLAB MS-H380-Pro
Precision balance	ACU HJ-150
Orbital shaker	Mikrotest MSCHH-A
Oven	DAIHAN WiseVen WOF-105
Vortex mixer	SCILOGEX MX-S
Centrifuge	Nüve NF 800R
Water bath	DAIHAN Scientific
pH meter	WTW inolab
UV/Vis spectrophometer	Thermo Fisher Scientific MULTISKAN SkyHigh
Autoclave	NUVE NC 40M

2.1.2. Media

2.1.2.1. Antibiotic preparation for maintenance of the microorganism

Ampicillin (Merck) was prepared by dissolving it in water to achieve a final concentration of 0.1 g/ml. Due to its heat sensitivity, the non-autoclaved ampicillin

was passed through 0.22 µm filters and transferred aseptically to sterile centrifuge tubes using a Bunsen burner flame. It was divided into portions with a final concentration of 100 µg/ml and stored at -20°C in a freezer.

2.1.2.2. IPTG preparation for P3HB production

IPTG (isopropyl-β-D-thiogalactopyranoside) (GoldBio) was dissolved in water to a final concentration of 100 mM. Due to its heat sensitivity, the non-autoclaved IPTG was passed through 0.22 µm filters and added aseptically to sterile conical centrifuge tubes. This was divided into portions with a final concentration of 0.1 mM and stored at -20 °C. For P3HB productions initiated with a 2% inoculum, IPTG was added aseptically to the P3HB medium under a bunsen burner flame and under sterile conditions once the OD value reached 0.6–0.8.

2.1.2.3. Luria Bertani (LB) medium for inoculum culture

10 g/L of trypton (Condalab), 5 g/L of yeast extract (Condalab) and 5 g/L of NaCl (Condalab) were added to distilled water. After dissolution using a magnetic stirrer, the volume was adjusted to the final volume and the solution was transferred to shake flasks of 250 ml in the desired quantities. The shake flasks were then sterilized in an autoclave at 121 °C for 15 minutes. Once the sterilized LB medium had reached room temperature, ampicillin to a final concentration of 100 µg/ml was added under aseptic conditions. As shown in Figure 5, the inoculum culture is demonstrated.

Figure 5. LB medium for inoculum culture.

2.1.2.4. P3HB medium

The P3HB medium is composed of 2 g/L tryptone, 1 g/L $MgSO_4 \cdot 7H_2O$ (Merck), 7 g/L KH_2PO_4 (Kimyalab), 1 g/L citric acid (Merck), 2 g/L yeast extract and either 10 g/L glucose (Condalab) or 10 g/L raisin waste (RW). The pH of the prepared medium is adjusted to 7 using 5 M NaOH (Merck). The prepared shot bottles are sterilized in an autoclave at 121 °C for 15 minutes and ampicillin to a final concentration of 100 µg/ml is added under aseptic conditions once the P3HB medium has reached room temperature. Figure 6 illustrates the P3HB medium.

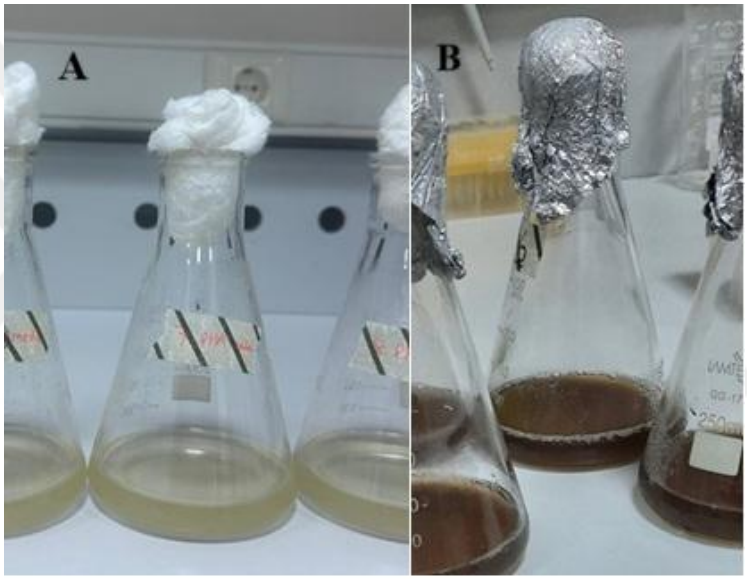


Figure 6. A) P3HB medium containing glucose and **B)** P3HB medium containing RW.

2.1.3. Microorganism

Recombinant *E. coli* Rosetta pQEAL1124 strain was obtained from the courtesy of Assist Prof. Dr. Yunus Ensari, Department of Bioengineering, Kafkas University (Figure 7).

Figure 7. rec. *E. coli* Rosetta pQEAL1124 strain used in this thesis**.**

2.1.4. Raisin waste

Raisin waste (RW) was used as carbon source for the strain. RW was obtained from PAGMAT Food Inc (Saruhanlı, Manisa).

2.2. Methods

2.2.1. Stock culture and maintenance of rec. *E. coli* Rosetta pQEAL1124

To prepare long-term microbial stocks, 10 ml of LB medium containing ampicillin and a 60% glycerol solution were sterilized separately in an autoclave at 121 °C for 15 minutes. After incubating the mixture at 37°C for 16 hours at 200 rpm, a 1:3 ratio mixture (500 µL glycerol: 1 mL culture) was prepared from the resulting microbial culture. The mixture was divided into sterile cryotubes under aseptic conditions and stored in a freezer at -20 °C and -86 °C for short-term and long-term use, respectively.

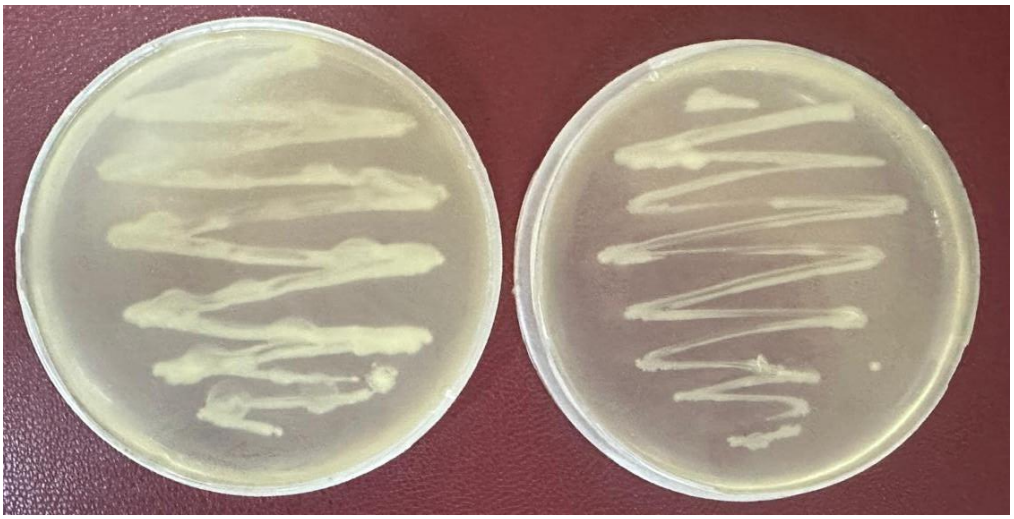


Figure 8. rec. *E coli* Rosetta pQEAL1124 stored in LB media.

Stock cultures were also maintained at +4 ºC using the same media supplemented with 10 g/L agar agar, autoclaved at 121°C for 20 minutes, cooled to 50°C, and 1 ml of 1000x sterile ampicillin stock solution was added, resulting in a final ampicillin concentration of 100 µg/ml. The medium was poured into sterile Petri dishes in appropriate quantities (approximately 20–25 ml), the lids were left partially open to allow moisture to escape, and the dishes were frozen at room temperature overnight. After freezing, the Petri dishes were sealed airtight and stored at 4°C for 15 days. Three inoculation loops of microorganisms were passaged into newly prepared Petri dishes at 15-day intervals. The Petri dishes were incubated at 37°C for 48 hours, then covered with parafilm and stored at +4°C (Figure 8).

2.2.2. Preparation of raisin waste

RW includes grape waste, crushed grapes, and grape juice with only the pulp remaining, along with the skins, as indicated in Figure 9A. The samples were dried at 60°C until a constant weight was achieved, then stored at room temperature. In order to determine the most appropriate concentration and form of the RW, different concentration values (1/4, 1/6, 1/8 and 1/10 w/v) and different forms (10-mm-size-RW and powdered-RW) of RW were studied. The powdered RW and the 10-mm-size-RW used in this thesis were shown in Figure 9B and 9C, respectively. In order to get the powdered form, a grinder was used. All RWs were autoclaved to release reducing sugars at 121 ºC for 15 minutes. After this step, solid-liquid separation was handled

using a centrifuge at 4100 rpm for 20 min. The selection of the most appropriate concentration and form was chosen based on the highest amount of released reducing sugar. The selected concentration and form were used as carbon source in all P3HB production studies.

Figure 9. A) RW includes grape waste, crushed grapes, and grape juice with only the pulp remaining, along with the skins, **B)** The powdered RW, **C)** 10-mm-size-RW.

2.2.3. Determination of the cell growth curve

To obtain the cell growth curve, production was carried out in 250-ml shake flasks containing 100 ml of LB broth in orbital shaker at 175 rpm for 60 hours. In experiments initiated with 2% inoculum, OD values were measured at 600 nm using a spectrophotometer at varying times (every 30 min or every hour) depending on the cell growth rate. All studies were conducted in triplicate. Equation 1 and Equation 2 were used to calculate the doubling time (td) and specific growth rate (μ):

$$t_d = \frac{0,693}{\mu}$$ Equation (1)

$$\mu = \frac{\ln OD_2 - \ln OD_1}{t_2 - t_1}$$ Equation (2)

OD_2 and OD_1 refer to the optical density (@600nm) at the end and beginning of the log phase, respectively; t_2 and t_1 refer to the times at which this optical density was obtained.

2.2.4. Dry cell weight determination

The dry cell weight (DCW) was determined by transferring 50 ml of P3HB medium culture into 250 ml shake flasks. After adding a 2% inoculum and 100 µg/ml of ampicillin, the cultures were incubated in a shaking incubator at 37 °C and 175 rpm for 48 hours. Once production was complete, the culture was transferred into conical centrifuge tubes. Subsequently, the culture was centrifuged at 4,100 rpm for 20 minutes and the supernatant was discarded. The samples were washed twice with distilled water, then dried in an oven at 60 °C overnight. Following drying, the DCW was calculated by subtracting the tare. All studies were conducted in triplicate.

2.2.5. Determination of the effects of psychochemical parameters on P3HB production

2.2.5.1. The effect of pH

A 250 ml shake flask was used to prepare 50 ml of P3HB medium. Due to the composition of the production medium, the pH value was acidic (pH = 4). After addition of 10 g/L of RW, the pH value set to a final value of 7 and 9. Following the addition of 100 µg/ml ampicillin, the preparation of a 2% inoculum and the subsequent addition of 0.1 mM IPTG. The mixture was then incubated at 37 °C and 175 rpm in a orbital shaker for 24 hours. Following a 24-hour period, samples were obtained and

DCW, P3HB, and yield analyses were conducted. All studies were conducted in triplicate.

2.2.5.2. The effect of carbon and nitrogen sources

Five different production conditions were applied to evaluate the effect of carbon and nitrogen sources in the production environment on cell growth and production. Production was carried out in 250 ml shake flasks containing 50 ml of P3HB medium (with 10 g/L of glucose). The P3HB medium contained 2% inoculum and was supplemented with 0.1 mM IPTG and 100 μg/ml ampicillin. It was then incubated at 37 °C and 175 rpm for 24 hours. Following a 24-hour period, samples were obtained and DCW, P3HB, and yield analyses were conducted. All studies were conducted in triplicate.

1. nonC-medium: Prepared with all contents identical to the control group, except that glucose (the carbon source) was removed from the media. Production was conducted for 24h.
2. nonT-medium: Prepared with all contents identical to the control group, except that one of the nitrogen sources (tryptone) was removed from the media. Production was conducted for 24h.
3. nonYE-medium: Prepared with all contents identical to the control group, except that one of the nitrogen sources (yeast extract) was removed from the media. Production was conducted for 24h.
4. 12h-medium: Production in this group was carried out in stages. For the first 12 hours, cell growth was maintained in a standard medium containing all components. At the end of 12 hours, the cells were collected by centrifugation (4100 rpm, 20 min) and transferred into a fresh PHA medium without yeast extract and tryptone, and production was continued in this medium for another 12 hours.
5. Control medium: The production media was prepared as standard to contain glucose, yeast extract, tryptone, and other basic components. It was used as control group. Production was conducted for 24h.

2.2.5.3. The effect of IPTG and RW addition

Four productions were carried out to determine the effect of time for adding IPTG and RW on P3HB production. A medium containing 10 g/L RW in 50 mL shake flasks was prepared. The medium were inoculated with a 2% inoculum and ampicillin was added at a concentration of 100 µg/mL. To determine the optimal incubation time, production was carried out for 24 and 48 hours at 37 °C and 175 rpm. To determine the most efficient time for the addition of RW, it was added at the start of the production process and when the OD value reached 0.6–0.8. Similarly, to determine the optimal time for IPTG addition, this component was added at the start of production and when the OD values were between 0.6 and 0.8 which stands for approximately 2,5-3 h after the beginning of the production. All studies were conducted in triplicate and summarized in Table 7.

Table 7. The runs that were conducted in this thesis in order to determine the effects of the time of the addition of RW and IPTG.

Name of the run	RW Addition Time	IPTG Addition Time
t0-t0	at the beginning	at the beginning
t0-t3	at the beginning	when OD was 0,6-0,8
0-t0	-	at the beginning
t3-t3	when OD was 0.6-0.8	when OD was 0.6-0.8

2.2.5.4. IPTG molarity determination

There are several papers reported the effects of different IPTG concentrations on P3HB production. In order to determine the effect of IPTG molarity, 250 ml shake flasks were prepared, each containing 50 ml of P3HB medium with 10 g/L RW. After adding 100 µg/mL of ampicillin to each flask, a 2% inoculum was added to each shake flask. Once the OD values of the production media had reached 0.6–0.8, different IPTG concentrations (0.1, 0.5 and 0.8 mM) were tried under the standard incubation conditions (at 37 °C, 175 rpm, 24 hours). Following a 24-hour period, samples were obtained and DCW, P3HB, and yield analyses were conducted. All studies were conducted in triplicate.

2.2.6. Response surface methodology

Response surface methodology (RSM) is used to develop, improve, and optimize processes based on various statistical and mathematical techniques. The main objective is to determine the conditions that provide the highest yield by providing a large amount of information with a small number of experiments (Keskin-Gündoğdu et al., 2016). In this thesis, optimized the optimum RW sugar concentration (5-15 g/L), tryptone (0-4 g/L), and agitation rate (50-300 rpm).

The Response Surface Methodology (RSM), Central Composite Design (CCD) was used to optimize production conditions. RW and tryptone levels, along with agitation rate, were selected as bioprocess variables that could potentially affect P3HB levels (Y, g/L) as the main factors. The three different factors and experiment sets obtained using CCD (Design Expert, 7.0.0, Stat-Ease Inc., USA) are presented in Table 8.

Table 8. Sets of experiments applied in shake flasks within the thesis.

Experiment set number	**Factor1: X_1: RW (g/L)**	**Factor2: X_2: Tryptone (g/L)**	**Factor3: X_3: Agitation rate (rpm)**
1	10	2	175
2	15	2	175
3	10	2	175
4	10	2	50
5	13	0.8	100
6	10	0	175
7	10	2	175
8	7	0.8	100
9	13	0.8	249
10	13	3.2	100
11	10	2	300
12	13	3.2	249
13	7	3.2	100
14	7	0.8	249

15	10	2	175
16	10	4	175
17	10	2	175
18	7	3.2	249
19	5	2	175
20	10	2	175

The values of the variables at different levels are shown in Table 9. Twenty sets of experiments were used in the RSM study to determine the optimal values of the bioprocess variables that would increase the P3HB yield the most. All experiments were repeated three times, and the average values of these experiments are presented.

Table 9. Levels of factors used in the central composite experimental design**.**

Factor	Name	Level			
		-1	+1	-α	+ α
X_1	RW (g/L)	7.027	12.97	5	15
X_2	Tryptone (g/L)	0.81	3.19	0	4
X_3	Agitation rate (rpm)	100.67	249.33	50	300

According to the determined factors, the mathematical equation of P3HB amount was modeled with a second-degree quadratic polynomial equation;

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j$$

In this equation, *Y* represents the predictive measured response; β0 represents the intercept; and βi, βii, and βlj are the regression coefficients of the model. Design Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, USA) software was used for the RSM approach and the estimation of the modified quadratic model. Analysis of variance (ANOVA) tests were applied to understand the relationship between variables in P3HB production.

2.2.7. P3HB extraction

2.2.7.1. Conventional extraction

To obtain the P3HB, 5 mL of the culture medium was transferred to centrifuge tubes. The cells were then centrifuged at 4,000 rpm for 30 minutes. After centrifugation, the supernatant was carefully removed, and 5 mL of a 14% sodium hypochlorite solution was added to the cell pellet. The tubes were then incubated at 50 °C for one hour to obtain a homogeneous mixture. The cells were then centrifuged again at 4,000 rpm for 35 minutes to remove the sodium hypochlorite. The resulting pellet was resuspended in 5 mL of distilled water and then centrifuged again at 4,000 rpm for 35 minutes. Then, 3 mL of pure acetone and 3 mL of pure ethanol were added, and the pellet was washed with each solution for 20 minutes. After washing, 10 mL of pure chloroform was added to the remaining pellet, which was then incubated at 50 °C for 12 hours. After this period, the chloroform was removed, and 2 mL of pure sulphuric acid (H_2SO_4) was added to the pellet. The resulting mixture was then incubated in a water bath at 100 °C for 10 minutes. During this process, the P3HB polymers in the tubes convert to crotonic acid. After incubation, the tubes were removed from the water bath, cooled to room temperature and the absorbance was measured at 235 nm using a spectrophotometer. A schematic representation of the experimental procedure is provided in Figure 10. Following the period, samples were obtained and DCW, P3HB, and yield analyses were conducted. All studies were conducted in triplicate.

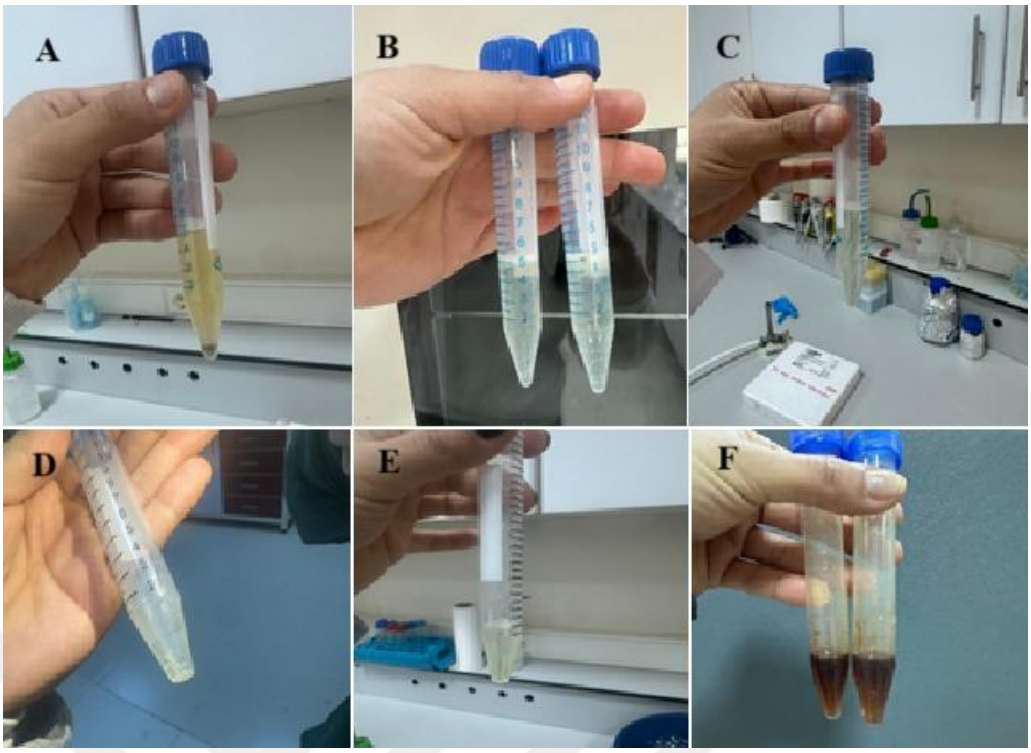


Figure 10. A) 5 mL of the culture medium, **B)** 5 mL of a 14% sodium hypochlorite solution was added to the cell pellet, **C)** Removal of sodium hypochlorite, **D)** Evaporation of chloroform, **E)** Addition of 2 mL of pure sulfuric acid, **F)** Conversion of P3HB to crotonic acid.

2.2.7.2. Green solvent extraction

In order to compare the green solvent extraction methods with conventional extraction method, 2 different terms were used. Extraction yield was used to indicate the amount of P3HB obtained per unit cell and the term 'relative extraction yield' was a term derived by accepting the yield obtained by the conventional method as 100% and comparing it with the results of the extraction methods that use green solvents.

$$Extraction\ Yield\ \% = \ 100\ x\ \frac{Extraction\ P3HB\ (g)}{DCW\ used\ for\ the\ extraction\ (g)} \qquad \text{Equation (3)}$$

$$Relative\ Extraction\ Yield = \frac{Extraction\ yield\ using\ green\ solvent}{Extraction\ yield\ of\ conventional\ method} \qquad \text{Equation (4)}$$

2.2.7.2.1. 1,3-dioxolane extraction

Following completion of the production process, the cells were centrifuged and dried overnight at 60 °C. They were then broken down mechanically into smaller pieces using a mortar and pestle. Then, 0.1 g of the dry cell biomass was incubated in a shaking water bath at 80 °C for 6 hours with 2 mL of 95% 1,3-dioxlane. Subsequently, 3 volumes of distilled water were added to precipitate the P3HB. The P3HB was then collected by centrifugation at 4,100 rpm for 25 minutes, after which it was washed twice with water. It was thereafter dried at 60 °C overnight. All studies were conducted in triplicate.

2.2.7.2.2. Ethyl levulinate extraction

To separate the cells from the production medium, 2.5 ml of the medium was centrifuged at 4,100 rpm for 20 minutes and the resulting supernatant was discarded. Then, 0.2 g of the cell pellet was added to 10 ml of ethyl levulinate solution, and the mixture was incubated at 150 °C for 60 minutes. The suspension was then cooled to 50 °C and filtered under vacuum using a Buchner funnel. The clear solution was gently stirred and slowly added to 30 mL of distilled water. The P3HB polymer was then centrifuged at 4,100 rpm for 30 minutes, dried at 60 °C for 48 hours and weighed on a precision balance. All studies were conducted in triplicate.

2.2.7.2.3. Dimethyl carbonate extraction

Subsequent to production, 0.1 g of wet biomass was added to 4 mL of 2.5% (w/v) dimethyl carbonate (DMC). The glass tubes were then incubated in a magnetic stirrer at 90 °C for 120 minutes. The mixture was then centrifuged at 4100 rpm for 30 minutes, after which the P3HB-containing supernatant was carefully separated. This was then added to 12 mL of ethanol to precipitate the P3HB. The mixture was left at 4 °C for 12 hours to complete the precipitation process. The precipitated P3HB was collected by vacuum filtration and air-dried to obtain the P3HB. All studies were conducted in triplicate.

2.2.8. Analytic methods

2.2.8.1. Reducing sugar determination using DNS method and determination of standard curve

The 3,5-dinitrosalicylic acid (DNS) method was used to detect reducing sugars. This method involved preparing the RW sample in two different sizes. The RW was first cut to 10 mm using a cutting tool and then ground into a powder using a grinder. Both sizes of RW were prepared at concentrations of 1/4 g/mL, 1/6 g/mL, 1,8 g/mL and 1/10 g/mL, and were then autoclaved. Analysis was performed using a DNS solution (10 g/L DNS, 2 g/L phenol, 0.5 g/L sodium sulphite and 10 g/L sodium hydroxide) (Merck), as a 40% potassium sodium tartrate solution (Condalab). 3 mL of the sample were added to 3 mL of the DNS solution and kept in a boiling water bath at 100 °C for 15 minutes. Once the samples turned red/brown, 1 mL of the 40% potassium sodium tartrate solution was added to stabilize the colour. After cooling to room temperature, the absorbance values of the tubes were measured at a wavelength of 575 nm using a spectrophotometer. Moreover, the reducing sugar amounts of fermentation medium were also determined using 3 ml of fermentation medium. The same procedure was applied to glucose solutions prepared at different concentrations in order to create a standard curve. All studies were conducted in triplicate.

2.2.8.2 P3HB quantification and determination of standard curve

In order to create a standard curve P3HB graph, 0.05 g of commercial P3HB (Merck) was weighed and 100 mL of chloroform was added to dissolve the P3HB granules. The homogenized solution was diluted 1/100 and P3HB stock solution was prepared at a concentration of 5 µg/mL (Ensari, 2012). From this stock solution, 1, 2, 3, 4, 5, 5, 6, 7, 8, 9 and 10 mL were taken and transferred to glass test tubes. After the chloroform was completely evaporated, 5 mL each pure sulfuric acid was added to the tubes to obtain solutions containing 1 to 10 µg/mL P3HB. The prepared tubes were placed in a hot water bath at 100°C for 20 minutes. During this process, the P3HB granules were converted to crotonic acid and the tubes were allowed to cool at room temperature. Absorbance measurements of the cooled samples were performed in a spectrophotometer at 235 nm wavelength.

To determine the P3HB concentration, 5 mL of fermentation medium was transfered into pre-sterilized colonical centrifuges tubes. The cells were then centrifuged at 4,000 rpm for 30 minutes. After centrifugation, the supernatant was carefully removed, and 5 mL of a 14% sodium hypochlorite solution was added to the cell pellet. The tubes were then incubated at 50 °C for one hour to obtain a homogeneous mixture. The cells were then centrifuged again at 4,000 rpm for 35 minutes to remove the sodium hypochlorite. The resulting pellet was resuspended in 5 mL of distilled water and then centrifuged again at 4,000 rpm for 35 minutes. Then, 3 mL of pure acetone and 3 mL of pure ethanol were added, and the pellet was washed with each solution for 20 minutes. After washing, 10 mL of pure chloroform was added to the remaining pellet, which was then incubated at 50 °C for 12 hours. After this period, the chloroform was removed, and 2 mL of pure H_2SO_4 was added to the pellet. The resulting mixture was then incubated in a water bath at 100 °C for 10 minutes. During this process, the P3HB polymers in the tubes convert to crotonic acid. After incubation, the tubes were removed from the water bath, cooled to room temperature and the absorbance was measured at 235 nm using a spectrophotometer. All studies were conducted in triplicate.

2.2.9. Sudan Black staining

Cells were stained with Sudan Black dye to observe P3HB production microscopically. In the Sudan Black staining procedure, first, 10 µL was taken from the bacterial culture, spread in a thin layer on a slide, and left to dry. The slide was stained with Sudan Black B (0.3% w/v in 60% ethanol) for 10 minutes. Shortly after washing with distilled water, it was stained again with a 0.5% safranin solution for 10 seconds and then thoroughly washed with distilled water. The slide was examined under a light microscope, and black/dark blue P3HB granules were observed (Figure 11).

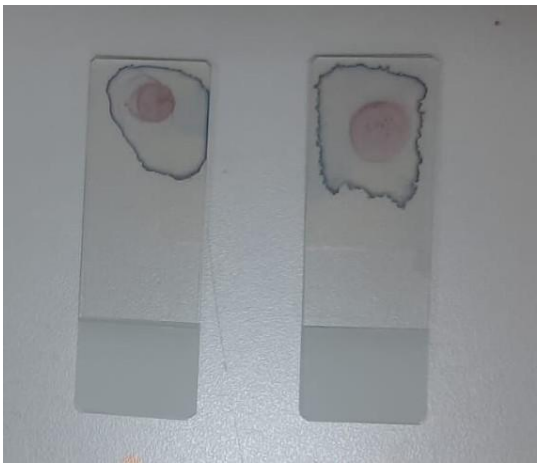


Figure 11. Application of Sudan Black staining.

2.2.10. Yield and volumetric productivity calculations

The yield ($Y_{P/X}$, % g P3HB/g DCW) and the volumetric productivity (Q_P, g/L/day) values were calculated using Equation 5 and Equation 6, respectively (Bailey and Ollis, 1986);

$$Yield = Y_{P/X} = \frac{P}{X} * 100 \qquad \text{Equation (5)}$$

$$Volumetric\ productivity = Q_P = \frac{Produced\ P3HB}{Production\ period} \qquad \text{Equation (6)}$$

where P is the produced P3HB amount (g/L) and X is the DCW (g/L).

2.2.11. Statistical analysis

All the experiments were performed and analyzed in triplicate. All data used in this thesis were subjected to analysis of variance (ANOVA) and analysis of data was presented as mean ± standard error of means. The statistical significance was determined at α level of 0.05 indicating the probability of obtaining such results by chance. To summarize, the results were considered statistically significant with *P* values < 0.05.

SECTION THREE

RESULTS AND DISCUSSION

This section reports the results and analyses of the experimental studies.

3.1. Growth curve of *rec E. coli* Rosetta pQEAL1124

Cell growth (OD_{600} nm) of rec. *E. coli* Rosetta pQEAL1124 was monitored at various time intervals (30 minutes, 1 hour) depending on its growth rate over a period of 60 hours (Figure 12).

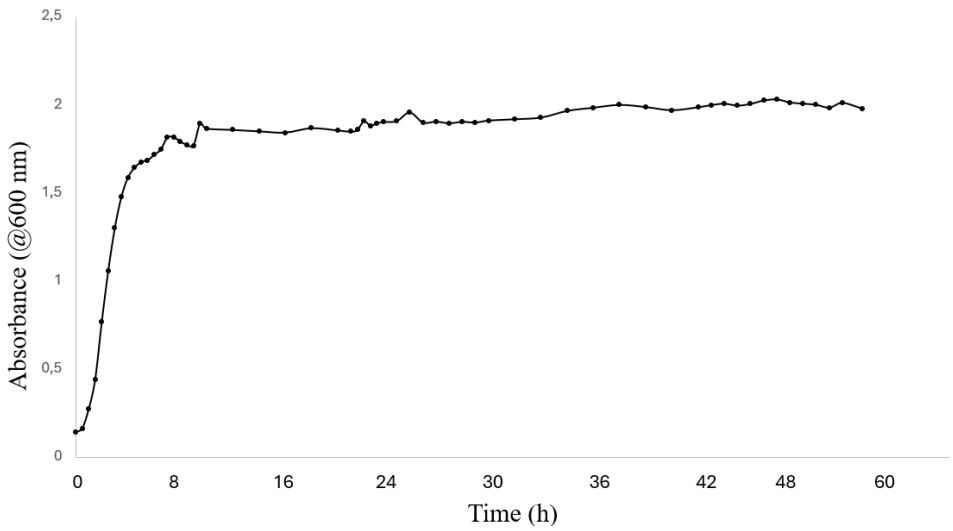


Figure 12. Growth-time curve of rec. *E. coli* Rosetta pQEAL1124 used in this thesis.

Figure 12 indicates that rec. *E. coli* Rosetta pQEAL1124 did not undergo a lag phase; it exhibited logarithmic growth during the initial 3.5 hours and subsequently entered a stationary phase after 7-8 h. The doubling time (t_d) of the microorganism was approximately 1 h, and the specific growth rate (μ) was determined to be 0.67 h^{-1} (Table 10). The growth time graph for rec. *E. coli* Rosetta pQEAL1124 was obtained using a P3HB production medium containing glucose. Subsequently, RW was used instead of glucose in the production medium. All optimization studies conducted with RW confirmed that rec. *E. coli* Rosetta pQEAL1124 completes its growth in the log phase within an average time range of three to four hours. These values are consistent with those reported in the literature for *E. coli* (Wang et al., 2023). Another study by

Khushoo et al. (2004) showed that *E. coli* grows in the logarithmic phase within an average timeframe of 3.5–5 hours when cultured in different media. Similarly, Galloway et al. (2003) reported that *E. coli* reached the logarithmic phase in 4 hours.

Table 10. Growth kinetics of *rec. E. coli* used in the thesis

μ (1/h)	t_d (h)
0.67	1.03

3.2. P3HB standard curve

Figure 13 shows the standard curve of P3HB. In this graph, the Y-axis represents the absorbance values and the X-axis represents the P3HB concentrations. The calculated coefficient of determination (R^2) was 0.9884, which is very close to 1, indicating a strong linear relationship between absorbance and P3HB concentration. This suggests that the standard curve is reliable and that the experiment was conducted successfully.

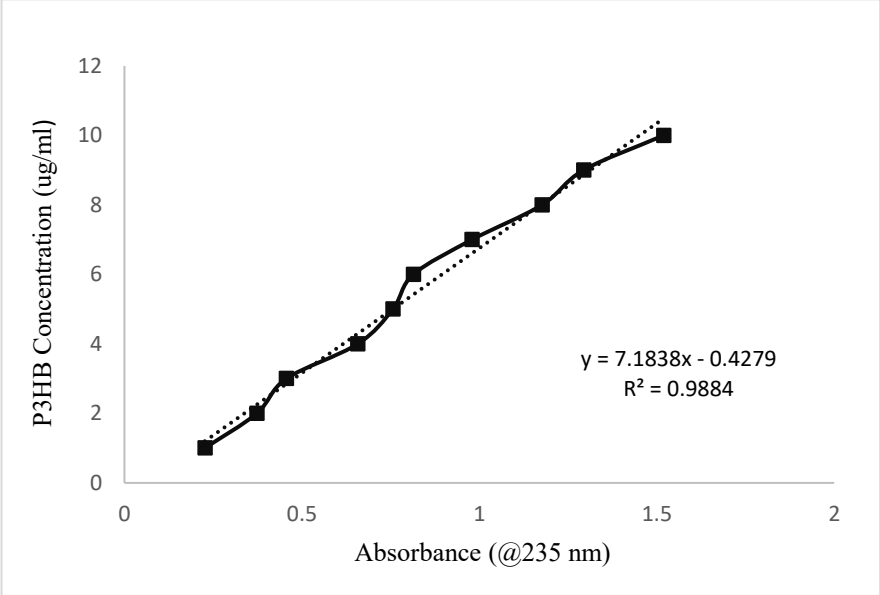


Figure 13. P3HB standard curve.

3.3. DNS Standard Curve

A glucose standard curve has been created for reducing sugar analysis. Figure 14 shows the calibration curve obtained using the DNS method. A coefficient of determination (R^2) of 0.995 was obtained, indicating that the relationship between absorbance and P3HB concentration exhibits a high degree of linearity.

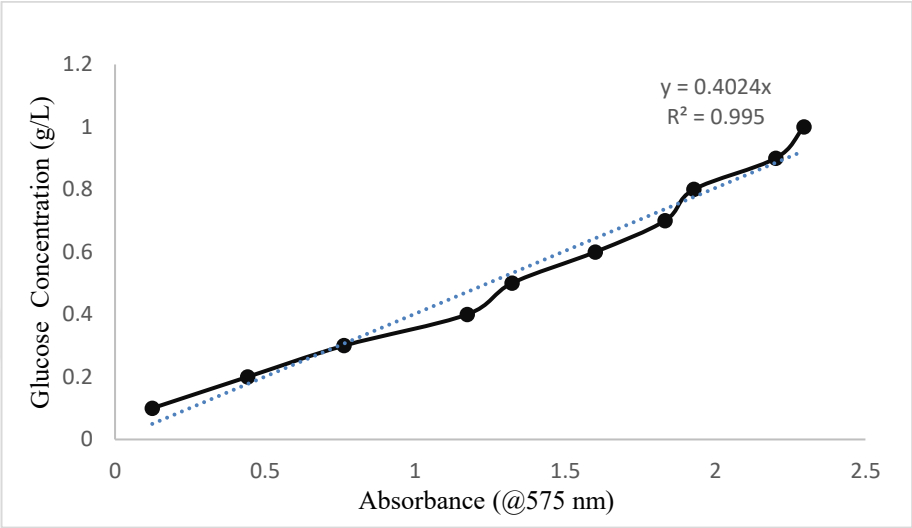


Figure 14. Glucose standard curve.

In this thesis, different RW concentrations (1/4, 1/6, 1/8 and 1/10 w/v) and RW forms (powdered or 10-mm-size) were used to determine the most appropriate RW form to be used in P3HB production studies. Comparing the results for reducing sugars, it was revealed that the highest reducing sugar content was obtained for the 10 mm size form using a concentration of 1/4 g/mL (Table 11).

Table 11. Reducing sugar content (g/L) for different RW concentrations and forms.

Concentration (g/mL)	10-mm-size	Powdered
1/4	102.61	69.60
1/6	54.08	33.62
1/8	21.34	29.21
1/10	43.43	21.46

It is well known that a decrease in particle size results in an increase in surface area resulting a better extraction and mass transfer. However, in this case, powdered

RW released less sugar than 10-mm-size RW. This result can be explained as, since mixing could not be performed during the autoclaving process, the powder particles accumulated due to hydrophobic effects, thereby preventing the expected surface area effect. Larger particles, on the other hand, could not aggregate and form clumps, thus exhibiting less hydrophobic effects. Similar results were also reported elsewhere (Vries et al., 2017; Nogueria et al., 2023). In this thesis, more sugar could be extracted from the 10 mm-size RW, which had a larger release area, compared to the powdered RW. Accordingly, it was decided to prepare RW at a size of 10 mm and a concentration of 1/4 g/ml in the P3HB production process.

3.4. Effect of pH in the production media

The DCW amounts, the P3HB productions and the yields at different pH values (7 and 9) were shown in Figure 15A, Figure 15B and Figure 15C, respectively. It can be seen from Table 12 that the DCW amount in the medium at pH 7 was found to be 25% higher than the production medium at pH 9. Moreover, the P3HB production in the medium initiated at pH 7 was calculated to be 5.63-fold higher than in the medium initiated at pH 9. Additionally, P3HB yield per gram of cells was calculated to be 4.26-fold higher. It can therefore be concluded that the DCW amount in production initiated at pH value of 7 was higher than the one initiated at pH value of 9. It can be concluded that the pH has a significant effect on the P3HB production.

Table 12. Effects of pH on biomass and P3HB concentrations, and yield.

pH value of the Media	DCW (g/L)	P3HB (g/L)	$Y_{P/X}$ (% g P3HB/g DCW)
7	2 ± 0.01	0.45 ± 0.0001	22.48 ± 0.9
9	1.5 ± 0.02	0.08 ± 0.0003	5.27 ± 0.5

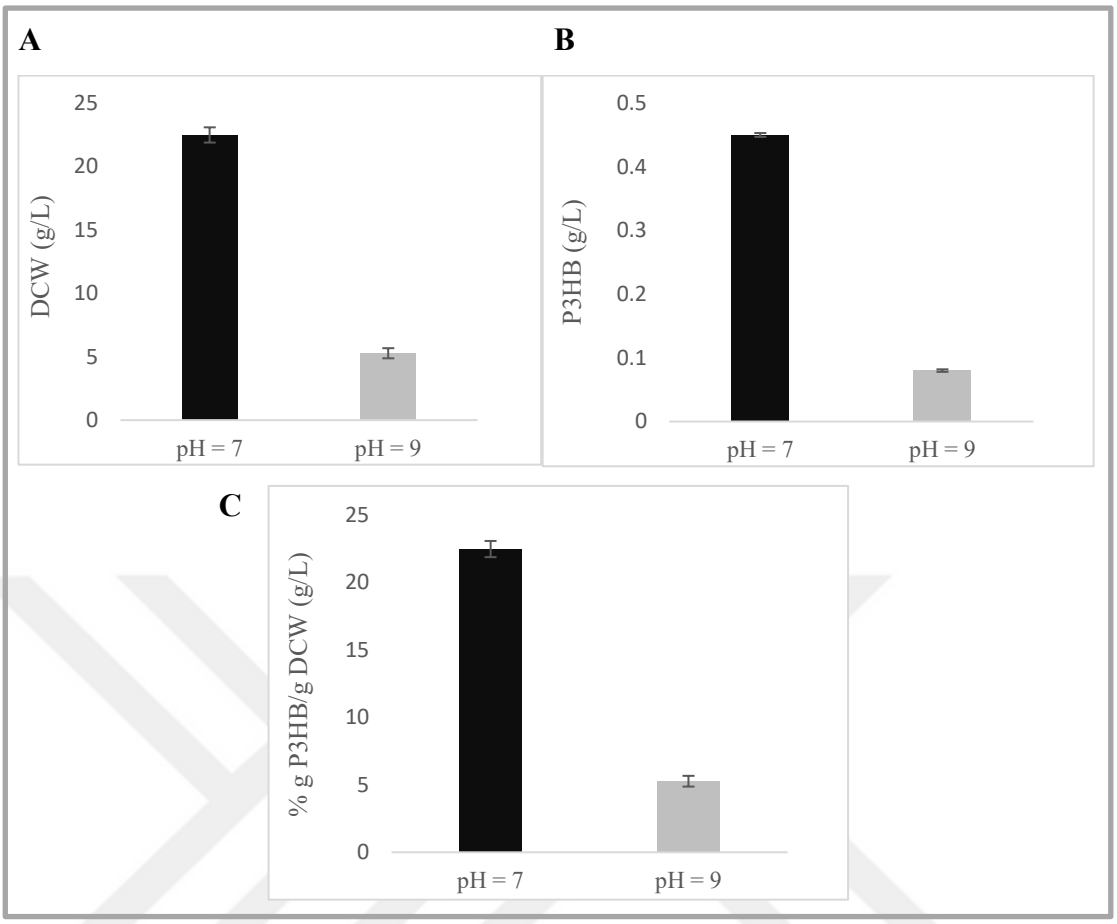


Figure 15. The effect of pH in the production media on **A)** biomass concentration, **B)** P3HB concentration and **C)** yield.

Various studies have thoroughly investigated the effect of the pH value of the production environment on P3HB production using recombinant microorganisms. In this context, a study was conducted using the rec. *E. coli* XL1-Blue strain, in which the pH of the medium was adjusted to 7 and an ammonium hydroxide solution was added automatically throughout the production process to maintain the pH at 7. It was reported that during the first 24 h of production period, maintaining the pH value at 7 was crucial for cell growth and P3HB accumulation (Napathorn et al., 2021). Similarly, Choi et al. (1999) used the same rec. *E. coli* XL1-Blue strain and set the initial pH of the medium to 6.9. They implemented a feeding strategy based on suddenly increasing the medium pH upon carbon source depletion, adding a 28% (v/v) ammonia solution to the medium when the pH exceeded 6.9 to balance it. This approach was presented as an effective control strategy for maintaining P3HB yield.

In another study, Patil et al. (2024) produced P3HB using *Bacillus megaterium* and *Ralstonia eutropha* strains, evaluating the effect of the production medium pH on DCW and product yield. Comparisons conducted in environments with pH values set at 5, 7, 9 and 11 and the study revealed that the highest DCW and P3HB yields were obtained at pH 7 for both bacterial types. Additionally, Wang et al. (2014) investigated the effect of the pH of the production medium on the kinetics of cell growth using a recombinant *E. coli* BL21 (DE3) strain, adjusting the pH to 6.5, 7.0, 7.5, 8.0 and 8.5. The specific growth rate was measured as 1.18, 1.16, 1.00, 0.98 and 0.92, respectively, at the end of a four-hour production period. These results suggest that cell growth is higher under pH conditions of 6.5 and 7.0, indicating that neutral and slightly acidic environments are more conducive to microbial growth in *E. coli*-based systems. It can also be concluded that at higher pH values (than 7), a decrease in the metabolic activities of microorganisms has been observed, and this situation negatively affects both cell proliferation and P3HB synthesis. According to Wang et al. (2014), the decrease in specific growth rate at increasing pH levels confirms that microbial growth is suppressed. This is associated with changes in cell membrane permeability, enzyme activity disruption, and slowed energy production mechanisms under alkaline conditions.

Similarly, Thapa et al. (2018) reported that pH 9 may support P3HB production in some *Bacillus* species, but optimal production yield is generally achieved around pH 7. Additionally, Ahn et al. (2000) noted that maintaining pH at 6.95 during fed-batch fermentations with rec. *E. coli* is a critical parameter for achieving high P3HB yield and cell concentration. As reported by Vijayalakshmi et al. (2013), membrane transport systems, enzyme substrate binding, and critical biocatalytic activities lose their effectiveness when the pH rises above the optimum pH, leading to a significant decrease in cellular growth and P3HB accumulation. The three-dimensional structure of enzymes and the ionization states in their active sites are disrupted due to rising pH, particularly reducing the catalytic ability of enzymes such as β-ketothiolase and P3HB synthase, which play a role in P3HB biosynthesis (Ali et al., 2024).

Taking all these studies together into account, it can be concluded that pH is an important factor affecting the performance of the microorganisms used in P3HB production. Neutral pH (7) conditions generally provide the most suitable environment for cell growth and product yield.

3.5. Effect of carbon and nitrogen sources on culture medium

In this study, the effects of the culture medium on P3HB production using rec. *E. coli* Rosetta pQEAL1124 were evaluated using five production environments with different carbon and nitrogen sources. Figure 16A, Figure 16B, Figure 16C shows that changes in environmental components significantly affect both cell growth and P3HB synthesis. In particular, the presence or absence of carbon and nitrogen sources created differences in biomass formation and the amount of P3HB per unit cell (Table 13).

Table 13. Dry cell weight and P3HB production amounts for 5 different media compositions.

P3HB Media	**DCW (g/L)**	**P3HB (g/L)**	**$Y_{P/X}$ (% g P3HB/g DCW)**
nonC- medium	0.53 ± 0.02	0.17 ±0.002	32.86 ± 0.9
nonT-medium	1.46 ± 0.03	0.26 ±0.001	16.15 ± 0.5
nonYE- medium	0.90 ± 0.02	0.22 ± 0.004	24.54 ± 0.7
12h-medium	1.03 ± 0.03	0.25 ± 0.001	23.94 ± 0.4
Control-medium	1.14 ± 0.01	0.33 ± 0.003	28.56 ± 0.8

nonC-medium:same as the control group, except lacking glucose as the carbon source, nonT-medium: same as the control group, except lacking tryptone as a nitrogen source, nonYE-medium: Same as the control group, except lacking yeast extract as a nitrogen source,12h-medium: first 12 h in standard medium, then transferred to PHA medium without yeast extract and tryptone for the next 12 h., control medium: Standard medium containing glucose, yeast extract, and tryptone.

The highest DCW, at 1.46 g/L, was obtained in the non-T medium, which is approximately 1.28-fold higher than in the control medium. However, the control medium yielded the highest P3HB concentration at 0.33 g/L, whereas the non-T medium yielded 21% less. The highest P3HB yield was obtained in the nonC-medium at 32.86%, approximately 15% higher than the control group. However, the total cell amount in this medium was the lowest. The yield of 23.94% obtained in two-stage production over 12 hours was approximately 16% lower than that of the control medium. Although the nonC-medium provided the highest yield, the P3HB production remained low. In contrast, the control medium, which had the highest DWC content, provided the highest P3HB production.

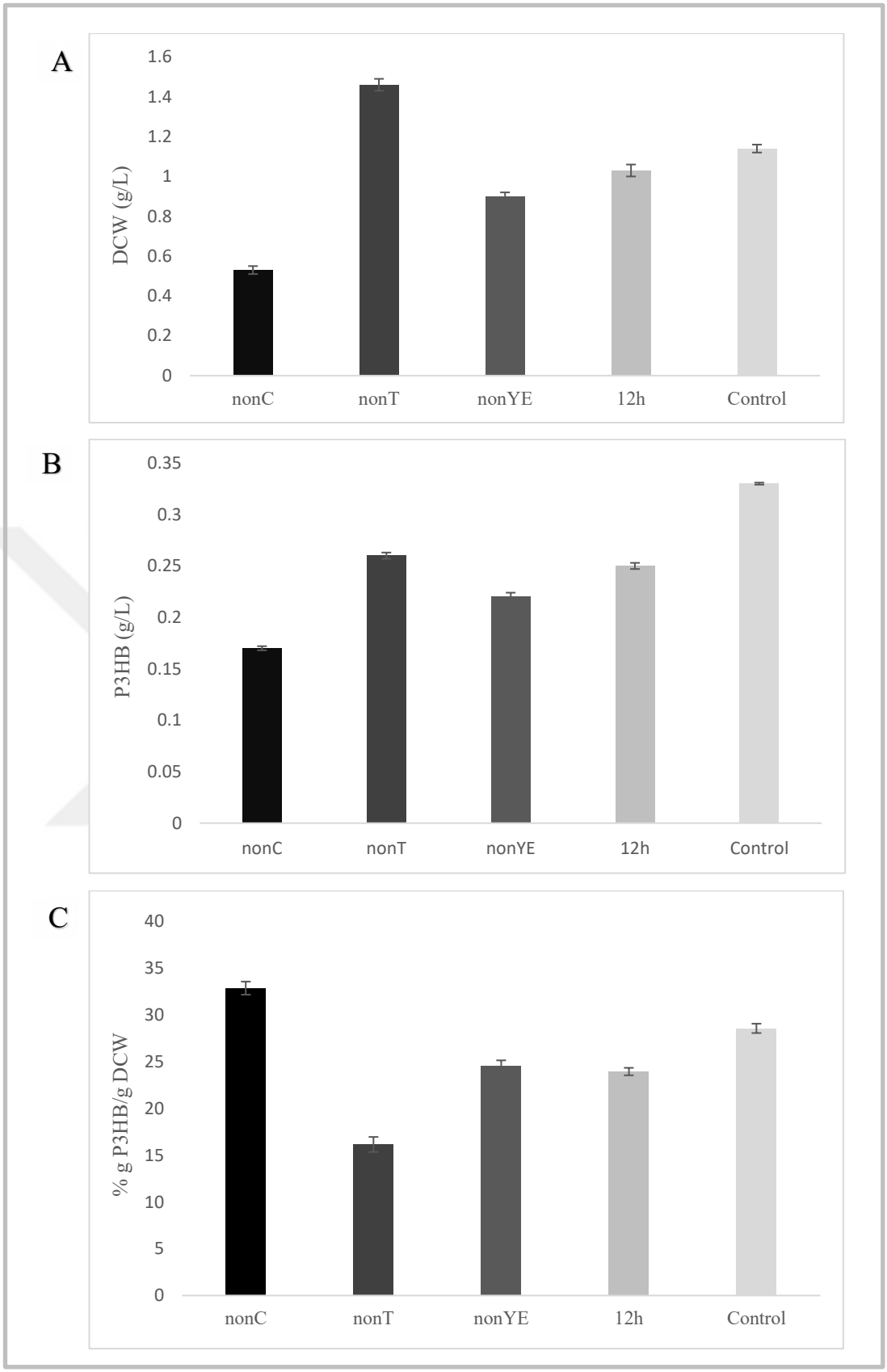


Figure 16. The effect of carbon and nitrogen sources on **A)** biomass concentration, **B)** P3HB concentration and **C)** Yield.

Wang et al. (2009) reported that P3HB synthesis accelerates in environments with high concentrations of carbon and nitrogen, and that P3HB metabolism functions as a stress response mechanism in several microorganisms. This study is in accordance with this thesis. This situation can be explained as an adaptive response to meet energy and carbon requirements. In a related study, Khosravi-Darani et al. (2013) found that fermentation using *Cupriavidus necator* in a medium containing 25 g/L glucose and 1.5 g/L ammonium sulfate resulted in high cell density and polymer yield. In conclusion, although P3HB yield increases under carbon limitation, total production is limited by a decrease in biomass.

The results in this thesis in non-T medium studies indicate that, under nitrogen-limited conditions, cells priorities growth and suppress storage polymer production. Similarly, Xu et al. (2019) compared cell growth and P3HB accumulation under nitrogen limitation and nitrogen surplus conditions, reporting that nitrogen promotes cell growth while reducing P3HB accumulation. In another study, Zhou et al. (2022) reported that nitrogen limitation negatively affects P3HB accumulation. However, several studies in the literature have indicated that nitrogen limitation is an important trigger, particularly at the onset of P3HB production (Cui et al., 2017; Wu et al., 2022). This contrasting result may be attributed to variations in the metabolic pathways among different microorganisms.

In this study, the results obtained indicate that the absolute amounts of carbon and nitrogen sources significantly affect microbial growth and P3HB accumulation. Notably, the production medium employed in the control group facilitated sufficient cell proliferation and substantial P3HB synthesis, establishing a metabolically balanced environment. These results suggest that the recombinant *E. coli* strain can efficiently synthesize P3HB when carbon and nitrogen levels are optimized. Therefore, it is necessary to use a balanced carbon and nitrogen source to support cell growth and efficient P3HB production. In this regard, the control medium used in this study provided the most optimal and efficient conditions and further studies were carried out using the control medium.

3.6. Determination of conditions of P3HB production media

In the study conducted to determine the optimum conditions of the P3HB production environment and to evaluate the effects of adding RW and IPTG at different time periods, the amount of DCW, P3HB and production yields at 24 and 48 hours were compared depending on the production time.

Table 14. The effect of addition time periods of RW and IPTG for 24 h of P3HB production.

Name of the run	**DCW (g/L)**	**P3HB (g/L)**	**$Y_{P/X}$ (% g P3HB/g DCW)**
t0-t0	1.83 ± 0.01	0.45 ±0.003	24.74 ± 0.8
t0-t3	2.05 ± 0.01	0.55 ±0.004	26.68 ± 0.5
0-t0	1.88 ± 0.02	0.26 ± 0.005	13.67 ± 0.7
t3-t3	1.00 ± 0.03	0.24 ± 0.001	24.35 ± 0.4

t0-t0:RW addition time at the beginning, IPTG addition time at the beginning, t0-t3: RW addition time at the beginning, IPTG addition time when OD was 0.6–0.8, 0-t0: not RW addition, IPTG addition time at the beginning. t3-t3: RW addition time when OD was 0.6–0.8, IPTG addition time when OD was 0.6–0.8.

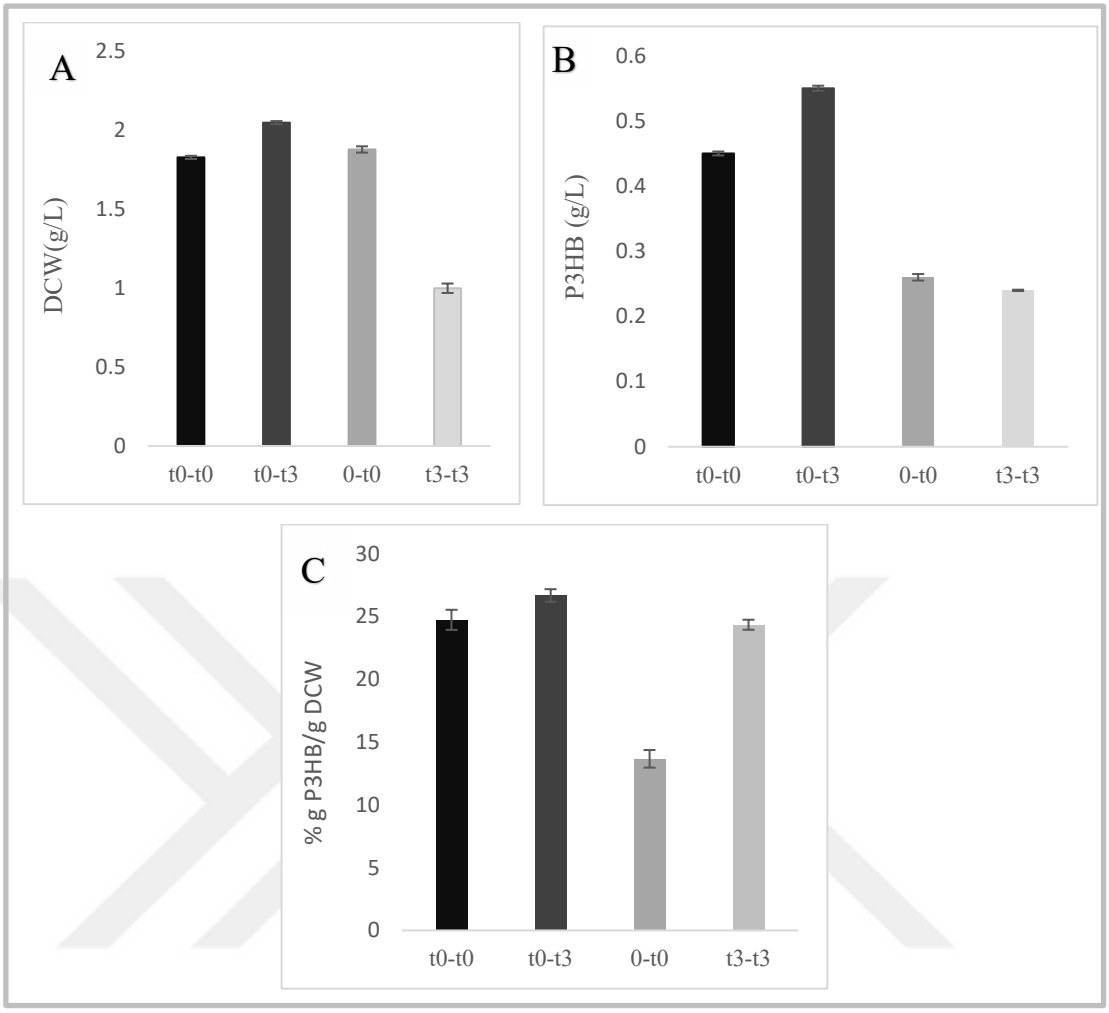


Figure 17. Effect of addition times of RW and IPTG for 24 h production on **A)** biomass concentration, **B)** P3HB concentration and **C)** yield.

Table 15. The effect of addition time periods of RW and IPTG for 48 h of P3HB production.

Name of the run	**DCW (g/L)**	**P3HB (g/L)**	**$Y_{P/X}$ (% g P3HB/g DCW)**
t0-t0	1.72 ± 0.02	0.09 ±0.001	5.46 ± 0.8
t0-t3	2.00 ± 0.04	0.17 ±0.003	8.57 ± 0.9
0-t0	1.76 ± 0.03	0.09 ± 0.001	6.15 ± 0.5
t3-t3	0.68 ± 0.01	0.08 ± 0.001	8.51 ± 0.7

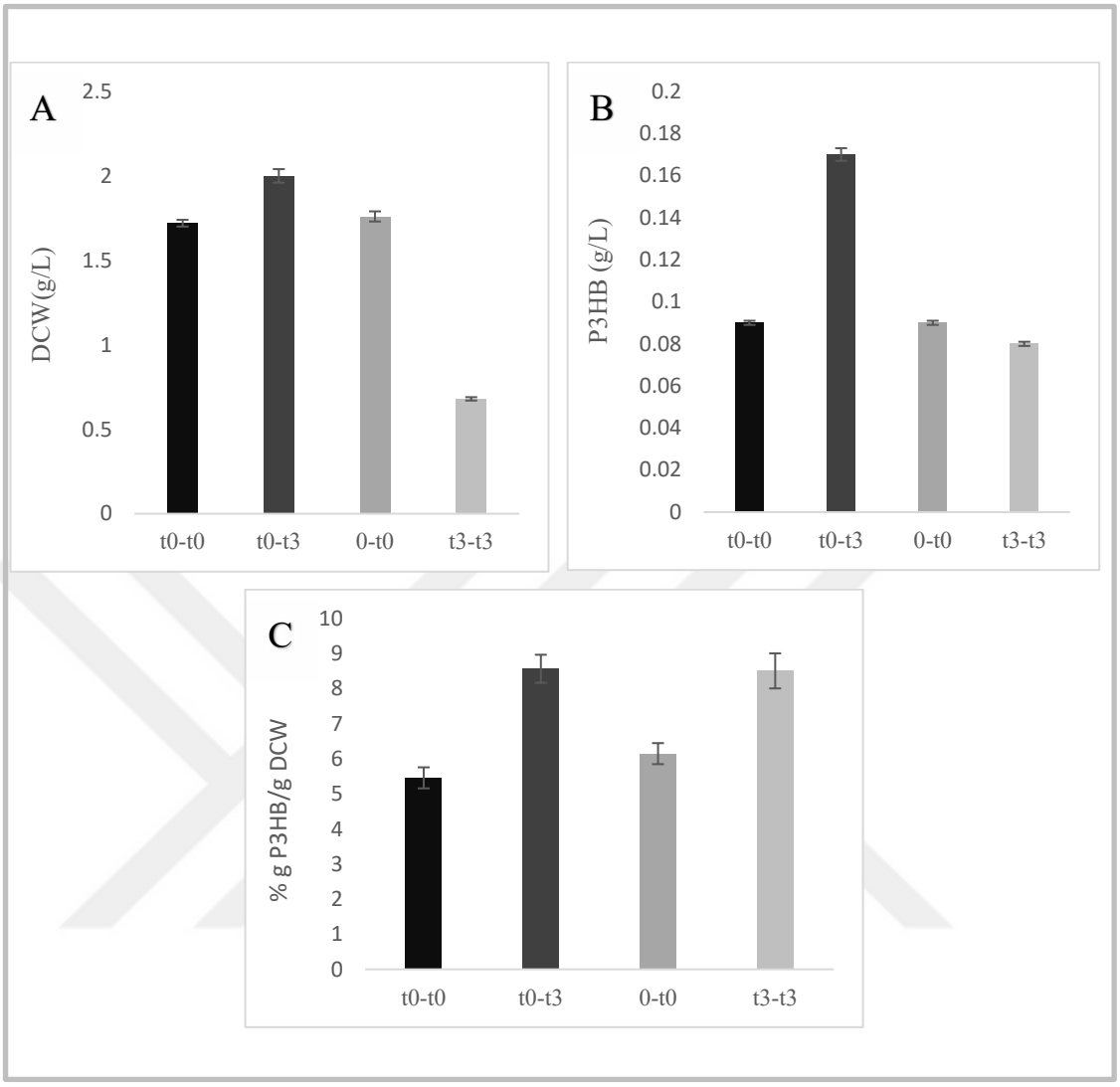


Figure 18. Effect of addition times of RW and IPTG for 48 h production on **A)** biomass concentration, **B)** P3HB concentration and **C)** yield.

This study evaluated the performance of the rec. *E. coli Rosetta* pQEAL1124 strain in producing P3HB based on the addition of RW and the P3HB-inducing agent IPTG at different times. The highest results were obtained after 24 hours of cultivation when RW was added at the beginning of the culture and IPTG was added when OD values were in the range of 0.6-0.8 (t0-t3) (Table 14). Under these conditions, the DCW was measured as 2.05 g/L and the P3HB content as 0.55 g/L. These values corresponded to an increase in biomass of approximately 12% and an increase in P3HB production of approximately 22%, respectively, compared to the condition in which both RW and IPTG were added at the beginning (t0-t0). Furthermore, the P3HB yield increased by 8%, corresponding to a value of 26.68%.

These results are consistent with the literature, which shows that adding IPTG at the log phase (when OD values are in the range of 0.6-0.8) was more effective for recombinant protein expression (Terpe, 2006; Wang et al.,2015; Hokamura at al.,2015; Zhuang et al., 2014). Administering IPTG at the beginning of production was reported to reduce yield by creating metabolic challenges due to the early induction of cell growth (Schein, 1989). When IPTG was applied when t= 0, P3HB yield was measured as 13.67%, which was nearly half of the yield obtained when IPTG was applied in the log phase (OD=0.6-0.8).

Moreover, when RW was administered during the log phase (OD=0.6-0.8), DCW was 1.00 g/L, P3HB content was 0.24 g/L, and the yield was 24.35%, as shown in Figure 17A, Figure 17B, Figure 17C. Under these conditions, decreases of approximately 51% in biomass and 56% in P3HB were observed compared to the initial RW addition. These results support the idea that using a carbon source late in the process negatively affects cell growth and PHA accumulation, and that an irregular supply of the carbon source reduces productivity, as reported in the literature (Khanna and Srivastava, 2005; Braunegg et al., 2004).

As shown in Table 15, there was a general decline in the 48-hour production results. Figure 18A, Figure 18B, Figure 18C indicates that highest values were obtained when RW was administered first and IPTG later: DCW was 2.00 g/L, P3HB was 0.17 g/L and the yield was 8.57%. However, when these values were compared with those from the 24-hour production period, P3HB decreased by around 69% and the yield decreased by 68%. This decrease can be explained by factors such as nutrient limitations, metabolic stress and product suppression experienced during prolonged culture conditions (Madison and Huisman, 1999). Generally, literature studies conducted with rec. *E. coli* reported that the optimal time for P3HB production was between 24 and 30 hours (Obruca et al., 2014; Lee, 1996). To summarize, the experimental results indicated that the optimal conditions for P3HB production was found to be to add the RW carbon source at the start of the production and the IPTG during the log phase. Under these conditions, the volumetric productivity obtained was 0.02 g/L/h and 0.003 g/L/h during 24 h production and 48 h production, respectively. Based on these results, the optimal P3HB production duration was determined to be 24 hours.

In conclusion, the timely applications of IPTG and the carbon source RW were found to have significant effect on P3HB production for rec. *E. coli* Rosetta pQEAL1124. In particular, IPTG administration during the log phase (when OD was in the range of 0.6-0.8) induced cells to reach sufficient biomass, balancing growth and polymer synthesis (Terpe, 2006). Similarly, providing the carbon source early in the culture period promoted optimal cell growth and created a favourable environment for biopolymer production (Khanna and Srivastava, 2005).

3.7. IPTG molarity determination

It is well-known that the optimal IPTG concentration can vary depending on the bacterial strain, culture medium, and working conditions used (Kim et al., 2021). Thus, doing induction experiments at different concentrations is important for figuring out the best conditions for the production system.

The molarity of IPTG (isopropyl β-D-1-thiogalactopyranoside), which is commonly used for induction in P3HB production, varies widely in the literature, generally ranging from 0.1 mM to 0.8 mM (Kim and Lee, 2021; Wang et al., 2023; Jung et al., 2020). Values within this range vary depending on different working environments and microorganism types. However, many studies, particularly the ones that use *E. coli* strains, indicate that 0.1 mM IPTG was the most common and preferred concentration for providing effective induction while minimizing the metabolic stress on cells (Wang et al., 2023; Kim et al., 2021; 2022; Inan et al., 2016). In this thesis, IPTG concentrations of 0.1, 0.5 and 0.8 mM were selected to investigate the effect of IPTG concentrations on P3HB production yield (Table 16).

Table 16. Effect of different IPTG concentrations on P3HB production.

IPTG Concentration	**DCW (g/L)**	**P3HB (g/L)**	**$Y_{P/X}$ (% g P3HB/g DCW)**
0.1 mM	1.96 ± 0.02	0.39 ±0.001	19.92 ± 0.8
0.5 mM	2.59 ± 0.04	0.30 ±0.006	11.60 ± 0.6
0.8 mM	1.99 ± 0.01	0.25 ± 0.003	12.33 ± 0.7

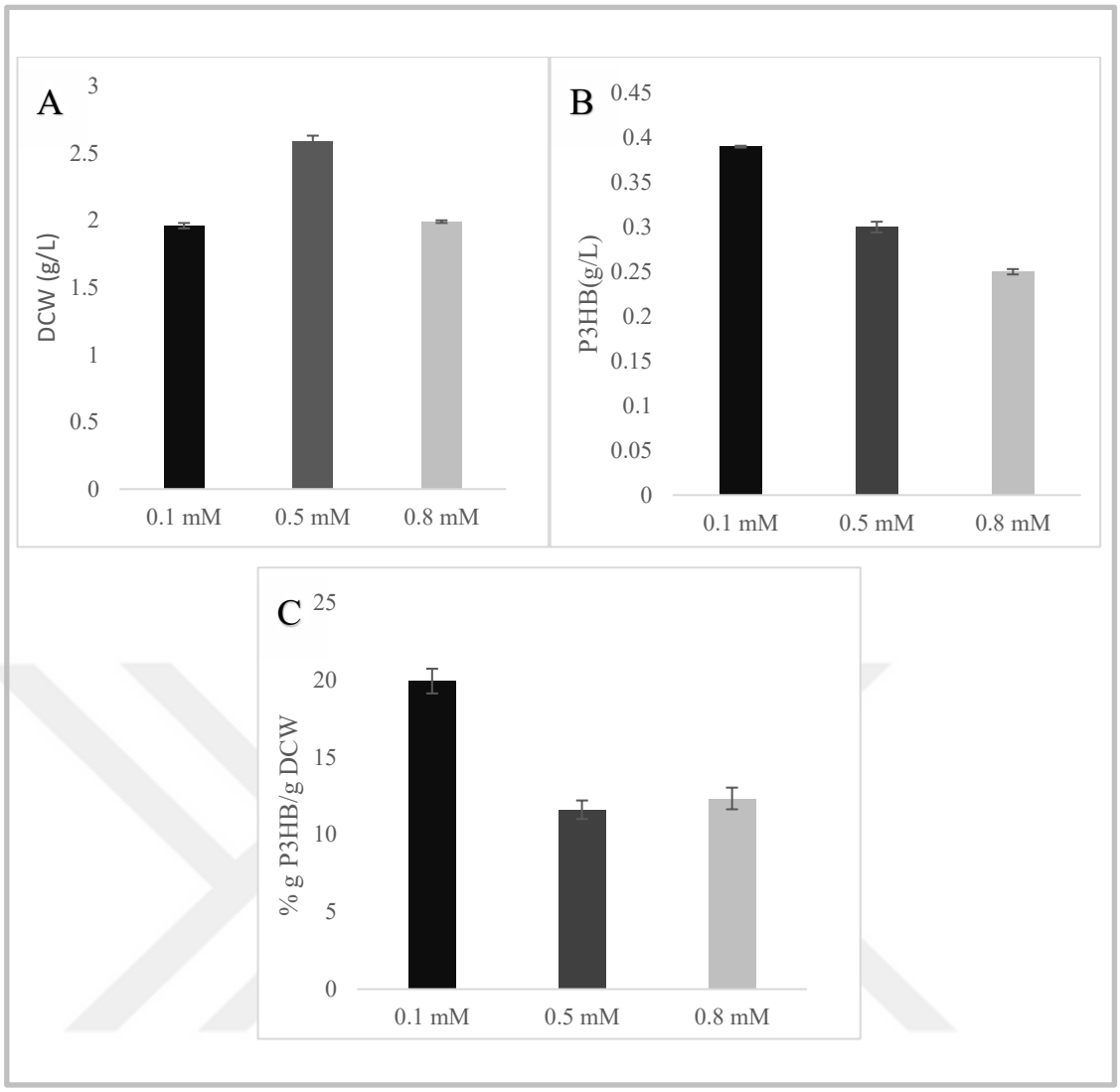


Figure 19. Effect of different IPTG molarity on **A)** biomass concentration; **B)** P3HB concentration; **C)** yield.

In this study, the effects of the commonly used and considered optimal 0.1 mM IPTG concentration and higher concentrations of 0.5 mM and 0.8 mM on production yield were investigated (Figure 19A, Figure 19B, Figure 19C). The results obtained showed that the 0.1 mM IPTG concentration was more efficient in terms of P3HB production, yielding 19.92% efficiency, compared to 11.60% efficiency at 0.5 mM and 12.33% efficiency at 0.8 mM. This finding is consistent with the literature suggesting that excessive IPTG induction may induce stress in cells and negatively affect product yield by increasing metabolic load (Lin et al., 2025). It is known that higher IPTG concentrations can negatively affect cell growth, particularly in recombinant proteins, leading to the accumulation of incorrectly folded proteins (Inan et al., 2016; Wang et al., 2023). This situation reduces the efficiency of the production process and increases

bioprocess costs. Additionally, higher IPTG amounts can be economically disadvantageous, as the use of this chemical directly increases production costs at industrial scale.

The results obtained in this thesis confirm that 0.1 mM IPTG is a sufficient and efficient induction concentration for recombinant production and support the views expressed in the literature. Consequently, this study demonstrates that lower IPTG concentrations are economically and biotechnologically advantageous. Low induction levels both protect cell health and optimize production efficiency. Thus, 0.1 mM IPTG was chosen as the most appropriate concentration for further studies.

3.8. P3HB granule observations

P3HB granules produced in cultures supplemented separately with RW and glucose in P3HB media are shown in Figure 20A, Figure 20B, respectively. The pink color represents the cells, while the purple color indicates the P3HB granules. As observed, distinct P3HB granules were clearly visible in both glucose- and RW-containing media. The microscopic images obtained in this thesis are consistent with findings reported in the literature concerning both the presence and the intracellular localization of P3HB granules within bacterial cells. For instance, Sato et al. (2007) demonstrated that PHB granules produced in recombinant *E. coli* strains could be effectively stained using Sudan Black dye and subsequently visualized under a light microscope, allowing clear observation of the granules. Similarly, Narayanan et al. (2020) employed Sudan Black staining to successfully detect and analyze P3HB granules in their experimental cultures, further supporting the reliability of this staining method for confirming intracellular polymer accumulation. These corroborative results strengthen the validity of the microscopic observations presented in this thesis.

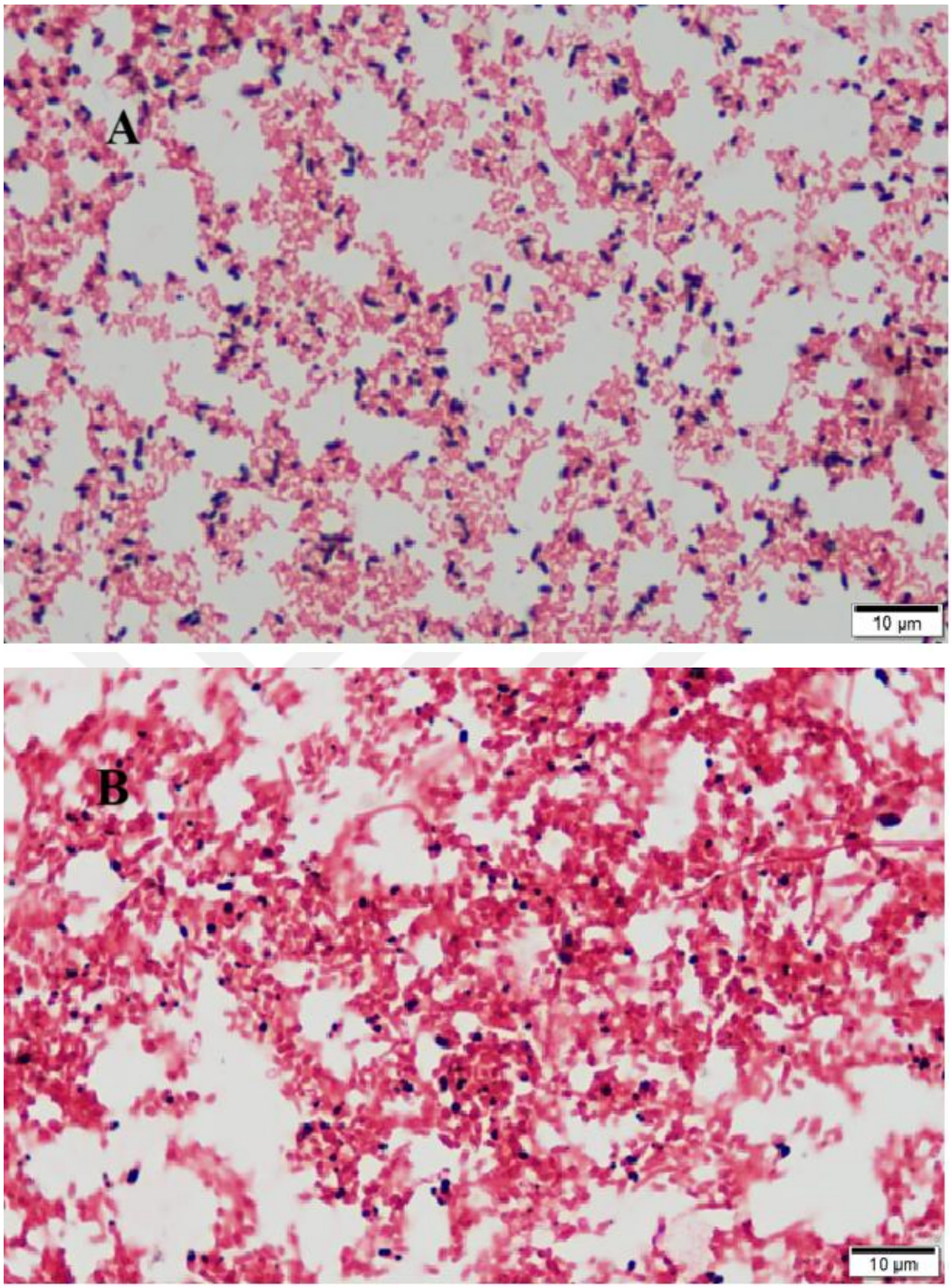


Figure 20. rec. *E. coli* Rosetta PQEAL1124 (pink) and P3HB granules (black) stored within the microorganism in P3HB production media supplemented with **A)** glucose, **B)** RW.

3.9. Optimization of P3HB production conditions using RSM

In order to maximize P3HB yield, a statistical optimization approach was applied in batch culture in shake flasks using *E. coli* Rosetta pQEAL1124 strain. The determination of the optimal values for RW, tryptone, and agitation rate was performed using CCD. Using 8 fact, 6 axial, and 6 center points, the responses obtained from the 20 runs created using CCD and the theoretical responses calculated according to the model are summarized in Table 17. A modified second-order model for bioprocess conditions was created as follows:

$$Y = -133{,}76 + 11{,}06\,X_1 + 79{,}54\,X_2 + 5{,}07\,X_3 - 0{,}06\,X_1X_3 + 0{,}38\,X_2X_3 - 31{,}14\,X_2^2 - 0{,}01\,X_3^2$$

In this equation, Y represents the expected amount of P3HB, while X_1, X_2, and X_3 represent the coded values of the amount of RW, the amount of tryptone, and the agitation rate, respectively.

Table 17. Responses obtained based on three different factors (P3HB amount g/L).

Experiment set (run)	X_1 (RW, g/L)	X_2 (Tryptone, g/L)	X_3 (Agitation rate, rpm)	Observed response (P3HB amount, g/L)	Predicted response (P3HB amount, g/L)
1	10	2	175	0.49	0.48
2	15	2	175	0.51	0.48
3	10	2	175	0.49	0.48
4	10	2	50	0.24	0.23
5	13	0.8	100	0.34	0.36
6	10	0	175	0.34	0.31
7	10	2	175	0.41	0.48
8	7	0.8	100	0.34	0.34
9	13	0.8	250	0.28	0.29
10	13	3.2	100	0.35	0.34
11	10	2	300	0.29	0.28
12	13	3.2	250	0.40	0.42
13	7	3.2	100	0.34	0.32

14	7	0.8	250	0.38	0.32
15	10	2	175	0.54	0.48
16	10	4	175	0.39	0.40
17	10	2	175	0.49	0.48
18	7	3.2	250	0.47	0.45
19	5	2	175	0.46	0.48
20	10	2	175	0.48	0.48

The ANOVA analysis of the model was performed using Fisher's *F* test, and the results are shown in Table 18. According to the statistical analysis results, the second-degree regression model was found to be significant ($p < 0.05 = 0.0001$). When the response and the regression coefficient of the model (R^2 value) are above 0.8, this is considered a strong correlation (Deniz et al., 2021). The R^2 value of 0.91 obtained in this study shows that the model explains 91% of the actual results and provides a good fit to the P3HB amount. The lack of fit value found to be 0.86 shows that the model is not affected by noise and provides a good fit. Additionally, the sufficient sensitivity value as seen in Table 19 indicates that the created model is consistent with the experimental data.

Table 18. ANOVA results of the response and characteristics of the modified model.

Source	Sum of squares	Degree of freedom	Mean square	F value	p-value Prob > F	
Model	133816.7	7	19116.67	17.69	<0.0001	significant
X_1-RW	0.34	1	0.34	0.000319	0.9860	
X_2-Tryptone	9126.51	1	9126.51	8.44	0.0132	
X_3-Agitation rate	2432.3	1	2432.3	2.25	0.1594	
X_1X_3	1544.57	1	1544.57	1.43	0.2550	
X_2X_3	9100.35	1	9100.35	8.42	0.0133	
X_2^2	28235.41	1	28235.41	26.12	0.0003	
X_3^2	91656.71	1	91656.71	84.79	<0.0001	
Residual	12970.35	12	1080.86			

Lack of fit	4763.56	7	680.51	0.41	0.86	not significant
Pure error	8206.79	5	1641.36			
Total	146787.1	19				

Table 19. Data from the model created with RSM.

Std. Dev.	32.88
Mean	398.39
C.V. %	8.25
R^2	0.91
Adjusted R^2	0.86
Predicted R^2	0.80
Adeq. Precision	11.89

The ANOVA results of the second-degree regression model show that the effect of tryptone amount and agitation rate on P3HB production is greater than that of RW. The interaction between the three factors on P3HB amount reveals the effect of bioprocess variables in 3D graphs as shown in Figure 21. Agitation rate above 175 rpm have caused a decrease in P3HB production. For optimal P3HB production, adequate agitation of the fermentation medium is necessary because agitation distributes air bubbles homogeneously throughout the medium, reduces temperature distribution differences within the bioreactor, and increases the transfer rate of nutrients. However, excessive agitation leads to high shear rates, negatively affecting both culture physiology and the rheological properties of the medium, thereby impairing enzyme production (Maiorano et al., 2020). Despite the effect of agitation, the effect of carbon source (RW) concentration on P3HB was found to be quite low as seen in Table 16. This is thought to be due to the varying concentrations of mixed sugars in the RW (glucose, fructose, and sucrose in amounts ranging from 10% to 15%) and the presence of other unidentified components in the RW. Similar results have been observed in other studies using hydrolysates with mixed compositions, such as corn stover (Kong et al., 2025) and sugarcane bagasse (Oyewole et al., 2024). Additionally, Lemos et al. (1998) and Liu et al. (2025) demonstrated that different

metabolic pathways resulting from the degradation of different carbon sources, along with the different enzymes and byproducts produced in these pathways, may have various effects on P3HB amount.

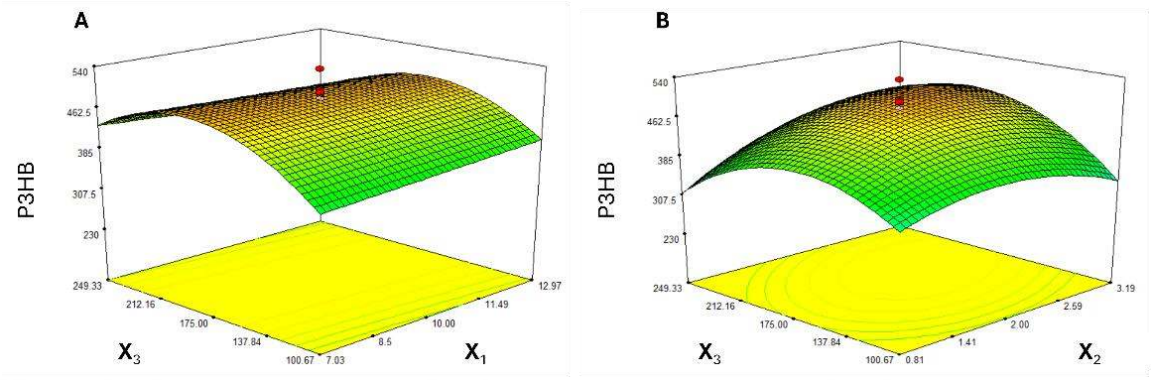


Figure 21. Three-dimensional response surface graph showing the effect of **A)** X_1 and X_3, **B)** X_2 and X_3on the amount of P3HB produced by rec. *E. coli* Rosetta pQEAL1124.

For the optimization of P3HB production, conditions providing the maximum predicted response and a maximum desirability value of 0.86 were selected as 7.03 g/L RW, 2.46 g/L tryptone, and 194 rpm. According to the modified second-order model, the predicted P3HB amount under these optimal conditions was calculated to be 491 mg/L at the end of 24 hours. Similarly, Tan et al. (2014) reported that high biomass and product yields were achieved in environments where carbon levels were optimized to 10 - 20 g/L and nitrogen levels to 1–2 g/L. In another study using rec. *E. coli*, the maximum P3HB production was reported to be 310 mg/L using a synthetic medium containing glucose and tryptone (Matsumoto et al., 2025). In another study performed with rec. *E. coli* and using corn straw, rice straw, and spent mushroom as carbon sources, 0.33 g/L P3HB production was achieved (Kong et al., 2025). The amounts of P3HB obtained from various production conditions of recombinant *E. coli* strains found in the literature are presented in Table 20 in comparison with this thesis study. Additionally, in another recently published study using *Azotobacter vinelandii* OP as the microorganism and hydrolyzed raisin waste as the carbon source, the P3HB yield at 72 hours was determined to be 1.0 g/L, with a productivity of 0.36 g/L/day (Andler et al., 2024). Although the P3HB amount obtained in this study is higher than that of this study, the productivity of this study is higher at 0.49 g/L/day.

Table 20. P3HB biosynthesis obtained from different *E. coli* strains.

rec. *E. coli* strain or inserted gene name	Carbon Source (g/L)	Nitrogen Source (g/L)	Agitation Rate (rpm)	DCW (g/L)	P3HB (g/L)	References
E. coli JM109	Glucose, 20	Tryptone 2	120	2.96	0.31	Matsumoto et al., (2025)
E. coli DHpACSTQKH	corn straw, rice straw, and spent mushroom	-	150	1.09	0.33	Kong et al., (2025)
PhaCAR	Glucose 10	Tryptone 2	120	3.04	0.38	(Furukawa et al., 2025)
LR1010 (ptsG)	Glucose and xylose 3.75	Yeast extract 1	200	2.3	0.26	Li et al., (2007)
BW25113 (pKCAB)	Glucose	-	150	0.95	0.45	Jian et al., (2010)
W ΔcscR	Sucrose 20	Tryptone 2	400	2.52	0.32	Arifin et al., (2011)
E. coli Rosetta pQEAL1124	RW 7.03	Tryptone 2.46	194	1.13	0.49	In this thesis

3.9.1. Model Validation

In order to validate the model, experiments were conducted in triplicate under optimized conditions in 50 ml production medium in 250 ml shake flasks. According to the model, the experimental results are expected to be in the range of 0.41 – 0.57 g/L. The predicted P3HB amount was 0.49 g/L, while the experimentally obtained result was determined to be 0.47 g/L. This indicates that the developed model is reliable. Thus, it has been confirmed that the model can be used for predicting bioprocess variables.

3.10. P3HB extraction

The most commonly used extraction method for P3HB is to use an acid for cell disruption, followed by chloroform to dissolve the P3HB. Thus, recent studies highlight the utilization of green solvents for P3HB extraction. In this study, 1,3-dioxolane, ethyl levulinate, and DMC were studied separately for P3HB extraction and compared with conventional methods. Table 21 shows the results of conventional, 1,3-dioxolane, ethyl levulinate, and DMC extraction methods applied to dried cells after the P3HB production.

Table 21. Comparison of P3HB extraction methods

P3HB Extraction Method	Extraction yield (%, g P3HB/ g DCW)	Relative extraction yield
Conventional	47 ± 3.1	100
1,3-dioxolane	33 ± 5.1	70.21
Ethyl levulinate	0	0
DMC	0	0

It is well known that conventional P3HB extraction methods, while enabling the production of high-purity products, pose significant risks in terms of environmental toxicity, human health, and occupational safety. In this thesis, the conventional method achieved the highest PHB recovery rate of 47% (Table 21). In the literature, Koller et al. (2014) reported that chloroform-based extraction methods provide high recovery and purity but also have significant limitations regarding environmental sustainability. Among the alternative solvents tested in this study, only 1,3-dioxolane resulted in a meaningful P3HB recovery (Figure 22), with an extraction yield of 33%. Although this rate is lower compared to the conventional method, considering the environmental benefits of green solvents, it can be regarded as a promising alternative. Similarly, Samorì et al. (2015) described 1,3-dioxolane as a promising green solvent due to its ability to operate at low temperatures and interact with PHB granules. In contrast, extractions carried out with ethyl levulinate, and DMC did not yield any P3HB. Dietrich et al. (2017) attributed this to the insufficient interaction of these solvents with the semi-crystalline structure of P3HB. Furthermore, solubility issues arising from the

structural characteristics of PHB are directly related to parameters such as solvent polarity, hydrogen bonding capacity, and process temperature.

Figure 22. P3HB obtained using 1,3-dioxolane as the green solvent.

In this context, various studies in the literature have reported high yields using green solvent applications. For instance, Wongmoon and Napathorn (2022) extracted P3HB from *Cupriavidus necator* using 1,3-dioxolane under high temperature (80°C) and continuous shaking conditions, achieving a recovery rate of up to 99.3%. Similarly, Gnaim et al. (2023) produced P3HB from avocado seeds using *Cobetia amphilecti* and employed ethyl levulinate as a green solvent. They reported that the obtained P3HB exhibited superior properties compared to the conventional chloroform-based extraction method, with a recovery rate of 97.4%. In another study, Mongili et al. (2021) extracted P3HB from recombinant *E. coli* using DMC and ethanol with wet biomass, achieving an extraction efficiency above 67%.

Although green solvents offer promising options for sustainable PHB production due to their low toxicity and biodegradability, they are still behind traditional methods in terms of efficiency. In this context, improvements in parameters such as solvent/biomass ratio, temperature optimization, and the integration of pretreatment steps could enhance the effectiveness of green solvents. Additionally, evaluating alternative extraction strategies such as binary solvent systems or ionic liquids holds significance for future studies.

CONCLUSION & RECOMMENDATIONS

Manisa is one of the major raisin production centers in Turkey, resulting in the generation of significant amounts of RW. Currently, this waste is mostly discarded into the environment without proper utilization or used in low-value applications. However, due to its organic composition and carbon content, it holds considerable potential as a valuable carbon source in microbial production processes. The environmental impacts of conventional petroleum-based plastics, their resistance to natural degradation, and inadequate waste management practices have heightened the importance of bioplastics within sustainable environmental policies. In recent years, technological advances in the production of biodegradable and biocompatible bioplastics have expanded both the diversity and application areas of these materials.

In this thesis, P3HB production was successfully achieved for the first time using the recombinant *Escherichia coli* Rosetta pQEAL1124 strain with raisin waste as a carbon source. Using the conventional extraction method, 0.47 g/L of P3HB was obtained. In addition, the study investigated the use of environmentally friendly green solvents as an alternative extraction strategy and compared their performance with the conventional method. Among these, 1,3-dioxolane enabled P3HB recovery with a yield of 33%, highlighting its potential as a sustainable extraction approach. These findings demonstrate that raisin waste can serve as an economical and renewable substrate for P3HB production. Moreover, process optimization through Response Surface Methodology (RSM) revealed that the maximum P3HB production was achieved under the conditions of 7.03 g/L raisin waste, 2.46 g/L tryptone, and 194 rpm.

In this context, enhancing extraction efficiency using green solvents requires detailed and meticulous optimization of critical parameters such as pretreatment steps, solvent-to-biomass ratio, temperature, extraction time, and solvent concentration. Each of these factors directly affects extraction performance and, if not optimized, may lead to significant yield losses. Furthermore, to improve the efficacy of green solvents, it is recommended to explore their combination with other solvents and integrate such systems into the extraction process strategically. Comprehensive studies on different solvent types and combinations should be conducted, alongside systematic optimization of extraction parameters. This approach can simultaneously increase

solvent efficiency and support environmental sustainability by developing more effective and greener extraction methods.

Moreover, to further enhance P3HB yield, it is essential to optimize the microorganism through genetic modifications and to activate cell disruption mechanisms that facilitate the release of P3HB granules from the cells. Such strategies can significantly improve the overall extraction efficiency and total yield. Finally, for the successful translation of laboratory-scale results to industrial applications, careful scale-up studies must be performed. The integration of green solvent-based extraction systems into biotechnological production processes is achievable only through these multidimensional and comprehensive approaches.

In conclusion, this study contributes significantly to sustainable waste valorization and bioplastic production by providing a novel perspective on environmentally friendly and sustainable production processes. Converting local agricultural waste, such as raisin residues, into value-added bioproducts offers a promising model for reducing environmental waste and generating high-value materials. Future work focusing on the development and optimization of green extraction techniques will promote more sustainable bioplastic production with reduced environmental impact and enhanced productivity, facilitating their broader industrial adoption.

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