POLYHYDROXYALKANOATE-BASED SUBSTRATE BAGS FOR SUSTAINABLE MUSHROOM CULTIVATION

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UNIVERSITI SAINS MALAYSIA

2025

POLYHYDROXYALKANOATE-BASED SUBSTRATE BAGS FOR SUSTAINABLE MUSHROOM CULTIVATION

by

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Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

March 2025

ACKNOWLEDGEMENT

Honouring the formal recognition of those who contributed significantly to developing my master's thesis, I consider it appropriate to convey my appreciation in a fashion that corresponds with the exceptional nature of this scholarly expedition. I thank my esteemed thesis adviser, Professor Dr. K. Sudesh Kumar, for providing valuable guidance and knowledge throughout my academic journey. Under your astute guidance, the enigmatic domain of concepts underwent a profound metamorphosis into a methodically organized storyline. Furthermore, your perseverance converted the complex network of obstacles into manageable puzzles. Distinguished colleagues from the Ecobiomaterial Research Laboratory at Universiti Sains Malaysia, your contributions transcended academic discourse and fuelled the intellectual fervour that underpins my thesis with enlightening exchanges. I am profoundly grateful for your encouragement of the embers of inspiration and stimulation of my intellectual curiosity. Sincere appreciation is extended to my family and friends, who are the unsung protagonists of this story. Your capacity to understand and aid with the discussions about my thesis is highly praiseworthy. Your steadfast confidence in my abilities has effectively averted the threat of procrastination. I wish to extend my sincere gratitude to the following individuals for generously sharing their experiences and serving as the foundation of my research: Dr. Manoj Lakshmanan, Dr. Tan Hua Tiang, Miss Vinishaa Raju, Mr. Lim Wei Yang, Mr. Fook Lun Qing, Miss Sharwini Raveentharan, Miss Thachayani Saravanan, Mr. Dinesh Kumar, Mr. Rinnesh Mohan, and all others who voluntarily contributed their insights. The invaluable aspect of authenticity that your collective contributions endowed into my work is something I could not have accomplished on my own. Sincere appreciation is extended to everyone

for contributing to this research's authenticity and exceptional quality. In summary, I express my sincere appreciation to every individual who has contributed to this thesis, regardless of the scale of their contributions, for assuming the roles of pivotal figures in this scholarly undertaking. With optimistic anticipation, I foresee a future convergence of our scientific endeavours and an exploration of the enigmas inherent in existence.

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LIST OF ABBREVIATIONS

3H4MV	3-hydroxy-4-methylvalerate
3HB	3-hydroxybutyrate
3HB-CoA	3-hydroxyacly-CoA
3HHp	3-hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3HHx-CoA	3-hydroxyhexanoyl-CoA
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
4HB-CoA	4-hydroxyacly-CoA
5HV	5-hydroxyvalerate
BE	Biological efficiency
C: N	Carbon to nitrogen ratio
CME	Caprylic methyl ester
CPKO	Crude Palm Kernel Oil
DM	Dry matter
DCW	Dry cell weight
DNA	Deoxyribonucleic acid
DSC	Differential Scanning Calorimetry
FabG	3-ketoacyl-ACP reductase
GC	Gas chromatography
GPC	Gel permeation chromatography
HA	Hydroxyacyl
HA-CoA	Hydroxyacyl-CoA

HPLC	High-performance liquid chromatography
LDPE	Low-density polyethylene
MEGA	Molecular Evolutionary Genetic Analysis
M_n	Number-average molecular weight
MSM	Mineral salt medium
M_w	Weight-average molecular weight
M_w/M_n	Polydispersity index
MCL	Medium-chain-length
NADPH	Reduced form nicotinamide adenine dinucleotide phosphate
NCBI	National Centre for Biotechnology Information
NR	Nutrient-rich
OD_{400nm}	Optical density at wavelength 400 nm
OD_{600nm}	Optical density at wavelength 600 nm
OD_{800nm}	Optical density at wavelength 800 nm
P(3HB)	Poly(3-hydroxybutyrate)
P(3HB-*co*-3HHx)	Poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3HB-*co*-4HB)	Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate)
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PE	Polyethylene
PP	Polypropylene
PHA	Polyhydroxyalkanoate
PhaA	Beta-ketothiolase

PhaB	NADPH-dependent acetoacetyl-CoA reductase
PhaC	PHA synthase
PhaJ	*(R)*-specific enoyl-CoA hydratase
PhaG	3-hydroxyacyl-ACP-CoA transferase
PhaY	PHA hydrolase
PhaZ	PHA depolymerase
PTFE	Polytetrafluoroethylene
RWS	Rubberwood sawdust
SD	Standard deviation
SCL	Short-chain-length
SMS	Spent mushroom substrate
T_g	Glass transition temperature
T_m	Melting temperature
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UHMW	Ultrahigh-molecular-weight
UV	Ultraviolet
WCO	Waste cooking oil
UCO	Used cooking oil

LIST OF SYMBOLS

α	Alpha
~	Approximately
β	Beta
Da	Dalton
°C	Degree Celsius
wt%	Dry weight percent
GPa	Gigapascal
g	Gram
h	Hour
∞	Infinity
kDa	Kilodalton
kPA	Kilopascal
kV	Kilovolt
L	Litre
N	Newton
Abs	Absorbance
MPa	Megapascal
µL	Microliter
µm	Microgram
cm	Centimetre
mg	Milligram
mL	Millilitre
mM	Millimolar

min	Minute
mol%	Mole percent
±	Plus-minus
%	Percentage
psi	Pounds per square inch
(R)	Rectus isomer
rpm	Revolutions per minute
s	Second
(S)	Sinister isomer
×	Times
V	Volt
v/v	Volume per volume
w/v	Weight per volume

LIST OF APPENDICES

BEG SUBSTRAT BERASASKAN POLIHIDROKSIAKANOAT UNTUK PENANAMAN CENDAWAN SECARA LESTARI

ABSTRAK

Plastik, yang terkenal dengan sifat serba bolehnya, telah menjadi bahan utama dalam pembungkusan beg substrat cendawan dan memainkan peranan penting dalam sistem pertanian moden. Ciri-ciri seperti ketahanan, keupayaan mengekalkan kelembapan, serta kos yang rendah menjadikannya bahan pilihan utama dalam memastikan keadaan pertumbuhan yang optimum. Walau bagaimanapun, penggunaan plastik secara meluas dalam industri ini menimbulkan implikasi alam sekitar yang serius, khususnya akibat pelupusan yang tidak lestari seperti pembakaran terbuka, yang menyumbang kepada pencemaran sisa plastik jangka panjang. Permintaan global yang semakin meningkat terhadap cendawan turut mempercepatkan pengumpulan sisa plastik, sekali gus memperburuk kesan negatif terhadap alam sekitar. Polihidroksialkanoat (PHA), sejenis poliester bakteria yang boleh terbiodegradasi, menawarkan alternatif yang mampan kepada beg substrat plastik konvensional. PHA mempunyai keupayaan untuk terurai sepenuhnya dalam persekitaran semula jadi dan boleh disintesis dalam keadaan terkawal, seperti melalui pembatasan sumber nitrogen. Kajian ini menilai kesesuaian pelbagai jenis PHA sebagai bahan pembungkusan substrat bagi penanaman Pleurotus ostreatus (cendawan tiram). Strain kejuruteraan Cupriavidus necator Re2160/pHT1-CBP-M-CPF4 telah digunakan untuk mensintesis poli(3-hidroksibutirat-ko-13 mol% 3-hidroksiheksanoat) daripada minyak masak terpakai dalam bioreaktor berskala 13 L. Polimer yang dihasilkan menunjukkan sifat fizikokimia yang setanding dengan plastik konvensional, menjadikannya berpotensi sebagai alternatif yang berdaya guna dalam aplikasi pertanian. Beg substrat

berasaskan PHA telah dihasilkan dan diuji dalam sistem penanaman cendawan tiram. Penilaian pasca penuaian merangkumi analisis kecekapan biologi, hasil tuaian, dan komposisi proksimat cendawan yang diperoleh. Selain itu, kadar biodegradasi filem PHA dikaji dalam pelbagai nisbah campuran substrat cendawan terpakai (SMS) dan tanah, dengan keputusan menunjukkan kadar degradasi yang lebih tinggi dalam persekitaran yang kaya dengan SMS. Kajian ini turut mengenal pasti dua spesies bakteria degradasi PHA, iaitu Acidovorax sp. dan Schlegella sp., yang diasingkan daripada filem yang mengalami degradasi separa. Penemuan ini memberikan pemahaman yang lebih mendalam mengenai mekanisme biodegradasi PHA dan berpotensi mempercepatkan proses pereputan melalui penambahan SMS. Kajian ini menegaskan potensi penggunaan beg PHA sebagai alternatif lestari kepada beg substrat plastik konvensional dalam industri penanaman cendawan. Dengan mengintegrasikan bahan biopolimer yang boleh terbiodegradasi ke dalam sistem pertanian, penyelidikan ini menyumbang kepada pengurangan pencemaran plastik serta memperkukuh amalan pertanian yang lebih mesra alam. Hasil kajian ini juga menyediakan asas saintifik bagi aplikasi masa hadapan polimer boleh urai dalam konteks industri dan penyelidikan, selaras dengan konsep ekonomi kitaran dalam pengurusan sisa agro-industri..

POLYHYDROXYALKANOATE-BASED SUBSTRATE BAGS FOR SUSTAINABLE MUSHROOM CULTIVATION

ABSTRACT

Plastics, renowned for their versatility, have become the predominant material for packaging mushroom substrate bags, playing a crucial role in modern mushroom cultivation. Their durability, moisture retention, and cost-effectiveness make them essential for maintaining optimal growing conditions. However, the extensive use of plastics in mushroom cultivation raises significant environmental concerns, as improper disposal—often through incineration—contributes to persistent plastic waste pollution. This issue is further exacerbated by the increasing global demand for mushrooms, which inadvertently amplifies plastic consumption and environmental burden. Polyhydroxyalkanoates (PHAs), a class of biodegradable bacterial polyesters, present a promising sustainable alternative to conventional plastic substrate bags. Notably, PHAs are fully biodegradable and can be synthesized under controlled conditions, such as nitrogen limitation. This study evaluates various PHA types for their suitability as substrate bags in *Pleurotus ostreatus* (oyster mushroom) cultivation. Using the engineered *Cupriavidus necator* Re2160/pHT1-CBP-M-CPF4 strain, poly(3-hydroxybutyrate-*co*-13 mol% 3-hydroxyhexanoate) was synthesized from waste cooking oil in a 13 L bioreactor, demonstrating material properties comparable to conventional plastics. PHA-based substrate bags were fabricated from the produced polymer and tested in oyster mushroom cultivation. Post-harvest assessments included biological efficiency, yield, and proximate analysis of the mushrooms. Additionally, the degradation of PHA films was examined in varying ratios of spent mushroom substrate (SMS) and soil, revealing accelerated degradation

in SMS-rich environments. Furthermore, two distinct PHA-degrading bacterial strains, *Acidovorax* sp. and *Schlegella* sp., were isolated from partially degraded films, providing valuable insights into enhancing PHA biodegradation efficiency. This study underscores the potential of biodegradable PHA bags as an environmentally responsible alternative to traditional plastic substrate bags. By integrating sustainable biomaterials into mushroom cultivation, this research contributes to reducing plastic waste and advancing sustainable agricultural practices. The findings provide a foundational framework for future applications of biodegradable polymers in industrial and scientific contexts, promoting a circular economy in agro-industrial waste management.

CHAPTER 1
INTRODUCTION

1.1 Overview of the study

Plastics have a substantial influence on modern life thanks to their economic viability, remarkable durability, and lightweight nature. They exhibit resilience to deterioration and have exceptional thermal and electrical insulating qualities. The versatility of plastic materials allows for the creation of a variety of valuable products, leading to several social benefits. The widespread expansion of plastic manufacturing in several sectors is expected to result in a significant 70% rise in plastic output by 2050 (Praveenkumar et al., 2024). Simultaneously, the global growth of fungiculture is increasing due to the growing demand for mushrooms, driven by their excellent nutritional value. Mushrooms include dietary fibre, polysaccharides, heteroglycans, peptidoglycan, and proteoglycans, which are essential sources of critical vitamins (Bains et al., 2021). Several studies have shown that different types of mushrooms can help prevent and treat chronic conditions like cardiovascular disease, diabetes, cancer, obesity, and high cholesterol (Ghani et al., 2019). Since 2000, the global mushroom business has grown fivefold, with current output at 44 million tonnes, driven mainly by Asia (Bijla & Sharma, 2023). Fungiculture is becoming more effective in many places because of its short harvest intervals, low input needs, and economically feasible production methods (Higgins et al., 2017).

Plastic materials play a crucial role in contemporary mushroom production methods, involving the use of trays, bottles, and bags (Panjikkaran & Mathew, 2013). Yamanaka's research highlights the significant reliance on little plastic bags, which made up over 80% of worldwide mushroom production in 2013, particularly in Southeast Asia (Yamanaka, 2017). These bags are typically made of polyethene (PE)

and polypropylene (PP) and are crucial for the growing process. After harvesting, these plastic substrate bags are often improperly disposed of (Haimid et al., 2013). According to Panjikkaran and Mathew, cultivation of *Pleurotus ostreatus* (oyster mushroom) produces around 18 g of non-biodegradable plastic bags for every 450 g of mushrooms (Panjikkaran & Mathew, 2013). Unfortunately, these plastic bags are either burned or thrown away with the used mushroom substrate, adding to the increasing pile of non-biodegradable waste (Mamiro et al., 2014). Furthermore, these discarded plastics undergo physiochemical changes, breaking down into microplastics, which worsens environmental contamination (Zhang et al., 2021). The increasing demand for mushrooms is causing environmental plastic pollution. To tackle environmental concerns and encourage sustainable mushroom production, a sustainable alternative is known as polyhydroxyalkoante (PHA).

PHA, akin to petroleum-based polymers such as PP and PE, have physical properties that enable them to decompose in natural settings (Lee, 2000; Sudesh, 2013). PHA possess a myriad of characteristics that distinguish them as "green plastics" as they are derived from renewable biological sources, synthesized through biological processes, and exhibit biodegradability, compostability, and biocompatibility (Koller, 2021). PHA are classified into three main types: short-chain-length (SCL-PHA) with monomer molecules containing 3 to 5 carbon atoms, medium-chain-length (MCL-PHA) with monomer molecules including 6 to 14 carbon atoms, and a mixture of both SCL-PHA and MCL-PHA (Sudesh, 2013). The precise monomer content in the polymer structure determines the physical characteristics of PHAs. poly-3-hydroxybutyrate [P(3HB)] is a form of SCL-PHA homopolymer composed of units of monomer with 4 carbon atoms. SCL-PHA homopolymer is known for its high crystallinity, high melting temperature (T_m), and brittle nature

(Reinecke & Steinbüchel, 2009). Incorporating MCL monomers of 3HB during copolymerization changes the characteristics of the polymer, creating a softer and more elastic material than P(3HB) (Noda et al., 2005; Turco et al., 2021). In addition, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] is considered an economically feasible copolymer compared to other PHA forms since it closely resembles commonly utilized polymers such as PP and low-density polyethene (LDPE). The properties of P(3HB-*co*-3HHx) biopolymer change depending on the amounts of 3-hydroxyhexanoate (3HHx), resulting in a spectrum of characteristics ranging from very rigid and less elastic to more flexible and stiffer forms (Asrar et al., 2002). Moreover, PHA with increased molecular weights (M_w) have enhanced mechanical characteristics, rendering them advantageous for real-world uses (Aoyagi et al., 2003). Besides, Research has shown that P(3HB-*co*-3HHx) is biodegradable in various aerobic environments, including seawater, freshwater, soil, and compost (Morse et al., 2011). Under aerobic conditions, it breaks down into water and carbon dioxide, while anaerobic degradation, facilitated by P(3HB) depolymerase, produces water and methane (Abe, 1999). In seawater, P(3HB-*co*-3HHx) degrades within approximately six months (Tang et al., 2022). Its degradation rate is influenced by factors such as environmental conditions, microbial activity, monomer composition, temperature, and humidity.

Although PHA are seen as a possible substitute for regular plastics, their production expenses are still notably more than those of standard petrochemical plastics. The expense of raw materials in manufacturing has been a significant challenge for PHA production, contributing significantly to its overall production costs (Tyagi & Sharma, 2021). Researchers have investigated ways to decrease the biosynthesis expense of PHA by replacing the pure substrate with sustainable waste

by-products from different sectors (Aslan et al., 2016). Prior research has explored using alternate carbon sources such as palm oil (Rao et al., 2010), agricultural wastes (Amulya et al., 2015), crude glycerol (Cavalheiro et al., 2012), and molasses (Rathika et al., 2019) to enhance the production of PHA economically. Numerous research studies have highlighted the prospect of waste cooking oil (WCO) as a raw material to synthesise the process of PHA due to its cost-effectiveness and sustainability as a carbon source (Fernández et al., 2005; Fook et al., 2024; Haba et al., 2007; Kourmentza et al., 2018; Verlinden et al., 2011). This research clearly shows that WCO is a dependable carbon source for successful PHA accumulation and economical PHA production.

This work aims to biosynthesize P(3HB-*co*-13 mol% 3HHx) by employing genetically engineered *Cupriavidus necator*, Re2160/pHT1-$C_{BP\text{-}M\text{-}CPF04}$, and utilizing WCO as an economical carbon source. The polymer is produced and utilized to fabricate mushroom substrate bags for growing *P. ostreatus*. An investigation is performed to ascertain the degradation rate in the presence of varying quantities of spent mushroom substrate (SMS) and soil. PHA-degrading bacteria are isolated and identified to confirm the effectiveness of PHA degradation in the presence of SMS. The nutritional composition of the cultivated *P. ostreatus* is also analyzed. This study thoroughly examines the production and application of PHA polymers for mushroom culture, as well as investigates their environmental impact through degradation studies. The collective results improve our comprehension of the correlation among PHA polymers, microbial breakdown, and the nutritional value of *P. ostreatus* cultivated using PHA-based mushroom substrate bags.

1.2 Justification of the Study

The global mushroom industry has experienced significant growth, driven by the increasing recognition of mushrooms as a nutritious and versatile food source. In Malaysia, mushroom consumption was projected to rise from 1.0 kg per person in 2008 to 2.4 kg per person by 2020, with production expected to increase by 67% by 2020 and potentially exceeding 50 million tonnes by 2025 (Abu Shamsi et al., 2023; Mwangi et al., 2022; Samsudin & Abdullah, 2019). To support this expansion, the Malaysian Agricultural Research and Development Institute (MARDI) has recommended government tax incentives for plastic-based mushroom substrate bags to boost local cultivation efforts (Haimid et al., 2013). However, the widespread use of petroleum-based plastic bags in mushroom farming exacerbates plastic pollution, posing environmental concerns. Given this challenge, advocating for polyhydroxyalkanoate PHA-based mushroom substrate bags presents a promising sustainable alternative. PHAs, known for their exceptional biodegradability and physical properties comparable to conventional plastics, offer an eco-friendly solution to mitigate the environmental impact of plastic waste in mushroom cultivation. This study aims to develop and evaluate mushroom cultivation using PHA-based substrate bags, with a strong emphasis on the sustainability of PHA production and its application. Additionally, it seeks to assess the biodegradation of PHA-based mushroom substrate bags in the presence of spent mushroom substrate, highlighting their potential to support environmentally responsible agricultural practices.

1.3 Objectives of the Study

The objectives of the investigation are:

1. To biosynthesize and characterize the determined composition of PHA polymer from waste cooking oil polymer for fabrication of mushroom substrate bags.
2. To develop and evaluate mushroom cultivation using PHA-based substrate bags.
3. To assess the biodegradation of PHA-based mushroom substrate bags in the presence of spent mushroom substrate.

CHAPTER 2
LITERATURE REVIEW

2.1 Fungiculture

Mushrooms are fungi with conspicuous fruiting structures visible to the human eye and may be physically harvested (Sisodiya et al., 2023). Out of the estimated 1.5 million fungi, around 14,000 identified species worldwide produce fruiting bodies big enough to be classified as mushrooms and 200 edible species (Assemie & Abaya, 2022). A typical mushroom is composed of a cap, which acts as a protective cover for the spore- producing terrain, a stalk or stem that provides support for the cap, and an arrangement of gills or other spore-bearing structures (Katoch et al., 2023). Spores are crucial for the dissemination and proliferation of fungi; specialized cells, such as basidia or asci, create spores depending on the fungal group (Yadav et al., 2023). Fungi have essential ecological functions, especially as decomposers that break down organic materials (Liu et al., 2021). The systematic growth and production of mushrooms for several purposes, such as gastronomic, medicinal, and industrial uses, is referred to as fungiculture.

Mushrooms are predominantly cultivated for their medicinal properties (Anusiya et al., 2021). Different types of mushrooms include bioactive compounds that have positive impacts on health. To begin with, mushrooms possess β-glucans, which are polysaccharides that facilitate the activation of the immune system (van Steenwijk et al., 2021). Besides, mushrooms contain anti-inflammatory chemicals such as triterpenoids and ergothioneine, which help reduce inflammation (Tung et al., 2020). In addition, mushrooms are highly acknowledged for their anti-cancer effects. Out of the 2000 edible types of mushrooms, varieties like *Ganoderma*, *Lentinus*, *Grifola*, *Agaricus*, *Cordyceps*, *Pleurotus*, and *Hericium* contain specific bioactive

compounds known for their anticancer effects (Anusiya et al., 2021). Moreover, research has indicated that various types of mushrooms have compounds that could minimize cholesterol levels while enhancing blood flow, potentially improving cardiovascular health (Mustafa et al., 2022).

Besides, mushrooms have played an essential role in diet for years, and now they are finding their way as cosmetic ingredients, either as cosmeceutical or nutricosmetics. Various mushroom extracts and their secondary metabolites have been documented to exhibit significant biological activities, such as inhibiting tyrosinase, collagenase, elastase, and hyaluronidase (Taofiq et al., 2016). Mushroom extracts are commonly incorporated into moisturizers, serums, and masks to provide hydration, brighten the skin, and enhance its elasticity and texture. Besides, mushrooms are abundant in bioactive compounds such as polyphenols, polysaccharides, vitamins, carotenoids, and minerals, all known for their potent antioxidant properties (Mago et al., 2023). Some studies indicate that mushroom extracts such as β-glucan acts as an immunomodulator that can reduce the appearance of wrinkles (Sangthong et al., 2022).

Worldwide, the consumption of mushrooms is increasing due to a growing awareness of their health benefits and delicacies. Fungiculture, such as mushroom cultivation, has been a long-standing industry in Asia, particularly China, Japan, and Korea (Junior Letti et al., 2018). Other significant contributors to mushroom cultivation worldwide include the USA, the Netherlands, as well as Poland, Spain, France, Italy, Ireland, Canada, and the UK. Projections indicate that the global mushroom market is poised to reach US$ 69.3 billion by 2024, driven by a growing demand for healthier food choices among consumers (Thakur, 2020). The increase in the popularity of vegan and vegetarian diets has led to a significant rise in the demand

for mushrooms as a substitute for meat (Kaur & Kapoor, 2023). The fast expansion of fungiculture may be ascribed to the growing worldwide demand.

2.1.1 Mushroom cultivation

Mushroom cultivation is a complex procedure that needs meticulous attention to several processes to create an ideal environment for the growth and maturation of mushrooms. The primary practical steps of mushroom cultivation comprise a few significant steps, as shown in Figure 2.1.

The first consideration is the choice of a suitable mushroom species. Before deciding to cultivate a specific type of mushroom, it is crucial to ascertain whether it possesses organoleptic qualities deemed acceptable by the local population or the international market, provided that there is an abundance of suitable substrates for cultivation (Chang & Wasser, 2017). Additionally, it is crucial to ensure that the environmental conditions necessary for growth and fruiting can be met without incurring excessive costs for mechanical control. *Agaricus*, *Lentinulla*, and *Pleurotus* mushrooms are widely cultivated and commercially popular in several countries due to their desirable properties (Sande et al., 2019).

The formation of a robust mushroom spore comes next. Mushroom spawn refers to the medium in which the mycelium of a vegetative culture has developed and is utilized as the inoculum for the substrate during mushroom cultivation (Idowu et al., 2016). Mycelium mushroom spawn is typically available for purchase from a vendor, as numerous spawn-producing companies offer a variety of spawns for sale, including liquid spawns and grains spawns (Moreaux, 2017). Furthermore, it is strongly advised that mushroom cultivators prioritize the growing process if they can

procure spores of top quality and affordability for the desired mushroom species (Oei et al., 2005).

The subsequent procedure involves the preparation of a specific substrate. Typically, substrates used for growing edible mushrooms need to undergo different levels of pre-treatment to encourage the development of the mushroom mycelia while minimizing the growth of other microorganisms. In most instances, the prepared substrate will undergo sterilization by a 15 min heat treatment at a temperature of 121 °C and 15 psi to eradicate foreign microbes and eliminate soluble nutrients (Oei et al., 2005). Plastics are the favoured choice because of their ability to handle high levels of sterilization pressure and temperature, especially PP. The substrate should have abundant vital nutrients that are easily accessible to the mushroom while also being devoid of any poisonous chemicals that hinder the development of the spawn (Chang & Wasser, 2017). Various mushroom species need distinct growing substrates. For example, *P. ostreatus* may be successfully grown on many substrates, such as straw, sawdust, and coffee grounds, but shiitake mushrooms prefer to grow on hardwood sawdust (Kamthan & Tiwari, 2017). It is crucial to consider the moisture content, pH level, and effective exchange of gases within the substrate and the environment that surrounds it as significant physical parameters (Shu & Lung, 2004; Wang & McNeil, 1995). Once the substrate has been inoculated, it should be placed in a container or chamber and given sufficient time to completely colonize before adding humidity and light to facilitate the growth of mycelium (Yamanaka, 2017). The spawn running phase is when mycelia extend from the spawn and infiltrate the substrate. Optimal mycelial proliferation is crucial for the successful cultivation of mushrooms (JasiŃSka et al., 2017).

The last step involves the cultivation and harvesting of mushrooms. Under optimal environmental circumstances, which may depend on the spawn used, spontaneous germination is formed by fruiting bodies (Chang & Wasser, 2017). The harvesting of the fruiting body varies according to the species and spawn quality of the substrate (Knoll et al., 2009). After the mycelium has fully colonized the substrate, the substrate bags are opened to allow for proper air circulation, which is necessary for the growth and development of the fruiting bodies (Oei et al., 2005). Hence, transparent plastic bags are used by mushroom farmers to see the development of mycelium. Once the mushroom caps have fully expanded and before the gills release spores, they should be harvested by twisting and delicately pulling the steam until it detaches from the substrate (Rubatzky & Yamaguchi, 1997). However, it is essential to avoid harming the mushroom's mycelium.

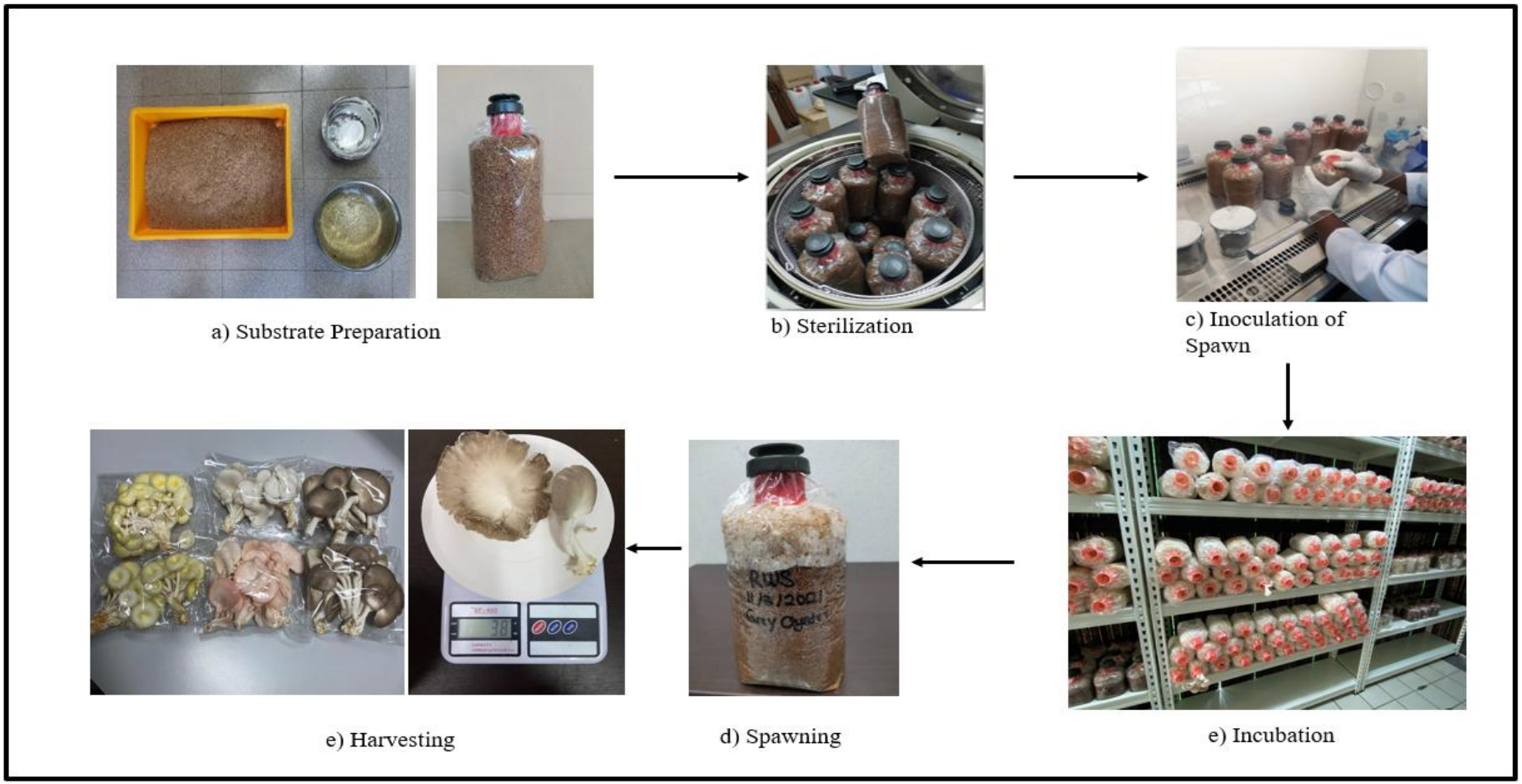

Figure 2.1: Sequential stages of mushroom cultivation, depicting (a) Substrate preparation, (b) Sterilization, (c) Inoculation of Spawn, (d) Incubation ,(e) Spawing and (f) Harvesting

2.1.2 Substrate for mushroom cultivation

The growth substrate is a fundamental component in mushroom cultivation, serving as the primary medium that provides essential nutrients for fungal growth and development. Mushrooms, as heterotrophic organisms, rely on organic substrates rich in carbon, nitrogen, and minerals for mycelial expansion and fruiting body formation (Suwannarach et al., 2022). The selection of an appropriate substrate is crucial, as it directly influences the yield, quality, and efficiency of production. Various types of substrates are used depending on the mushroom species, with common choices including lignocellulosic materials such as sawdust, straw, corn cobs, sugarcane bagasse, and agricultural residues (Kosre et al., 2021). Other organic materials, such as coffee grounds, spent brewery grains, and animal manure, can also be utilised, often in combination with supplementary additives like wheat bran or gypsum, to enhance the nutrient profile (Sydor et al., 2022)

The role of the substrate extends beyond nutrient provision; it also determines the physical and chemical conditions necessary for optimal mycelial colonisation. The structure of the substrate affects aeration, moisture retention, and water availability, all of which are critical for fungal metabolism and enzyme activity. Properly prepared substrates should have an adequate carbon-to-nitrogen (C: N) ratio, usually between 20:1 and 40:1, to support efficient decomposition and nutrient uptake by the mycelium. Additionally, maintaining a suitable moisture content, typically between 60% and 70%, is essential to facilitate the enzymatic breakdown of complex organic matter into simpler compounds that can be absorbed by the fungal cells.

The importance of the substrate in mushroom cultivation extends to its influence on contamination risk and overall crop success. Unprocessed or poorly

prepared substrates may harbour competing microorganisms such as bacteria, moulds, and unwanted fungi, which can outcompete or inhibit mushroom mycelium growth. To mitigate this, substrates undergo treatment processes such as pasteurisation or sterilisation, which help eliminate potential contaminants while preserving favourable microbial communities that may support mushroom development. Furthermore, the degradation of organic matter in the substrate leads to the release of bioavailable nutrients that stimulate fruiting body initiation and maturation, ultimately affecting the productivity and economic viability of cultivation.

In addition to its role in mushroom production, the use of agricultural and industrial by-products as substrates promotes environmental sustainability by reducing waste and converting biomass into valuable edible or medicinal fungi. The selection and preparation of an appropriate substrate are, therefore, integral to both the biological and economic aspects of mushroom cultivation. By optimising substrate composition, moisture content, and treatment methods, cultivators can enhance growth efficiency, minimise losses due to contamination, and improve overall yield and quality.

On the other hand, Spent mushroom substrate (SMS) is the residual organic material left after mushroom cultivation, consisting of partially decomposed lignocellulosic matter enriched with fungal metabolites, microbial communities, and residual nutrients (Qian et al., 2022). Effective management of SMS is essential to minimise environmental impact while maximising its potential for value-added applications. In Malaysia, SMS is commonly utilised as an organic fertiliser due to its improved nutrient profile and microbial activity, which enhance soil fertility and plant growth (Rosmiza et al., 2016). The partially decomposed organic matter enriches the soil with essential macronutrients such as nitrogen, phosphorus, and potassium, while

its high organic content improves soil structure, water retention, and microbial biodiversity (Mohd Hanafi et al., 2018). In other countries, SMS is widely repurposed as animal feed, particularly for ruminants such as cattle. Studies have shown that incorporating SMS into livestock feed can enhance digestibility and increase milk production in dairy cows, attributed to its fibre content, residual protein, and probiotic properties that support rumen fermentation (Martin et al., 2023). Additionally, SMS can be utilised for composting, biofuel production, and bioremediation, further contributing to sustainable waste management and circular bioeconomy initiatives (Leong, Varjani, et al., 2022; Martin et al., 2023) . The strategic utilisation of SMS not only reduces agricultural waste but also provides economic and environmental benefits, reinforcing its role as a valuable by-product in the mushroom industry.

2.1.3 *Pleurotus ostreatus*

One of the most widely cultivated mushrooms in the world *is P. ostreatus*, also called the oyster mushroom, as shown in Figure 2.2. *Pleurotus ostreatus* is famous for its fast and easy growth rate, capacity to grow in various environments, and delicious flavour and texture. Table 2.2 presents a nutritional comparison of *P. ostreatus* with other most commonly produced mushrooms worldwide (Hou et al., 2019; Vetter, 2019). *Pleurotus ostreatus* is the most typically cultivated and the best for first-timers discovering mushroom farming. Additionally, *P. ostreatus* exhibits rapid growth on various agro-wastes, including dried sugar cane leaves, sawdust, maize stover, banana leaves, palm cones, coffee husks, and wheat bran (Al Turki, 2021). These substrates can be supplemented with corn flour, rice bran, molasses, soybean, or kernel cake,

depending on the specific substrate material used (Rezania et al., 2017). Table 2.1 shows the detailed scientific classification of *P. ostreatus* (Wan Mahari et al., 2020).

Pleurotus ostreatus is a nutrient-dense product that is low in calories (Lesa et al., 2022). Studies have shown that the proximal chemical examination of *P. ostreatus* reveals a high protein content, typically ranging from 15% to 34.7% (Lesa et al., 2022). In addition, *P. ostreatus* contains a high amount of fibre, roughly 17 to 42 g per 100 g of mushroom. Moreover, *P. ostreatus* contains numerous vitamins, including niacin, riboflavin, and vitamin D (Fulgoni & Agarwal, 2021) and also contains abundant polysaccharides, such as β-glucans and chitin, which have potential health benefits, including immune system stimulation and blood sugar regulation (Kumari, 2020).

Moreover, *P. ostreatus* has antioxidant properties that may help protect against oxidative stress and reduce the risk of chronic diseases such as cancer, diabetes, and heart disease (Roncero-Ramos & Delgado-Andrade, 2017). In addition, *P. ostreatus* is rich in anti-inflammatory properties, which help reduce inflammation and improve symptoms of inflammatory conditions such as arthritis and asthma (Gunawardena et al., 2014). *Pleurotus ostreatus* has been shown to have antimicrobial properties, which help to protect against bacterial and fungal infections (Lesa et al., 2022). Some studies also indicated that *P. ostreatus* may have anti-cancer properties due to its ability to stimulate the immune system and inhibit the growth of cancer cells (Mishra et al., 2021). Lastly, *P. ostreatus* contains compounds that may protect against neurodegenerative diseases and improve cognitive function (Beelman et al., 2019).

(a) (b)

Figure 2.2: (a) Rubberwood sawdust-based mushroom substrate block. (b) Fruiting bodies of *P. ostreatus*.

Table 2.1: Scientific classification of *Pleurotus ostreatus*

Taxonomic description	Classification
Kingdom	Fungi
Phylum	Basidiomycota
Class	Agaricomycetes
Order	Agaricales
Family	Pleurotacceae
Genus	*Pleaurotus*
Species	*Pleaurotus ostreatus*

Table 2.2:Comparative analysis of nutritional analysis of *P. ostreatus* with widely cultivated varieties of mushrooms worldwide

Mushroom species	Crude protein (% DM)[a]	Crude fat (% DM)[b]	Carbohydrate (% DM)[c]	Energy (kcal/100 g DM)
Agaricus bisporus	14.0-36.3	0.8-2.5	50.9-74.0	303-325
Pleurotus ostreatus	7.0-23.8	0.5-5.4	51.9-85	416
Lentinula edodes	4.4-20.5	1.7-6.3	67.9-87.0	386
Volvariella volvacea	36.5	2.2	52.3	n.d [d]
Auricularia auricula	5.7-15.5	0.4-4.5	77-91	97-140

[a,b,c,d] Abbreviations: DM, Dry matter; n.d, no data; (Vetter, 2019)

2.1.4 Cultivation of *Pleurotus ostreatus* in Malaysia and the world

The cultivation of *P. ostreatus* in Malaysia generates considerable interest owing to its economic viability and nutritional benefits. The cultivation of *P. ostreatus* in Malaysia is mainly conducted on a small scale, frequently by smallholder farmers or domestic cultivators (Sridhar & Deshmukh, 2021). Over the past few years, there has been a surge in the commercial cultivation of *P. ostreatus* within Malaysia, resulting in some firms generating substantial mushroom yields for overseas distribution (Zaffrie et al., 2014). The increased demand for *P. ostreatus* in Malaysia can be attributed to its nutritional advantages and reputation as a delicacy. Besides, cultivating *P. ostreatus* in Malaysia presents an opportunity for environmentally

sustainable practices, as it involves utilizing agricultural waste materials, especially palm oil waste, that would otherwise be disposed of (Aubrey et al., 2022).

Pleurotus ostreatus is the predominant mushroom species cultivated in Malaysia, accounting for almost 90% of the total mushroom production. The production is projected to reach 65,000 tonnes by 2020 (Wan Mahari et al., 2020). The total value of *P. ostreatus* was expected to increase to RM 110 million in 2014, which denotes a surge compared to preceding years, signifying the expansion of *P. ostreatus* in Malaysia, with a particular concentration in regions such as Selangor, Pahang, and Johor (Haimid et al., 2013; Rosmiza et al., 2016). The areas mentioned earlier exhibit propitious ecological circumstances for cultivating *P. ostreatus*, characterized by optimal temperature and humidity levels and the presence of requisite raw materials such as sawdust, rice straw, and corn cobs, which are conventionally employed as substrates in mushroom cultivation. Notably, the production of mushrooms in Malaysia may have undergone alterations in recent times owing to diverse factors such as fluctuations in market demand, shifts in climatic patterns, and modifications in governmental regulations (Rosmiza et al., 2016).

As per previous research findings, the worldwide cultivation of mushrooms, including *P. ostreatus*, will be valued at 20.84 million tonnes by 2026 (Leong Ma, et al., 2022). The expansion of the *P. ostreatus* industry can be attributed to various factors, including the growing trend of vegan and vegetarian diets, increased consumer interest in natural and organic food items, and the numerous health advantages linked to the consumption of *P. ostreatus* (Predanócyová et al., 2023). Furthermore, other research highlights that the *P. ostreatus* market has experienced an increase in demand due to the COVID-19 pandemic (Maring et al., 2023). Various new diseases can be

attributed to the increased concern among consumers regarding health and wellness, leading them to seek foods that can enhance their immune systems.

2.2 Challenges in sustainable mushroom cultivation

Nearly 80% of mushroom farmers in Malaysia operate on a small scale. The mushroom export value in Malaysia amounted to RM 300 million in 2020, with expectations of an annual doubling in both consumption and production (Abu Shamsi et al., 2023; Haimid et al., 2013). Nevertheless, the significant production and usage in this sector raise a crucial inquiry about the long-term viability of these procedures. Although cultivating mushrooms sustainably presents notable benefits, it is not without difficulties. Multiple impediments obstruct the implementing and sustaining of environmentally friendly and socially responsible practices in the mushroom cultivation industry.

Plastic bag waste is one of the most significant wastes in the mushroom industry. Plastic bags, particularly those made of PE and PP, are often preferred for mushroom cultivation due to their favourable physical qualities. Besides, Yamanaka's study states that the main components utilized in the small plastic bags are sawdust and agricultural by-products, which are often inexpensive and readily accessible. Therefore, the use of plastic bags is preferable for mushroom culture since it requires a lesser amount of nutrients compared to bottle cultivation (Yamanaka, 2017).

Depending on the size of the cultivation, the kind of plastic used, and the culture technique, different amounts of plastic trash may be produced when growing mushrooms, resulting in plastic waste if not disposed of or recycled appropriately. Hence, large-scale cultivation operations could produce more plastic waste than smaller-scale operations. The amount of waste generated by the mushroom production

industry may appear comparatively low compared to other sectors. However, the quantities produced are estimated to be approximately equivalent to 1000 times the weight of one billion plastic straws previously utilized annually by Starbucks before their demise of using plastic straws in 2020 (Okuda, 2022). Undeniably, the growth in demand for mushrooms is directly correlated with the increase in plastic waste.

The plastic waste generated by mushroom cultivation is primarily disposed of by landfilling, on-site burial, illegal dumping, or on-site incineration, all representing significant environmental risks (Galati & Scalenghe, 2021; Wan Mahari et al., 2020). Plastic waste in landfills takes up extra space, contaminating waterways, soil, and air (Evode et al., 2021). Besides, plastic waste in landfills also can take hundreds of years to decompose, which may increase the area of landfills and reduce the readily available space (Chen et al., 2021). As plastic waste fragments into smaller particles due to environmental changes, it transforms into microplastics, posing a potential threat to human health by infiltrating the food chain and endangering wildlife (Bajt, 2021). At the same time, the incineration of plastic bags and containers used in mushroom cultivation contributes to carbon emissions, which causes global warming (Atiwesh et al., 2021).

Malaysia possesses significant potential for the mushroom business to evolve into a circular economy. Nevertheless, the mushroom industry has yet to achieve its potential as a circular economy and sustainable activity due to the prevalent usage of single-use plastic substrate bags.

2.3 Polyhydroxyalkanoate (PHA)

2.3.1 Background of PHA

Polyhydroxyalkanoate (PHA) represents a variety of biodegradable polymers synthesized intracellularly by almost 75 distinct species of Gram-positive and Gram-negative microorganisms as carbon and energy storage (Reddy et al., 2022). PHA is accumulated by microorganisms when subjected to limited nutrients, specifically in excess carbon and insufficient nutrients such as nitrogen, phosphorus, magnesium, or oxygen (Anderson & Dawes, 1990; Chavan et al., 2021). Formation of PHA is the metabolic conversion of the assimilated carbon sources into 3-hydroxyalkanoic acid (3HA) monomer units from the polymerization of a carboxyl group and a hydroxyl group into water-insoluble inclusions occur in the cytoplasm of the microorganism (Sridewi et al., 2006; Tan et al., 2020). According to earlier studies, since PHAs are insoluble in bacterial cells, microbes can store roughly 90% of PHA extracellularly without affecting the cells' osmotic pressure (Goswami et al., 2023).

P(3HB) was initially identified by Lemoigne in 1926 in *Bacillus megaterium* (Steinbüchel, 1991). Further investigations unveiled a multitude of monomer units, including 3-hydroxyhexanoate (3HHx), 3-hydroxyvalerate (3HV), 3-hydroxyheptanoate (3HHp), and over 150 other distinct constituents of PHA (Rehm, 2003). These PHA monomers exhibit a wide array of structural variations. PHA can be classified into homopolyesters and heteropolyesters based on the number of distinct building blocks or monomers present. Homopolyesters comprise only one monomer type, with P(3HB) being the quintessential example, characterized by its isotactic, linear, and thermoplastic nature (Koller, 2018). Conversely, heteropolyesters are copolyesters containing two different monomers, exemplified by P(3HB-*co*-3HHx). Moreover, the diversity of microorganisms producing PHA depends on the substrate specificity of

PHA synthase and the carbon source used, which subsequently influence the metabolic pathways of microbes (Loo & Sudesh, 2007). Consequently, PHA are classified based on the carbon atom count in their monomers, resulting in short-chain-length (SCL), medium-chain-length (MCL), and a combination of SCL-MCL (Sudesh & Iwata, 2008). SCL PHA comprises 3 to 5 carbon atoms, MCL-PHA ranges from 6 to 14, and SCL-MCL-PHA can feature anywhere between 3 and 14 carbon atoms per molecule (Sudesh, 2013). Typically, Figure 2.1 and Table 2.3 depict the general structures of PHAs (Lee, 2000).

Depending on the monomers used in their construction, PHA can exhibit a wide range of physical characteristics that can be tailored to specific needs (Mezzina et al., 2021). The physical and thermal properties of PHA depend on factors such as the monomer's nature, composition, and the polymer's M_w (Sudesh & Iwata, 2008). SCL-PHA typically display thermoplastic traits, including high crystallinity, stiffness, brittleness, a high T_m, and reduced elongation at break (Yu, 2007). However, copolymers combining SCL-PHA and MCL-PHA monomers, particularly SCL-MCL-PHA copolymers, often exhibit superior thermal and mechanical properties compared to homopolymers like SCL-PHA and MCL-PHA (Kellerhals et al., 2000). This variability extends to SCL-MCL-PHA copolymers, where the composition of MCL-PHA determines the range of properties, from increased durability with decreased flexibility to a more balanced blend of flexibility and durability (Asrar et al., 2002). For example, the inclusion of MCL-PHA monomers such as 3HHx into P(3HB) results in a P(3HB-*co*-3HHx) copolymer with elastomeric traits, including high elasticity, low crystallinity, and significant elongation at break. The flexibility of this copolymer can be adjusted based on the molar compositions of incorporated 3HHx (Tang et al., 2022).

Molecular weight significantly influences PHA's physical and mechanical strength (Hahn et al., 1994). PHA with higher M_w are recognized to exhibit superior mechanical properties and are favoured for application purposes (Sudesh & Doi, 2000). PHA possesses the distinctive characteristic of being entirely biodegradable, and heat resistant, particularly evident in its highly crystalline variants (Tan et al., 2020). PHA is receiving increasing attention as a potential alternative to synthetic plastics derived from petrochemicals due to its ability to undergo thermal processing for various applications (Sudesh & Iwata, 2008). PHA can be seamlessly processed using conventional machinery utilized in the production of petrochemical-based plastics. The melting behaviour of PHA mimics that of liquid crystalline polymers, facilitating the moulding of intricate and thin-walled structures, a feature of utmost importance in the fabrication of various applications (Koller, 2021). PHA production utilizes a diverse range of carbon sources, including agricultural waste products, industrial wastewater, food waste, fatty waste, as well as cane molasses, beet molasses, whey, plant oils, starch, cellulose, and hemicelluloses (Katagi et al., 2023). This array of substrates provides microorganisms with ample options for bacterial growth and polymer production, making PHA production highly beneficial.