PRODUCTION OF POLYHYDROXYALKANOATE (PHA) BIOPOLYMER FROM WASTE PRODUCTS OF FRUIT-BASED FOOD INDUSTRY, SRI LANKA

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Degree Master of Science in Environmental Management

Department of Civil Engineering

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Thesis/Dissertation submitted in partial fulfillment of the requirements for the degree Master of Science in Environmental Management

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ABSTRACT

Recently, conventional petrochemical-based plastic waste has been a global crisis, creating numerous environmental and economic challenges. Addressing this problem, the present study was aimed to assess the feasibility of bacterial polyhydroxyalkanoates (PHA) synthesis from locally available lignocellulosic fruit waste obtained from the fruit-based industry in Sri Lanka. The present study was focused on assessing the feasibility of bacterial PHA synthesis from locally available lignocellulosic fruit waste obtained from the fruit-based industry in Sri Lanka. This study investigated the viable of using papaya (*Carica papaya*), mango (*Mangifera indica*), watermelon (*Citrullus lanatus*), and pineapple (*Ananas comosus*) fruit wastes as substrates for PHA production. The pure strain of *Bacillus subtilis* bacterium was selected as the microorganism for 72 h fermentation process to produce PHAs under ambient temperature conditions. The bacterial strain cultured in modified LB agar media was stained with Sudan Black B (SBB) as a preliminary screen to confirm PHA production. Two pretreatments (Crude aqueous extract: CAE, and sulphuric acid treatment: SAT) were performed to analyze total reducing sugars in fruit waste. Comparatively the highest concentration of reducing sugar was obtained with SAT pretreatment method. Maximum average value of cellular dry weight (CDW) was obtained in papaya waste (2.15 ± 0.15) g/L at 60 h of fermentation and minimum value was obtained in watermelon (1.23 ± 0.06) g/L after 72 h of Fermentation. Fourier transform infrared spectra (FTIR) were performed for the obtained extracted samples, and PHA synthesis was confirmed by the corresponding peaks of PHA functional groups. The proof-of-concept stage was realized to promote PHA to be developed from fruit wastes.

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List of Abbreviations

3HB	3-hydroxybutyrate
3HD	3-hydroxydecanoate
3HDD	3-hydroxydodecanoate
3HH	3-hydroxyheptanoate
3HHx	3-hydroxyhexanoate
3HV	3-hydroxyvalerate
3HV	3-hydroxyvalerate
4HB	4-hydroxybutyrate
5HV	5-hydroxyvalerate
Acetyl CoA	acetyl coenzyme A
BioPE	biopolyethylene
CAE	crude aqueous extract
CDM	cell dry mass
CFU	colony forming units
CoA	coenzyme A
DCW	dry cell weight
DNS	3,5-Dinitrosalicylic acid
FTIR	Fourier transform infrared spectroscopy

LB media	luria-bertani media
lcl-PHA	long chain length PHA
mcl-PHA	medium chain length PHA
OD	optical density
P(3HB)	poly-3-hydroxybutyrate
P(3HP)	poly-3-hydroxypropionate
P(3HV)	poly-3-hydroxyvalerate
P(4HB)	poly-4-hydroxybutyrate
PHA	Polyhydroxyalkanoates
PHB	poly(3-hydroxybutyrate)
PHBHHx	copolymer of 3HB and (R)-3-hydroxyhexanoate(3HHx).
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PHHx	poly(3-hydroxyhexanoate)
PHV	poly(3-hydroxyvalerate)
PLA	polylactic acid
SAT	sulphuric acid treatment
SBB	sudan black B stain
scl-PHA	short chain length PHA
TPS	thermoplastic starch

CHAPTER 1

INTRODUCTION

Sustainable delivery of material needs for the growing world population is a challenging necessity. Plastics are used in almost each and every manufacturing industry, from automobiles to pharmaceuticals. Thus, the packaging industry is severely affected by tightened legislation on the restricted use and disposal of conventional plastics. Microplastic and Plastic pollution has been identified as a significant global environmental issue threatening the natural environment, biodiversity, health, and economies. The plastic crisis is one of the largest obstacles that our planet currently faces and thus needs to be solved with a sustainable solution. If it is not recycled or incinerated, most petroleum-based plastics accumulate in natural environments after disposal. Currently, millions of tons of plastic end up in the oceans annually with disastrous and pernicious consequences for seabirds, aquatic life, including marine & other ecosystems, and even for human health (de Silva et al., 2021).

Annually, approximately a total amount of 500,000 metric tonnes of plastic are imported into Sri Lanka. 7,000 metric tonnes of solid waste is generated in Sri Lanka, per day. In general, packaging waste that included PE and PP accounts for more than 50% all plastic waste (Plastic Waste Management - Sri Lanka, 2021; Samarasinghe et al., 2021).

Since the imports of petroleum-based plastics will be limited in the future and as a promising solution to plastic waste and environmental contamination, a vast potential has been identified in manufacturing biodegradable plastics at the industrial level. Unlike the traditional petroleum-based plastics, which come from polymerizing oil molecules by heating and treating, bioplastics come from natural sources, including crops and microbial by-products, a sustainable alternative to non-renewable energy sources such as petroleum. The manufacturing process of biodegradable plastics requires a fewer amount of energy compared to traditional plastics (Satoh et al., 1998).

Bioplastics are known to be biodegradable and/or bio-derived. But biodegradable and bio-derived are two different and independent classifications. Some fossil-derived plastics, such as BioPE (Biopolyethylene), are not biodegradable, while plastics such as PHA, TPS (thermoplastic starch), and PLA (polylactic acid) are biodegradable. Even-though PLA is a

biodegradable plastic, it is biodegraded under engineered, industrial environments. In terms of plastic properties, unlike PHA, PLA, and TPS, encounter barrier properties such as poor resistance to O_2 and water, respectively. Therefore, according to recent bioplastic studies, PHA is considered the "greenest" plastic (Nair et al., 2022).

PHAs are polyesters of various hydroxyalkanoates, synthesized in stressed conditions as intracellular granules (Figure 1.1) in a wide range of microbial species (Shah & Kumar, 2020). Vast range of microorganisms produce PHA in different conditions, which varies from pure cultures to halophilic bacterium and mixed cultures (de Souza Reis et al., 2020; Duque et al., 2014). PHA production from halophilic bacterium is also currently gaining an attention because of the non-contamination conditions (Mitra et al., 2020).

Polyhydroxyalkanoates (PHAs) are biodegradable, water-insoluble, non-toxic, and readily compostable thermoplastics that are mainly produced by microbial fermentation using carbon-based feedstocks, and have recently gained increased interest. (Dalton et al., 2022).

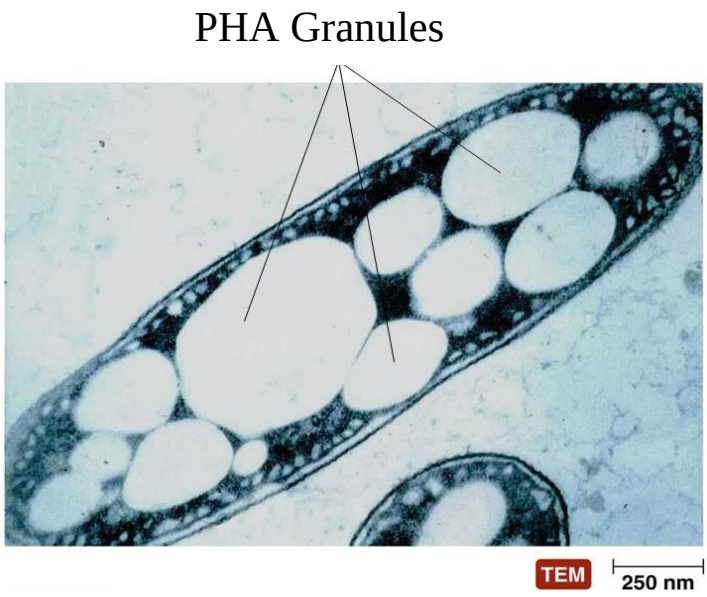


Figure 1.3. Transmission electron microscope image of accumulated PHA granules in the cell of *Azotobacter chroococcum* before recovery (dos Santos et al., 2017)

Excellent biodegradability is the most entrancing characteristic of PHA polymers. Packaging materials that are manufactured from PHA possess excellent coating and film-forming properties like that of polypropylene (PP).

Hence, the production of readily biodegradable polymers such as Polyhydroxyalkanoates (PHAs) can be considered one of the most appropriate and sustainable solutions for this issue. High production cost is considered the major stumbling block to the biosynthesis of PHAs. However, agricultural residues and industrial effluents (food waste, municipal solid waste, used cooking oils, etc.) are inexpensive, abundant, and nutrient-rich raw materials that can significantly reduce the total cost of production (Cavaliere et al., 2018; Stavroula et al., 2020). Production of PHAs has become increasingly popular as it contributes to environmental remediation and waste minimization.

In Sri Lanka, inexpensive feedstocks such as industry effluents from food industries also favored by PHA-producing microorganisms are produced in significant quantities and are currently being discarded otherwise. In general, there's a massive amount of fruit production losses in fruit-based food industry, which amount to 10-60% of the production (i.e. papaya 10–20%, mango 25–40%, pineapple 29–40%). Annually, 6,210,135 Kg of papaya and 795,362 Kg of pineapple are exported to the international market (EDB, 2019).

Rising fresh fruit utilization leads to an accumulation of fruit peels, rinds, seeds and the residue left over at the point of down-stream processing of fruit-based products in food industry. Recent exhaustive fruit production practices have generated a significant amount of fruit wastes, and improper handling of these fruit wastes can pose a risk towards the public's health and cause ecological problems. Fruit peels are considered the most common type of solid waste within the fruit processing industry (Dunuweera et al., 2021; Thiviya et al., 2022).

In this study, PHA production is achieved by utilizing fruit waste from discarded pieces produced in large amounts. The raw material is abundantly available in the fruit-based industry in Sri Lanka. Fruit waste fermentation is assumed to be a potential candidate as a feedstock rich in higher concentrations of fermentable sugars for PHA production. It can also be considered a feasible economic option for managing fruit waste in the food industry.

The main objective of this study is to experimentally determine the potential of mango, papaya, pineapple, and watermelon fruit waste as a feedstock (carbon source) to produce PHA by pure

strain bacterium. The experiments were conducted to obtain PHA from *Bacillus subtilis* bacteria species, using four types of pretreated fruit waste (Figure 1.2).

1. Mango (peel)
2. Watermelon (peel and seed)
3. Pineapple (core and peel)
4. Papaya (peel and seed)

Figure 1.4: Different types of Raw Fruit waste used for the study

Keeping the points mentioned above in view, the following objectives were addressed in the present study.

1. Screening for PHA production from the selected pure strain bacteria in modified LB agar medium.
2. Comparison of two pretreatment methods (Crude aqueous extract and Sulphuric acid treatment) that results in a higher concentration of fermentable reducing sugar
3. Identifying the potential of fruit wastes (mango, watermelon, pineapple, papaya) to be applied separately as the only carbon source for synthesis of PHA
4. Identifying the type of fruit residue and appropriate fermentation time which produces more biomass in the selected bacterium
5. Characterization of PHA produced by selected bacterium using different types of fruit waste by FTIR Spectroscopy.

For the novel aspects of this MSc dissertation, the potential of commercially important biopolymer (PHA) production using fruit residue (especially from mango, papaya, pineapple, and watermelon) obtained from the fruit-based industry in Sri Lanka from *Bacillus subtilis* pure strain has not been studied before. Furthermore, an area that has been neglected by researchers, studies on the comparison of biomass production by *Bacillus subtilis* using four fruit waste types (mango, papaya, pineapple, and watermelon) which are inexpensive feedstock for PHA production, is presented in this MSc dissertation with some findings which will benefit other researchers in their prospective work.

CHAPTER 2

LITERATURE REVIEW

2.1. Polyhydroxyalkanoates

The expansion of the production and consumption of conventional petrochemical plastics has significant visible and invisible socio-environmental impacts. The reason conventional plastics are objectionable is the difficulty in their disposal as they are xenobiotics recalcitrant to microbial degradation (Reddy et al., 2003).

In recent times, biodegradable plastics have become an attractive material that has a great importance in many areas of day-to-day life. Many case studies in the academic literature report biodegradable plastics as a successful alternative material of packaging to the commonly used polyethylene plastics (Din et al., 2020).

Bio-based polymers, such as polylactic acid (PLA), are made from bio-based monomers (i.e. Corn), which must then be polymerized through standard routes, including a hybrid chemical process (Bugnicourt et al., 2014). In contrast, Polyhydroxyalkanoates (PHA), the biopolymer referred to in this study, is considered a sustainable solution to this crisis as engaging property of being readily biodegradable in ambient environments (Elbanna et al., 2004).

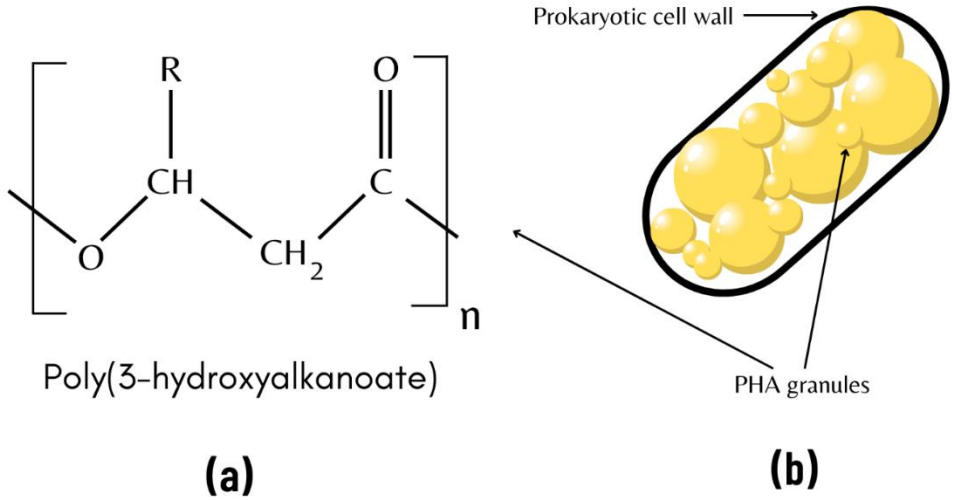


Figure 2.1: (a) General chemical structure of Polyhydroxyalkanoates (PHA); (b) An illustration that symbolizes a microbial cell containing PHA as granular inclusion bodies ("carbonosomes")

Hence, according to the present studies, PHA will be the “bioplastics of the future”, (Nyssa Nair et al., 2022). The first experimental realization of Polyhydroxyalkanoates was reported in 1920 by a French microbiologist, Maurice Lemoigne, observing the intracellular granule formation in gram-positive bacterium *Bacillus megaterium*. (Shah & Kumar, 2020).

The PHAs are a group of bio-based polyesters which are produced and accumulated intracellularly (Lyla, 2020) as secondary metabolites by numerous bacteria species as energy and reserves of carbon under nutrient-stressed conditions (Figure 1.1). Synthesis of PHA is initiated in the late log phase of the bacterial growth curve, whereas it is synthesized in stationary and exponential phases. These spherical lipid inclusions are generally produced with an excess of carbon source, whereas the nutrients such as Nitrogen, Phosphorous, Oxygen and Sulphur are deficient. Although the “lack of nitrogen” is the most common limitation for PHA synthesis, for some bacteria (*Azotobacter* sp.), the "lack of oxygen" is the most effective one since they can fix atmospheric Nitrogen (Mohapatra et al., 2017). However, several species (i.e. *Alcaligenus eutrophus, Alcaligenus latus*) synthesize PHA under non-stress conditions (Shah & Kumar, 2020).

2.2. PHA Classification

The monomer of PHA is hydroxyalkanoates, whereas the repeating unit is Poly(3-hydroxyalkanoate) (Figure 2.1). Due to the R group attached to the 3rd carbon, which varies from methyl (C-1) to tridecyl (C-13), there is a vast variation observed in the length and composition of the side chains is the reason for imparting a range of properties. This variability makes the PHA family of polymers (Table 01) suitable for a variety of potential applications (Verlinden et al., 2007). PHAs include poly-(R)-3-hydroxybutyrate (PHB), whereas the copolymer PHBV produced from (R)-3-hydroxybutyrate (3HB) and (R)-3-hydroxyvalerate (3HV), and a copolymer PHBHHx produced from (R)-3-hydroxyhexanoate (3HHx) and 3HB (Xiao & Jiao, 2011).

Table 01. Classification of PHAs and corresponding R groups; (Verlinden et al., 2007)

R-Group		Total No. of Carbon atoms	PHA Polymer	
Methyl	CH_3	C_4	Poly(3-hydroxybutyrate)	PHB
Ethyl	C_2H_5	C_5	Poly(3-hydroxyvalerate)	PHV
Propyl	C_3H_7	C_6	Poly(3-hydroxyhexanoate)	PHHx

PHAs are classified to two main categories based on the source of enzymes utilized in synthesis and the number of carbon (C) atoms exist in the monomers.

1. Short-chain length PHAs - (scl-PHAs) ranging from 3-5 carbon (C) atoms
2. Medium- chain length PHAs - (mcl-PHAs) ranging from 6-14 carbon (C) atoms

The properties of these two PHA categories depend on the number of carbon atoms and the chemical structure of the molecule. These are produced by different microorganisms (i.e. scl- *Cupriavidus necator*, *Alcaligenus latus;* mcl- *Pseudomonas putida*). Mcl is more elastic and less crystalline compared to scl. Thus, Mcl PHAs are further used in bio-medical products. However, scl PHAs (most studied scl PHA is PHB) are stiff and brittle with a crystalline structure which is difficult to process. Therefore, it is usually copolymerized with 3PHV, which results in PHBV; Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) in order to transform into a strong, flexible, and thermally stable bioplastic. Currently, PHBV is a widely used copolymer in storing and food packaging manufacturing (Shah & Kumar, 2020).

2.3. Biochemical Process of PHA Synthesis

Stressful conditions favor PHA production to meet microorganisms' energy needs during their starvation period (Koller, 2018b ; Ebu & Ray, 2021). When surplus carbon converts to a storage-chemical (i.e. PHB), and the PHB percentage in the cell can ascend from 10 - 80% of the cell dry mass (Fang et al., 2019). Four types of enzymes (Table 2) are comprehended in synthesis of PHAs, which differ in size, number of subunits, and substrate specificity (Wang et al., 2012; Koller, 2018a).

Table 2. Four types of enzymes comprehended in PHA synthesis with corresponding functional genes and subunits; (Shah & Kumar, 2020)

Type of Enzyme	Functional Gene	Category of PHA produced (mcl/ scl)	Number of subunits
Type I	PhaC	Scl	Single subunit
Type II	PhaC	Mcl	Single subunit
Type III	PhaC+PhaE	Scl	Dimers composed of 2 subunits
Type IV	PhaC+PhaR	Scl	Dimers composed of 2 subunits

PhaE and PhaR have 18% sequence similarity, but both function in a similar way by establishing contact between the enzyme and the enzyme and the hydrophobic polymer (Nambu et al., 2020). These enzymes are participated in the production of PHAs from Acetyl CoA (Figure 2.2).

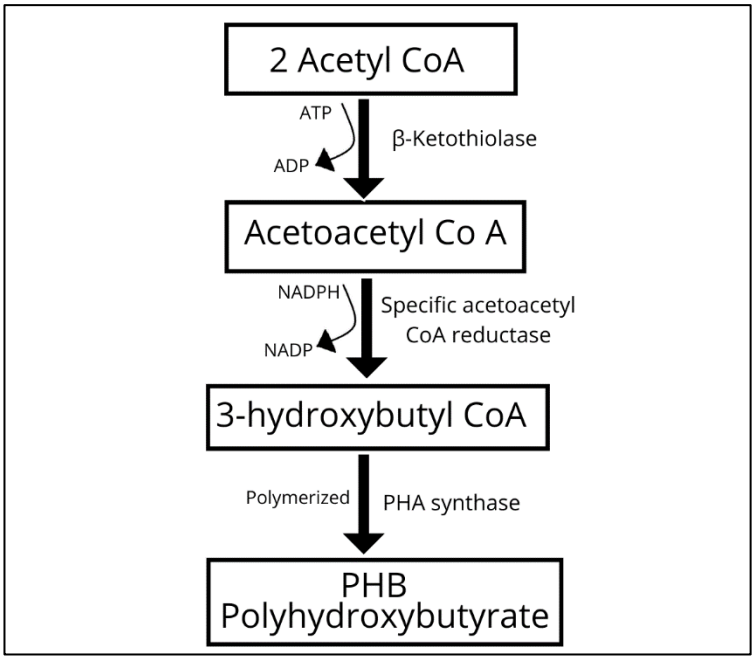


Figure 2.2: The biochemical pathway of PHB synthesis

2.4. Properties of PHAs

These polymers have attracted the attention of researchers and have been shown to have similar properties to petroleum-based plastics. PHAs have been compared to polypropylene (Table 03) as they have numerous common properties (Figure 2.3).

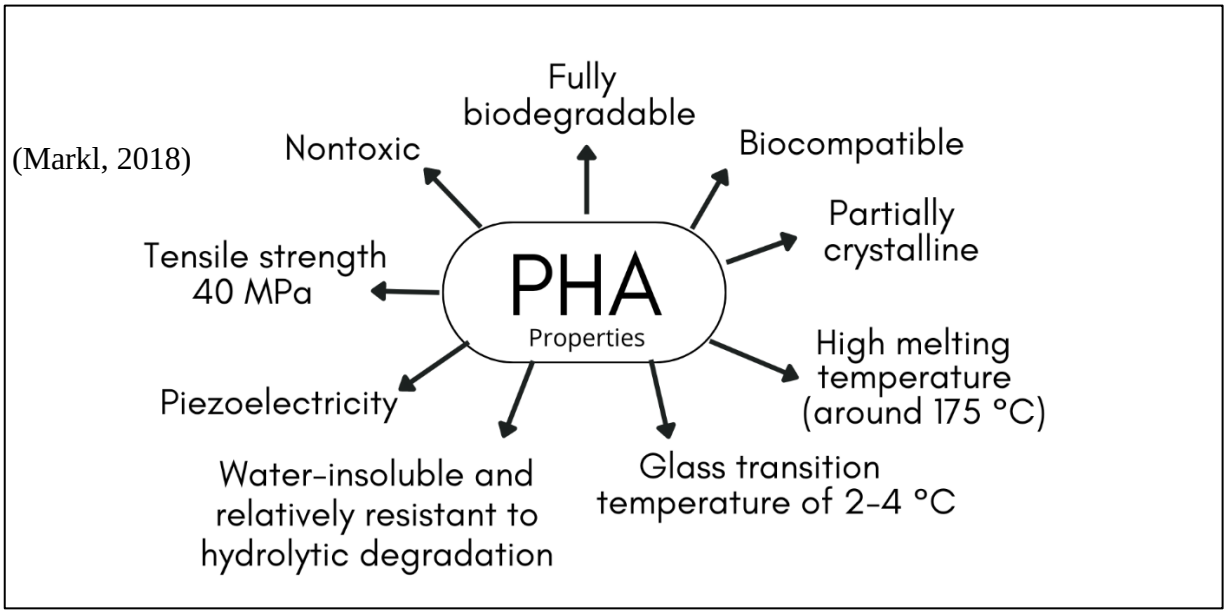


Figure 2.3: Properties of PHA; source: (Markl, 2018)

PHB shows low permeability to gases such as O_2, H_2O, and CO_2. It is resistant to hydrolytic degradation and insoluble in water, but is soluble in organic solvents such as chloroform and other chlorinated hydrocarbons. This is the reason for most PHB extraction processes use the organic solvent extraction method. PHB inside the cell is in a fluid, amorphous state. Although these properties make this polymer a better choice, the strength of PHB required to be improved. During mechanical processes, the brittleness of PHB makes it not resistant to stress. Furthermore, it has a high production cost, is thermally unstable during processing, and decomposes near its melting point.

Considering the general characteristics, PHAs are relatively resistant to hydrolytic degradation, soluble in chloroform and other chlorinated hydrocarbons, non-toxic, and less 'sticky' than traditional polymers when melted. It readily facilitates anaerobic biodegradation in sediments as it sinks into the water (dos Santos et al., 2017).

Table 03. Comparison of properties of PHB and Polypropylene (Source; Markl, Grünbichler & Lackner, 2018)

Property	**PHB**	**Polypropylene**
Crystalline Melting Point	175 °C	176 °C
Crystallinity	80 %	70 %
Molecular Weight (Daltons)	5·105	2·105
Glass Transition Temperature	4 °C	-10 °C
Density	1.25 g/cm3	0.90 g/cm^3
Flexural Modulus	4.0 GPa	1.7 GPa
Tensile Strength	40 MPa	38 MPa
Extension to Break	6 %	400 %
Resistance to UV	Good resistance	Poor resistance

2.5. PHA Applications

Polyhydroxyalkanoates (PHAs) have emerged as an intriguing biomaterial in recent years because of their wide range of potential applications in the industry.

PHAs have an extensive design space in different applications such as food, pharmaceutical, paper industries, bioplastic, agricultural and medical sectors. PHA is an eco-friendly polymer since it can be reused for PHA production by recycling, composting, and biodegrading PHA products (Hazer & Steinbüchel, 2007). PHB is compatible with mammals' tissues and blood in the medical sector. PHB monomers are general metabolites in human blood (Muhammadi et al., 2015).

As PHB is reabsorbed by the human body, it can be used in surgeries as a surgical implant, like suture thread to heal wounds and blood vessels. PHB can be used in pharmacology as microcapsules or as a material for packaging cells and tablets. It can also be used in deep-drawn applications for food packaging (i.e. bottles, laminated sheets, fast food, disposable cups), fishing nets, flower pots, sanitary ware, agricultural plates, and fibers in textiles (Bugnicourt et al., 2014).

PHAs are considered one of the newest biodegradable materials which is thermoplastic and used for food packaging applications (Din et al., 2020).

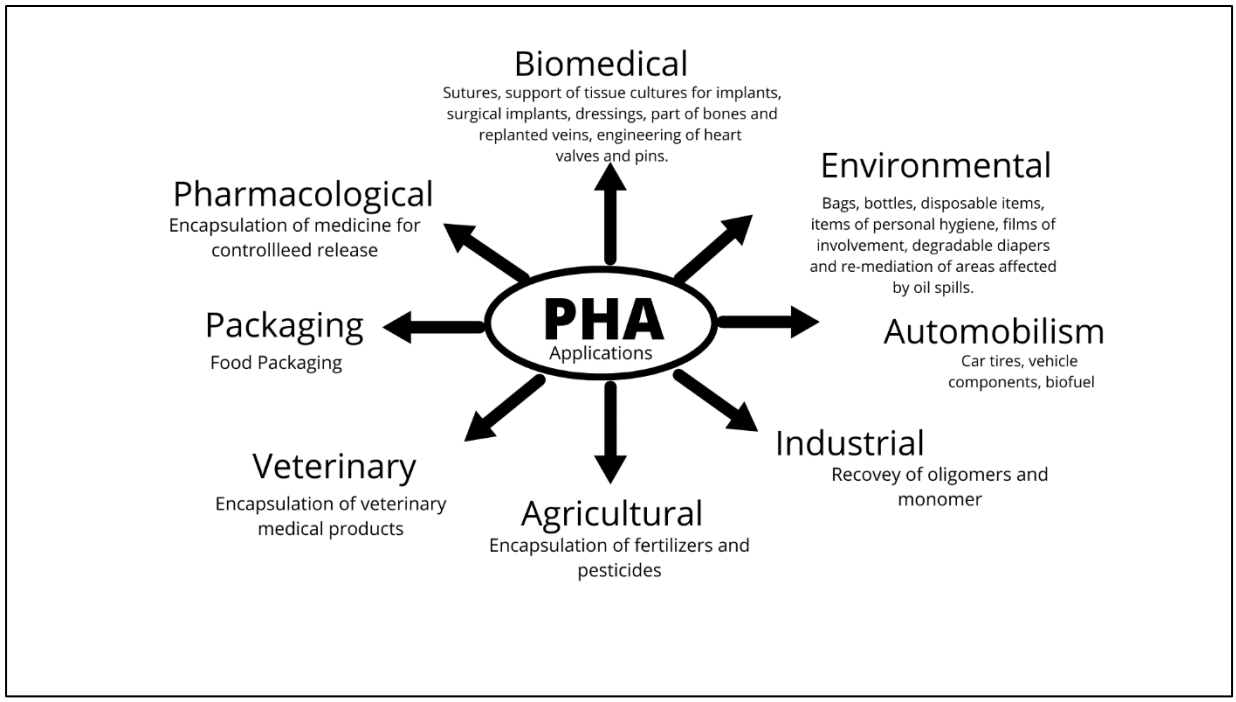


Figure 2.4: Industrial applications of PHA; source: (Bugnicourt et al., 2014)

PHAs possess a great variation of characteristics, and their biodegradability, sustainability, and biocompatibility signify that many industries would benefit from further utilizing them. The properties of PHA polymers can be designed for specific applications by incorporating specific combinations of different monomers into the polymer chain (Kaniuk & Stachewicz, 2021).
If more effort is brought to further analysis and research, PHAs may be a strong candidate for food packaging applications (Naser et al., 2021). Growing demand for alternative and renewable raw materials and use of biodegradable plastics, promotion of green purchasing policies, and awareness are intended to support the advancement of the PHA market.

2.6. Biodegradation of PHA

PHA is a readily biodegradable material that can be recycled for CO_2 and H_2O. The first step in the process of PHB biodegradation is film surface moderation by surface hydrolysis, and it increases the number of hydroxyl and carboxyl sites on the surface. Then the bacterial colonization starts with the rate of growth depending on pH and temperature. PHB is eventually degraded to 3-hydroxybutanoic acid, general metabolite in human blood, in applications such as in-vivo transplantation. PHB is thus melt processable and biocompatible, with none of the

cytotoxic reactions. The rate of degradation is accelerated with increasing pH and temperature, implying that ester hydrolysis is the mechanism of degradation. Numerous bacteria which are capable of rapid depolymerization, activate the biodegradation (Huang et al., 1990).
In anaerobic wastewater, soil, and seawater, P(HB-HV) is completely degraded after 6, 75, and 350 weeks, respectively (Muhammadi et al., 2015).

2.7. Selection of Bacterial Strain

PHA synthesis is majorly governed by factors such as species of bacteria, culture media, fermentation conditions, and recovery (Shah & Kumar, 2020). The major impediment in the biosynthesis of PHA production to replace conventional plastic is the higher production cost (Mohapatra et al., 2017).

In order to overcome that barrier and increase the efficiency, selecting the bacteria species is crucial as the bacteria species is considered the significant factor determining the efficiency of PHA production. The bacteria should be high yielding when a cheap substrate is provided, rapidly growing species and carry out cell lysis easily. PHAs from *Bacillus* sp. are closer than other genera to polypropylene. Since *Bacillus* sp. has no lipopolysaccharide layer, the extraction is comparatively easier. A higher growth rate can grow in cheap raw materials. The species also contains the stability of its replication and has the ability to maintain the plasmids (Maity et al., 2017). *Bacillus subtilis* is also found to produce the maximum amount of PHAs in a modified growth medium (3.09 gL^{-1}). Such yield appears to be the highest recorded for all isolated bacteria so far (Mohapatra et al., 2017).

Since *Bacillus subtilis* fits most of the appropriate features as a promising bacterium for PHA production (Fujikawa, 2006; Gomaa, 2014), which also lives at room temperature (Anjali et al., 2014), *Bacillus subtilis* species was designated as the suitable microorganism for the present study. The Pure strain of *Bacillus subtilis* was obtained from the Department of Pathology, Faculty of Medicine, University of Sri Jayewardenepura (Figure 2.5).

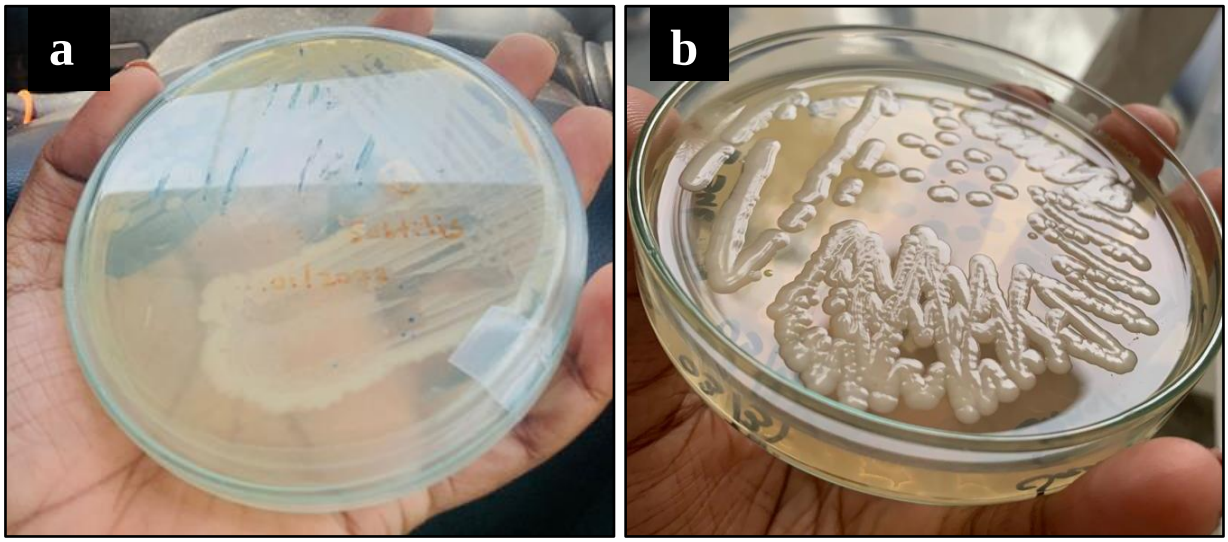


Figure 2.5: (a) *Bacillus subtilis* Pure culture received from the Faculty of Medicine, University of Sri Jayewardenepura (b) Sub-cultured *Bacillus subtilis* from original sample

2.8. Raw Materials Used in Past Studies

The quantity and rate of the biosynthesis of PHA not only depend on the enzymes involved but also on the physiological status of the bacteria and the feedstock provided (Shah & Kumar, 2020).

Biopolymers which are naturally synthesized (i.e. PHB, PHV) and copolymers (i.e. PHBV) can be fermented from a variety of substrates, from glucose and methane to carbon dioxide and hydrogen gas mixtures (Huang et al., 1990). Various studies were performed by bioscientists and bioengineers to produce and develop PHAs from different carbon sources as examples mentioned in Table 04.

Extensive use of PHAs is currently limited, majorly due to the high production cost (Levett et al., 2016). In terms of the production cost of Polyhydroxyalkanoates, 50% is due to the substrate cost and downstream processing (Shah & Kumar, 2020). Therefore, the utilization of an inexpensive plant-based substrate as the raw material to produce PHA will be a more effective and sustainable solution to overcome the limitations of PHA synthesis with a minimum cost of raw materials.

Table 04. Recent studies carried throughout the world with regarding to PHA synthesis using different type of low-cost carbon sources:

Carbon source relevant to PHA synthesis research	Reference
Waste rapeseed oil	Obruca et al. (2010)
Cane molasses	Gomaa (2014)
Waste frying oil	Benesova et al. (2017)
Orange peel	Umesh et al. (2018)
Sugar in date molasses	(Purama et al. (2018)
Pineapple peel and core	Sukruansuwan & Napathorn (2018)
Beer brewery wastewater containing maltose	Amini et al. (2020)
The fatty acids in Mexican avocado	Flores-Sánchez et al. (2017)
Pomegranate peels	Rayasam et al. (2020)
Broken discolored and unripe rice	Brojanigo et al. (2020)
Adsorbed residual oil in spent bleaching clay	Hairudin et al., (2021)
Grape Sugar extract	Kovalcik et al. (2020)
Crude glycerol	Mozumder et al. (2014)
Olive mill wastewater	Agustín Martinez et al. (2015)
Soyabean oil	Park & Kim (2011)

2.9. Residue of Tropical Fruits as a Substrate

In Sri Lanka, tropical fruit-based products are traded to the international market in a variety of forms including fruit jam, canned fruits, fruit juice, and various dried and frozen products. A large amount of agro-industrial waste is generated during the processing of canned products. Studies reveal that pineapple waste comprises 44.36% of the peel and 15% of the core compared to the total raw materials (Sukruansuwan & Napathorn, 2018). The current research was conducted to evaluate the potential of various fruit wastes for inexpensive biomass production of *Bacillus subtilis* bacteria. These tropical fruit wastes were selected according to local availability and inexpensiveness in Sri Lanka.

In present study, the fruit-residue materials such as mesocarps, seeds, and peels of pineapple (*Ananas comosus*), watermelon (*Citrullus lanatus*), papaya (*Carica papaya*), and mango

(*Mangifera indica*) were presented as the potential substrates for bacterial fermentation for PHA synthesis. These fruit wastes not only provide a source for fermentable sugars but also provides an appropriate nutrient composition containing most of the essential minerals and trace elements necessary for fermentation media (Table 05). Thus, the use of fruit waste as a raw material may also pave the way to avoiding the use of expensive synthetic mineral solutions for the production media.

These fruit residues are locally available organic-rich agricultural residues that can be used for microorganisms as energy and carbon sources for biomass production (Dunuweera et al., 2021). Nowadays, the value-added processing of tropical fruit waste can be a potential source of essential compounds such as fructose, glucose, sucrose, cellulose, fibers, phenol, pectin, hemicellulose, bromelain, lignin, vitamins, and other trace elements. (Sukruansuwan & Napathorn, 2018; Rico et al., 2020).

Therefore, the focus of this thesis was to fill a research gap by investigating the viability of utilizing lignocellulosic substrates of fruit residue with available fruit-based feedstock for value-added manufacturing. The research focused on the use of tropical fruit waste for PHA synthesis through the developing an effective fermentation method. However, pretreatment is required for fruit waste in general in order to degrade the lignin matrix of lignocellulosic biomass (Sukruansuwan & Napathorn, 2018). The composition and sugar content may vary with the fruit species' ripening stage, variety, subspecies, and morphotype.

Although Polyhydroxyalkanoates are a group of biopolymers found in heterotrophic bacteria, archaea, cyanobacteria, and plants, plants have the disadvantage of slow growth and are difficult to operate compared to bacteria (Thomson et al., 2010). Therefore, bacterial fermentation using inexpensive and locally available nutrient-enriched substrate would be an effective pathway for PHA production. However, valuable products can be developed from the derived biopolymer which is able to be used in the packaging industry and will be considered one of the most suitable, appropriate, and sustainable panacea. Hence, PHA has the potential to play a vital role in a sustainable future.

Table 05. Proximate Composition in Watermelon, Papaya, Pineapple and Mango Fruit Residue

Fruit Residue		Composition / g per 100 g of fruit dry weight basis)						Minerals present	Reference
		Moisture	Ash	Fiber	Protein	Fat	Carbohydrates		
Watermelon	Rind	92-94	3.2–18	24–46	7.4-18	1.1–2.6	28–85	Ca, Mg, K, Na, P, Fe, Mn, Zn	Morais et al. (2017); Olayinka, (2018); Rico et al. (2020)
	Seed	7.7-25	2.3–3.8	2.5–49	18-25	24–58	13–29		
Papaya	Pulp	89.74	0.31	1.8	0.9	0.37	7.56	Ca, Mg, K, Na, P, Fe, Mn, Zn	Marfo et al. (1986); Ojimelukwe et al. (2012); Rico et al. (2020)
	Seed	42.70	3.91	2.02	2.34	2.92	46.11		
Pineapple	Core	-	1.3–4.5	64–76	0.85-4.0	1.3–3.2	14	Ca, Mg, K, Na, P, Fe, Mn, Zn	Bartolomk et al. (1995); Rico et al. (2020); Sukruansuwan & Napathorn (2018)
	Peel	83	0.38–5.9	42–82	0.36-9.1	1.1-2.0	14–42		
Mango	Pulp	83.46	0.52	1.6	0.82	0.38	14.98	Ca, Mg, K, Na, P, Fe, Mn, Zn, Cu, Se	Ajila & Prasada Rao (2013); Lebaka et al. (2021); Maldonado-Celis et al. (2019)
	Peel	72.5		40–72.5	3.6	2.2	28.2		

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

(I.) List of Equipment

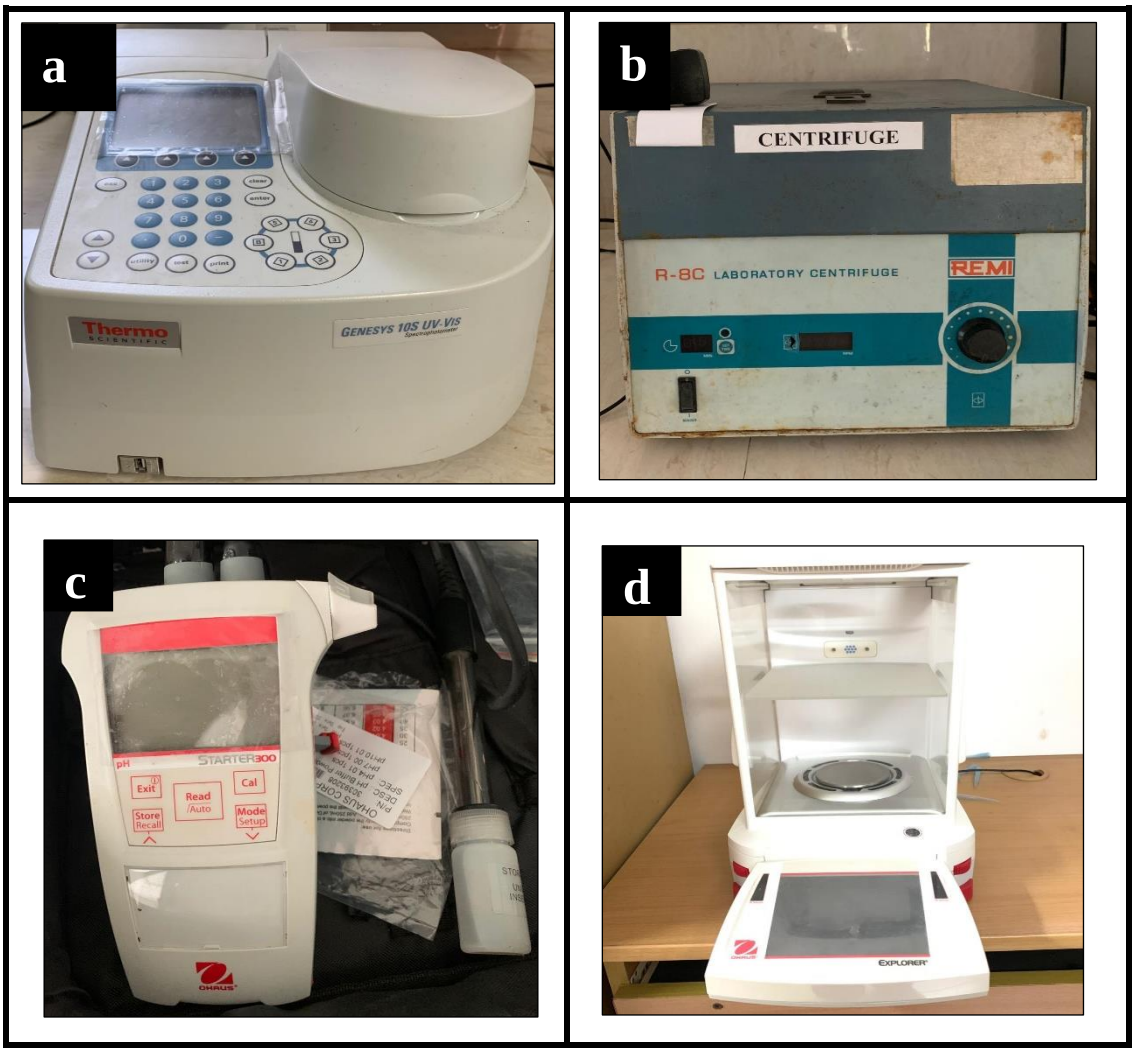
All the materials used in this study were obtained from Faculty of Engineering, University of Moratuwa (Table 6).

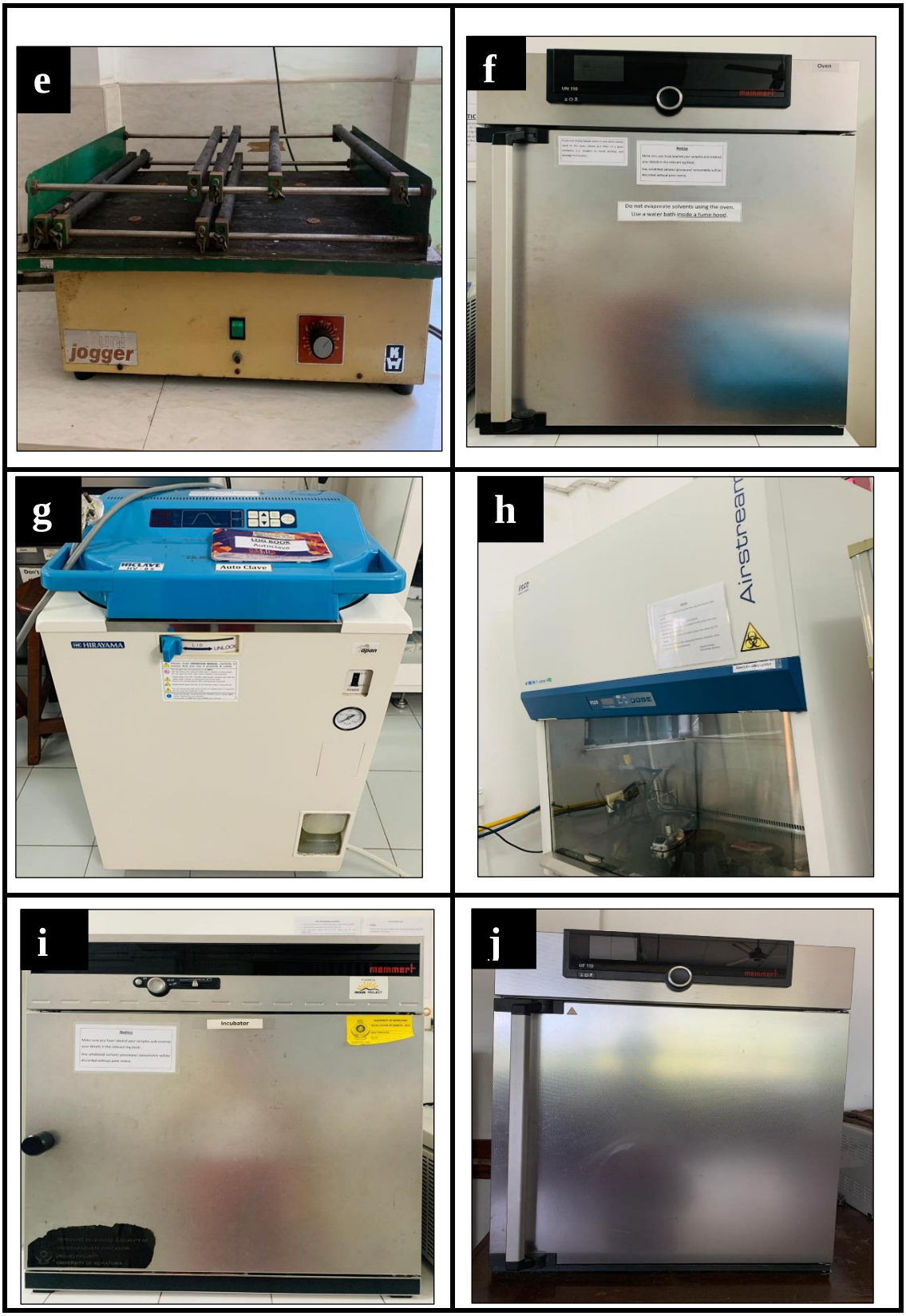
Table 06. List of Equipment used in the Study

Department	Equipment	Details
Department of Chemical and Process and Engineering	01.Autoclave Machine (Figure 3.1.g)	Brand: Hirayama Product Name: HICLAVE HVA series Model: HVA-85 Made in Japan
	02. Incubator (Figure 3.1.i)	Brand: Memmert Product Name: Memmert INB Laboratory incubator Made in Germany
	03. Micro Centrifuge (Figure 3.1.l)	Brand: Eppendorf ® Product Name: Centrifuge 424/5424R Model: EP5404000537 Made in Germany
	04. Compact Centrifuge	Brand: Hermle Product Name: Benchtop Centrifuge Model: Centrifuge Z 206 A Made in Germany
	05. Bio Safety Cabinet (Figure 3.1.h)	Brand: ESCO Product Name: Esco AIRSTREAM 5 Ft Class II Type A2 Made in Singapore
	06. Oven (Figure 3.1.f, & j)	Brand: Memmert Product Name: Universal Oven UF110 Made in Germany

	07. Oven (Figure 3.1.k)	Brand: LabTech Model: LDO-060E Made in Korea
Department of Civil Engineering	01. Laboratory Shaker/ Rotator (Figure 3.1.e)	Brand: KH Product Name: KH Uni jogger Made in Germany
	02. Spectrophotometer (Figure 3.1.a)	Brand: Thermo Product Name: Genesys 10S UV-VIS Made in Germany
	03. Analytical Balance (Figure 3.1.d)	Brand: Ohaus Product Name: Ohaus Explorer Made in Germany
	04. pH Meter (Figure 3.1.c)	Brand: Ohaus Product Name: Starter300 Made in Germany
	05. Centrifuge Machine (Figure 3.1.b)	Brand: REMI Product Name: R-8C Laboratory Centrifuge Made in India
	06. Laminar Flow Cabinet	Brand: BIOBASE Product Name: Horizontal Laminar Flow Cabinet Model: BBS-1100 Made in China
	07. Autoclave Machine	Brand: Selecta Product Name: Selecta Presoclave Made in Spain
	08. Incubator	Brand: Selecta Product Name: Selecta Master Laboratory Incubator Made in Spain
Department of Material Science and Engineering	01. Scanning Electron Microscope	Brand: ZEISS Product Name: Field Emission-Scanning Electron Microscopes (FE-SEM) Made in Germany
	02. Light Microscope	Brand: Meiji Techno Product Name: Economical Compound Microscope Trinocular Model: MX4300L Made in Japan

	03. Fourier Transform Infrared Spectrophotometer (FTIR)	Brand: Bruker Product Name: ALPHA II compact FT-IR spectrometer Made in Germany

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jogger
f
Oven
memmert
Notice
Do not evaporate solvents using the oven.
Use a water bath inside a fume hood.
g
Auto Clave
HIRAYAMA
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Airstream
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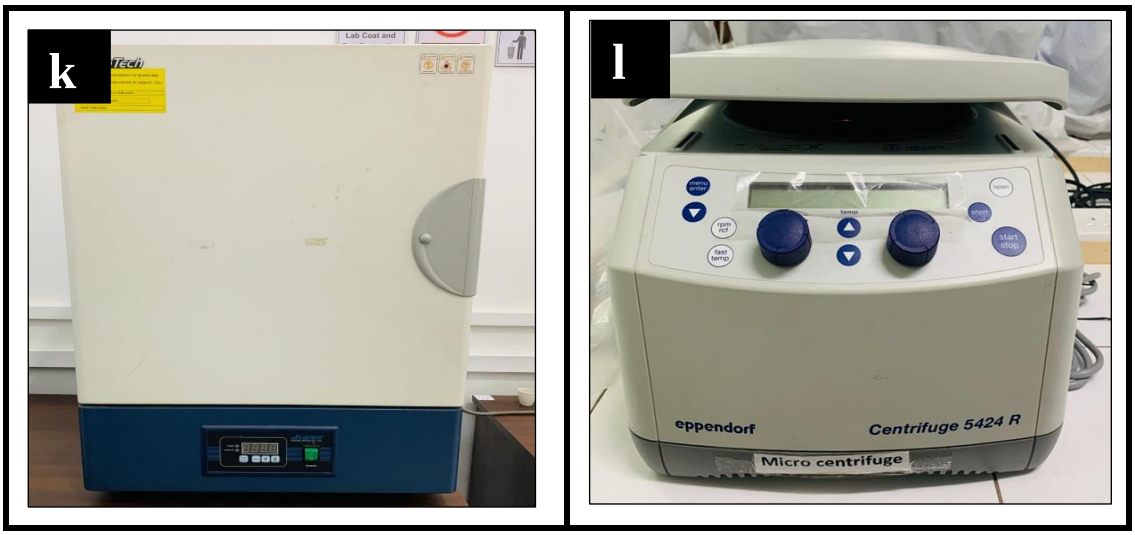

Figure 3.1: Equipment obtained from Faculty of Engineering, University of Moratuwa

(II). Microorganisms and Chemicals

Bacillus subtilis Pure strain (Department of Pathology, Faculty of Medicine, University of Sri Jayewardenepura, Sri Lanka) was used in the study. Cultures of the strain were maintained separately at -20 $^{\circ}$C (glycerol stock) and, at 4 $^{\circ}$C Luria-Bertani media (LB agar and broth). All the chemicals that are used for the experiments were of analytical grade. The fruits for the raw material were directly purchased from a supermarket (Keels Super Center), and from a fruit juice bar (Roots), at Katubedda, Sri Lanka.

3.2. Methods

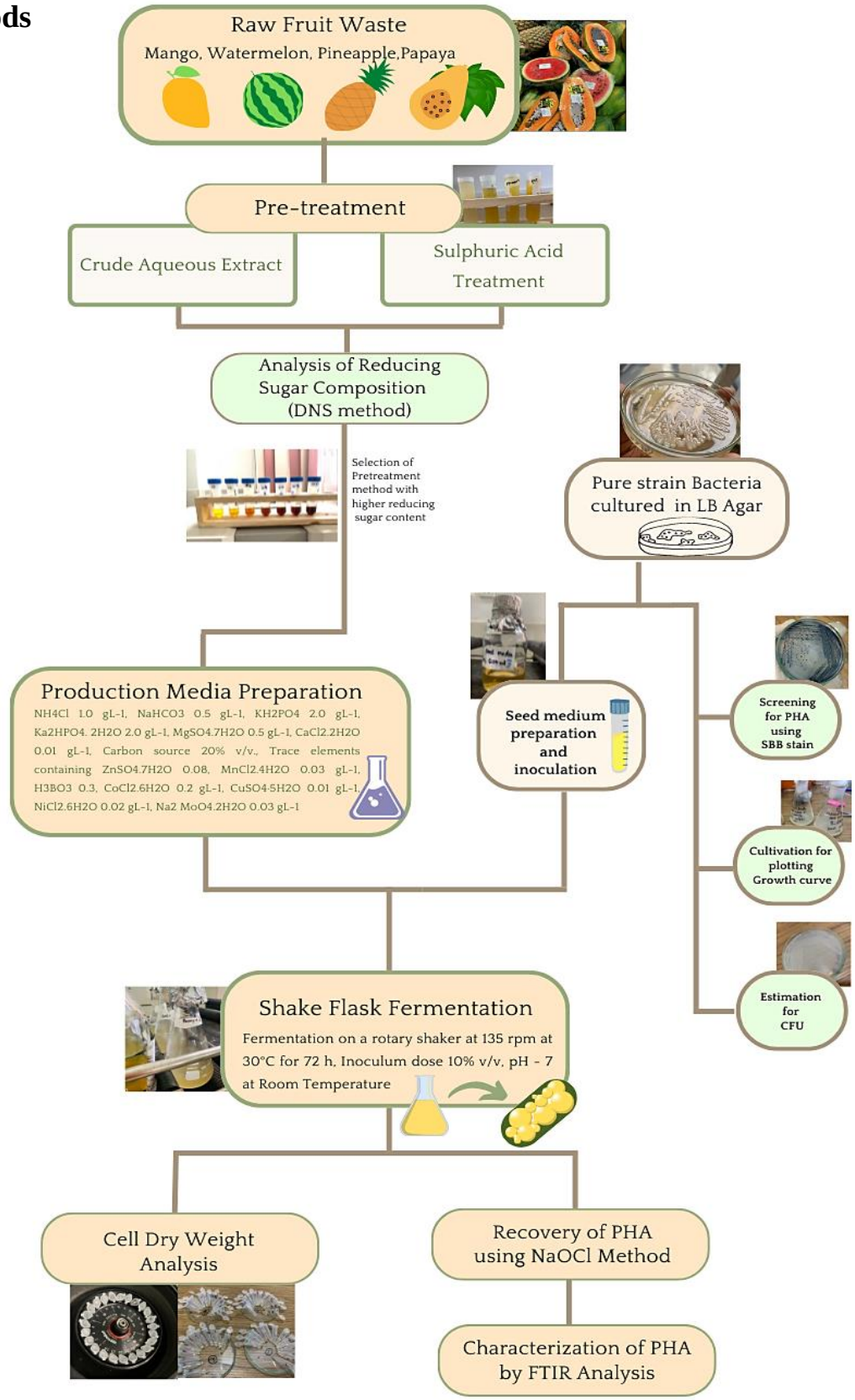

Figure 3.2: Graphical Methodology for PHA Production from Bacterial Fermentation using Fruit-Wastes

(I). Media Formulation for *Bacillus subtilis*

(i.) Preparation of LB Agar Culture (LB Agar) and Glycerol Stock

LB Agar plates were prepared as the culture media to grow and store the bacteria strain. 500 mL of LB Agar media was prepared by adding 7.5 g agar, 5 g tryptone, 5 g sodium chloride (NaCl), and 2.5 g yeast extract and added to a 1 L Duran bottle. Approximately 400 mL of distilled water was measured and added to the Duran bottle. Once the reagents were fully dissolved, the solution was topped up to 500 mL with distilled water, and the pH was adjusted to 7.0 by using sodium hydroxide (NaOH) solution (1N).

The solution was autoclaved to sterilize, prepared the LB agar plates pouring a thin layer of solution into the petri dishes to cover the bottom of the plate (approximately 10–20 mL per plate). The solution was left to cool down. Once cooled, the *Bacillus subtilis* cultures were prepared, incubated overnight at 35 °C, and subsequently stored at 4 °C. The LB agar plates containing bacteria were sub-cultured every 7–15 days. The glycerol stock was prepared to store the original bacteria strain and stored at -20 °C (Gomaa, 2014).

(ii). Inoculum Preparation in LB Broth

The original bacteria culture was introduced to the prepared agar and liquid LB medium and incubated overnight to determine the culture growth, CFU estimation and for further use in the fermentation process. For preparation of the inoculum (seed culture), the bacterium was cultured on LB broth for 24 h at 37 °C on a rotary shaker (150 rpm), and the culture in log-phase was used for the cultivation/ batch fermentation studies.

(II). Pretreatment of Carbon Source for Fermentation

Four types of fruit wastes, papaya (*Carica papaya*), watermelon (*Citrullus lanatus*), pineapple (*Ananas comosus*), and mango (*Mangifera indica*), were used as the carbon substrate for PHA synthesis. Two pre-treatment methods (crude aqueous extract- CAE and sulphuric acid treatment-SAT) (Sukruansuwan & Napathorn, 2018) were carried out in order to pre-process the fruit residue, which will be subsequently used to feed the production media by reducing the sugars content in fruit waste as the only carbon source.

(i). Crude Aqueous Extract

Fruit residues were separately mixed with distilled water with an equal quantity. The diluted mixtures were kept at 40 °C for 5hrs and were centrifuged for 10 min at 2000 rpm.

The clarified liquids (crude aqueous extract) were tested for fermentable sugar content by the DNS method (section. (i). Analyzing Reducing Sugar Composition in Fruit Residues) before using it as the carbon source for the production media and stored (at 4 °C) for future use.

(ii). Sulphuric Acid Treated Fruit Waste

Four types of fruit waste were milled and prepared 50 g samples of each kind of fruit waste, mixed and diluted (with an equal amount) 50 mL of distilled water. The diluted mixture was kept at 40 °C for 5 h, centrifuged at 2500 rpm for 10 min, and the clarified liquid was treated with 0.1 M sulphuric acid until the pH was adjusted to 3.0 and the solution was left to stand for 1.5 hours. The mixture was then centrifuged for 15 minutes (at 3000 rpm).

The fermentable sugar content was assessed by the DNS method for the supernatant, before using it as the carbon source and stored at 4 °C for future use.

(III). Procedure for Production of PHA

(i). Shake Flask Fermentation

(a). Seed Inoculum Preparation for Shake Flask Fermentation

A single colony was streaked from the stock culture and aseptically inoculated into seed medium (50 ml), (Peptone, 10 g/L; $MgSO_4.7H_2O$ 1 g/L and yeast extract, 2 g/L at pH 7) (Sukruansuwan & Napathorn, 2018) and allowed the culture to grow on rotary incubator shaker at 30 ˚C and 200 rpm for 24 h. The cultures were incubated at 37ºC for 24 hrs.

(b). Production Medium Preparation

Seed inoculum was taken and transferred with an inoculum dose of 10% v/v to four 500 mL conical flasks for sulphuric acid-treated fruit waste Papaya, Pineapple, Watermelon, and Mango pre-treated fruit waste, each containing 200 mL of sterilized production medium ($NaHCO_3$ 0.5 gL^{-1}, NH_4Cl 1.0 gL^{-1}, KH_2PO_4 2.0 gL^{-1}, Ka_2HPO_4. $2H_2O$ 2.0 gL^{-1}, $CaCl_2$. $2H_2O$ 0.01 gL^{-1} , $MgSO_4.7H_2O$ 0.5 gL^{-1}, Trace elements containing $ZnSO_4.7H_2O$ 0.08, $MnCl_2$. $4H_2O$ 0.03 gL^{-1}, H_3BO_3 0.3 gL^{-1}, $CoCl_2$. $6H_2O$ 0.2 gL^{-1}, CuSO4· 5H2O 0.01 gL^{-1}, $NiCl_2$. $6H_2O$ 0.02 gL^{-1}, $Na_2MoO_4.2H_2O$ 0.03 gL^{-1}) (Gomaa, 2014). Sulphuric acid pre-treated fruit waste was added as the only carbon substrate at 20% v/v. The cultured medium was incubated on a rotary shaker at 135 rpm at 30 °C for 72 h (Kovalcik et al., 2020). The pH was adjusted to 7.0 by 10% v/v NaOH or HCl of the final PHA production medium.

(c). Recovery of PHA Polymers

The sodium hypochlorite method was used as it produces the highest PHA recovery yield, and the degradation of PHA was minimal (Gobi & Vadivelu, 2015). Cell pellets were suspended in sodium solution and incubated (at 37 °C) for 1–2 h to complete digestion of cellular components except for PHA, whereby degrading proteins and lipids of the cell were degraded. (Anjali et al., 2014). To collect PHA, the mixture was centrifuged and the aqueous phase was disposed. The sediment was twice washed with distilled water, centrifuged, and analyzed to confirm PHA production with FTIR analysis.

(IV). Analytical Methods

(i). Analyzing Reducing Sugar Composition in Fruit Residues

The dinitro salicylic acid (DNS) method measured the total amount of reducing sugars in the pre-treated CAE and SAT fruit waste. 3,5-dinitrosalicylic acid (DNS) is broadly used in biochemistry to estimate the reducing sugars (Gusakov et al., 2011).

It detects the presence of the free carbonyl group (C=O) of reducing sugars. This involves the oxidation of the aldehyde functional group (in glucose) and the ketone functional group (in fructose) (Hu et al., 2008). The calibration curves were performed using glucose solution as a standard regent with known series of concentrations.

(a). Preparation of DNS Reagent

The reagent was prepared by dissolving 182 g of sodium potassium tartrate, 6.3 g of 3,5-dinitrosalicylic acid (DNS), and 262 mL of 2 M NaOH, finally adding to 500 mL of hot deionized water (50°C). 5g of sodium sulfite and 5g of phenol were added into the solution. The solution was stirred well, subsequently cooled to room temperature, and finally diluted with deionized water to 1000 mL to prepare the DNS reagent (Prasertsung et al., 2019).

(b). Standard Curve for Glucose

Standard Glucose solution was prepared 10 gL^{-1} in a 100 mL volumetric flask and a solution series was prepared by adding 0 mL, 0.1 mL, 0.2 mL, 0.4 mL, 0.6 mL, 0.8 mL, 1.0 mL of standard glucose solution to 7 clean test tubes (Yu et al., 2015). 1 mL of DNS solution was separately added to each test tube, and the test tubes were incubated in a boiling water bath (for 5 minutes). After allowing the tubes to cool to room temperature, 8 mL of distilled water was added to each and mixed well (Table 07). The absorbance of each solution was checked spectrophotometrically at a wavelength of 540 nm (Marsden et al., 1982). The experiments were performed in triplicates.

Table 07. Experimental and observation table for determination of reducing sugar by DNS method

No.	Standard glucose solution (mL)	Distilled water (mL)	DNS (mL)	Incubation	Distilled water (mL)	Concentration of glucose (µg/mL)
0	0.0	1.0	1	95 °C for 5 minutes	8	0
1	0.1	0.9	1		8	100
2	0.2	0.8	1		8	200
3	0.4	0.6	1		8	400
4	0.6	0.4	1		8	600
5	0.8	0.2	1		8	800
6	1.0	–	1		8	1000

(c). Measuring the Reducing Sugar Content of Fruit Waste Series

1 mL of each CAE and SAT pre-treated fruit waste was added to test tubes containing fruit waste of each type (papaya, pineapple, watermelon, and mango). 1 mL of DNS solution was added to each test tube, and then incubated in a boiling water bath for 5 minutes. Once the tubes were cooled to room temperature, 8 mL of distilled water was added to each test tube and mixed well. Afterwards, the absorbance of each solution was checked at a wavelength of 540 nm (Marsden et al., 1982).

(ii). Measurement of Moisture Content in Fruit Waste

The fruits were directly purchased from the supermarket, and fruit available fruit residues were collected from a fruit juice bar (Keels Super Center and Roots juice bar, Katubedda). Raw papaya (seeds and peel), raw pineapple (peel and core), raw watermelon (seeds and peel), and raw mango (peel) were hand-peeled and cut into 10 mm thick and 25 mm diameter slices (approximately). The weight of each raw sample was measured. Samples were washed, and gently blotted off with tissue paper to remove excess water (Yan et al., 2008). The weight of each raw sample was measured. Then the samples were oven dried at 2% relative humidity, 65ºC (Sukruansuwan & Napathorn, 2018), and maximum airflow. The moisture content of each fruit waste was measured by the weight difference after drying the fruit waste.

(iii). Cultivation/ Batch Fermentation of *B. subtilis*

LB Broth was prepared as the growth media for *Bacillus subtilis.* A loop full of bacteria culture was inoculated on a freshly prepared agar plate and incubated at 37 °C for 8–24 hours (Maier, 2000). A single colony was introduced to the 10 mL of LB Broth in a test tube and incubated overnight at 37 °C. 5 mL from 10 mL culture was introduced to each 250 mL LB broth in three conical flasks (500ml) and the culture was incubated in a rotary shaker at 135 rpm (Kim et al., 2011). The growth curves were plotted in order to measure the bacteria population in growth media periodically. The optical density was periodically measured at a wavelength of 600_{nm} for 14 hrs (Stevenson et al., 2016).

(iv). Estimation of Colony Forming Units of *Bacillus subtilis* (CFU)

For CFU estimation, the technique of Single plate-serial dilution spotting (SP-SDS) was used, which was carried out through a series of dilution plating in LB agar medium (Drop Plate method) in pre-sterilized plates (Hoben & Somasegaran, 1982). Serial dilutions (101–106) were prepared from 100 stock in LB broth 100 mL (two samples), and 10 µl aliquots of six dilutions were applied on divided sections over an agar-gel medium in glass petri dishes with the dimeter of 9 cm.

CFU enumeration was done after 18–48 h, colonies were marked on the reverse of the plate. The colony development pattern was recorded at different dilutions with the number of colonies countable (Thomas et al., 2015). Once the dilution level was recorded, which yields the acceptable colonies, the CFU per sector was counted. Colony-forming units per mL (CFU mL^{-1}) were calculated by applying the formula; $n \times 10 \times 10^{(d+1)}$ where n = number of colonies in 10 µl sample; d = dilution level which yields countable colonies. (The experiment was performed in triplicates for both samples).

(v). Qualitative Analysis

(a). Screening of *B. subtilis* for PHA Production

Petri Dish Preparation

Screening for PHA-producing bacteria was carried on using Sudan black B (SBB) staining method. The colonies stained in black-dark blue indicated PHA accumulation. (Zaki, 2018). SBB stain was prepared as a 0.3% solution (w/v) in 70% ethanol for 10 minutes(Ammar et al., 2021). For the solid agar method screening, the bacteria were streaked on a modified (Nitrogen limited) LB agar plate and incubated (at 37 °C) for 48 h (Mohapatra, Mohanta, et al., 2017). Sudan Black B-stain was spread over the colonies and kept undisturbed for 30 min. After 30 mins, the plate was de-stained by washing with 96% ethanol to remove excess stain from the colony. Nitrogen-limited modified LB Agar medium was prepared using 15gL^{-1} agar, 10 gL^{-1} NaCl, 0.5 gL^{-1} Yeast extract, and 0.5 gL^{-1} Tryptone (Mohapatra, Mohanta, et al., 2017).

Microscope Slide Preparation

Smears of *B. subtilis* were prepared on clean glass slides and heat fixed, then stained with SBB solution for 10 min. To decolorize the cells, the stained smears were immersed in xylene and followed by counter-staining with 0.5% safranin for 5 sec (Ammar et al., 2021). Stained samples were observed under a light microscope.

(v). Characterization of PHA by Fourier Transform Infrared (FTIR) Analysis

The characterization of the PHA produced was analyzed using Fourier Transform Infrared (FTIR) analysis instrument (Bruker, Brand: ALPHA; Made in Germany). FTIR analysis was used to determine the functional groups of the extracted PHA polymers. (Ammar et al., 2021) Infrared spectra of PHA samples produced using different types of fruit wastes as the only carbon source were recorded in the wavenumber range between 400 to 4000 cm^{-1} (Kemavongse et al., 2008).

(vi). Quantitive Analysis

(a). Dry Cell Weight (DCM)

Samples in triplicates were collected under aseptic conditions from the growing cultures periodically in different time intervals (24 h, 42 h, 60 h, and 72 h) from each fruit waste type (1 mL aliquots in 1.5 microcentrifuge tubes), centrifuged at 5000 rpm for 15min in 4 °C, washed with 0.5 mL of saline solution (NaCl, 0·9 g/L) and recentrifuged. The supernatant was discarded, and the pellets were dried at 85 °C for 36 h until a constant weight was reached in order to estimate DCM (Anjali et al., 2014).

CHAPTER 4

RESULTS AND RECOMMENDATIONS

4.1. Qualitative Analysis; Screening of *B. subtilis* for PHA Production

The pure strain of *Bacillus subtilis* was confirmed for PHA production using SBB as the preliminary staining method. When the bacteria cells were stained with SBB, the cells were clearly visible in dark blue-Black color in the agar plate (Figure 4.1), and from light microscope observation, PHA granules were visible in dark blue-black color.

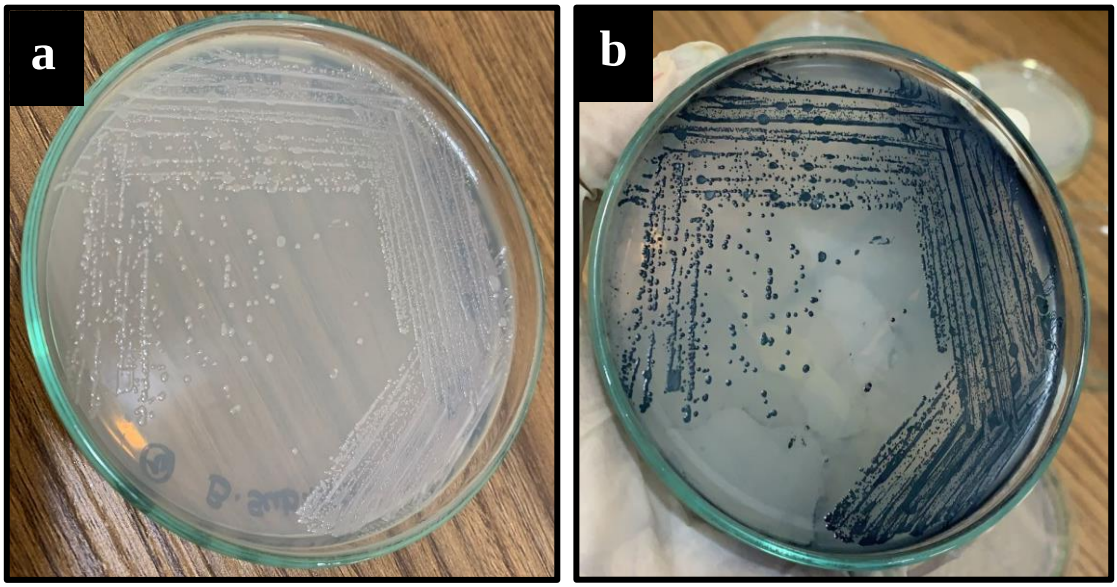

Figure 4.1: Confirmation of PHA production from SBB staining method in modified LB Agar media before (a), and after (b), SBB staining

The intracellular granules of this culture sometimes showed outside the cell membrane after being stained by SBB, when viewed microscopically, which suggests that the cell wall structure of the bacterium may have burst during smear preparation, and the intracellular granules were leaked out of the bacterial cells. In this respect, *Bacillus subtilis* pure strain obtained from the Faculty of Medicine, University of Sri Jayewardenepura, showed strong SBB positive after staining.

4.2. Light Microscope Images of PHA Production

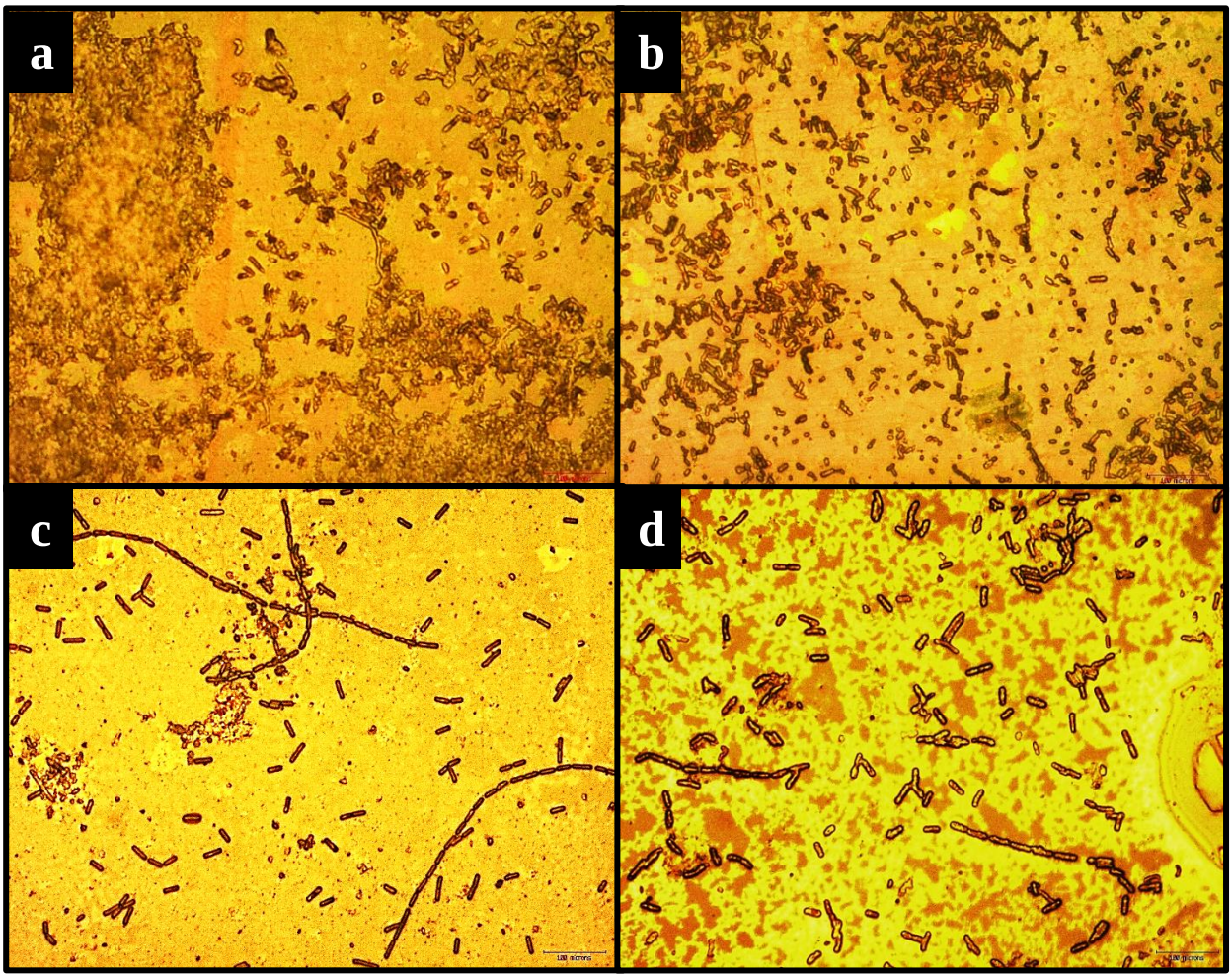

Figure 4.2: Light microscope images of PHA accumulating *Bacillus subtilis* using different fruit waste substrates (a); Papaya (b); Pineapple (c); Mango (d); Watermelon

4.3. Reducing Sugar Content in Fruit Waste

Reducing sugar contents of pre-treated fruit waste in each fruit waste type were obtained using the standard Curve for Glucose concentration from the DNS method. The reducing sugar concentration of Sulphuric acid treated Fruit waste was higher compared to the reducing sugar concentration of Crude Aqueous Extract. The reason should be the oxidization of non-reducing sugars (sucrose) into reducing sugars by sulphuric acid. Among the Acid treated fruit waste, mango contained the highest amount of fermentable carbon resources compared to papaya, pineapple, and watermelon fruit waste.

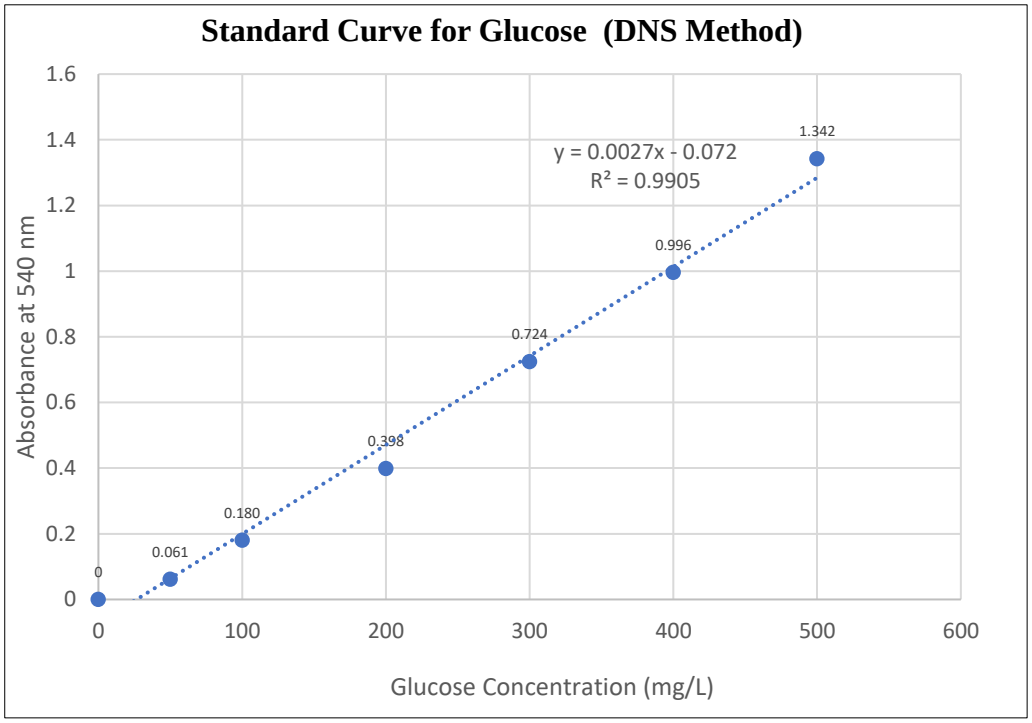

Figure 4.3: The glucose standard curve for reducing sugar estimation by DNS method

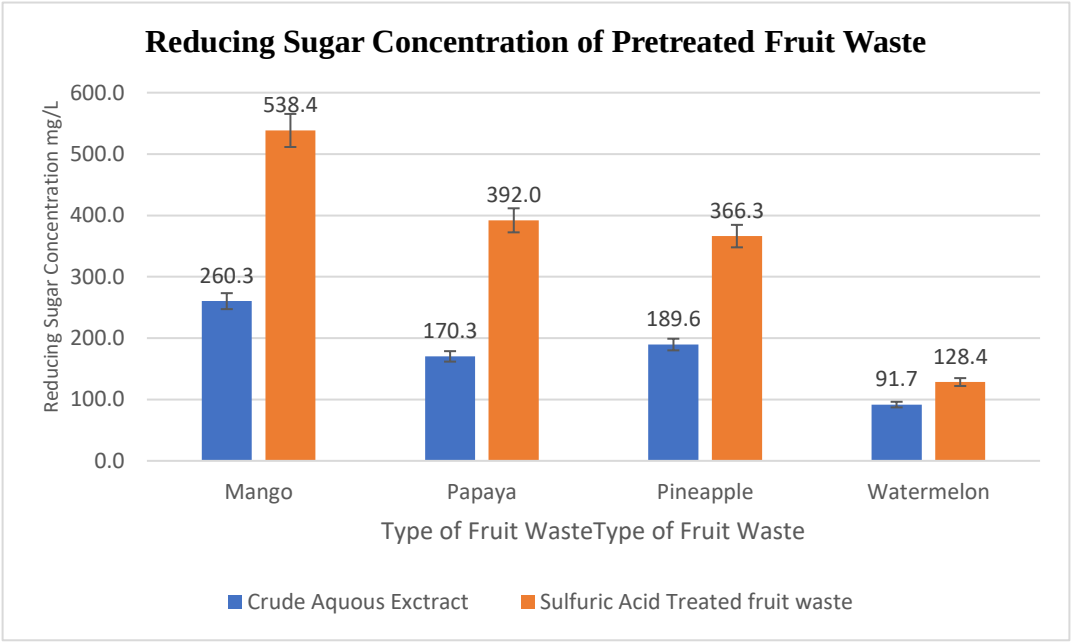

Figure 4.4: Reducing sugar content of fruit waste series were obtained using the standard Curve for Glucose concentration from DNS method

4.4. Growth Curve from Batch Cultivation

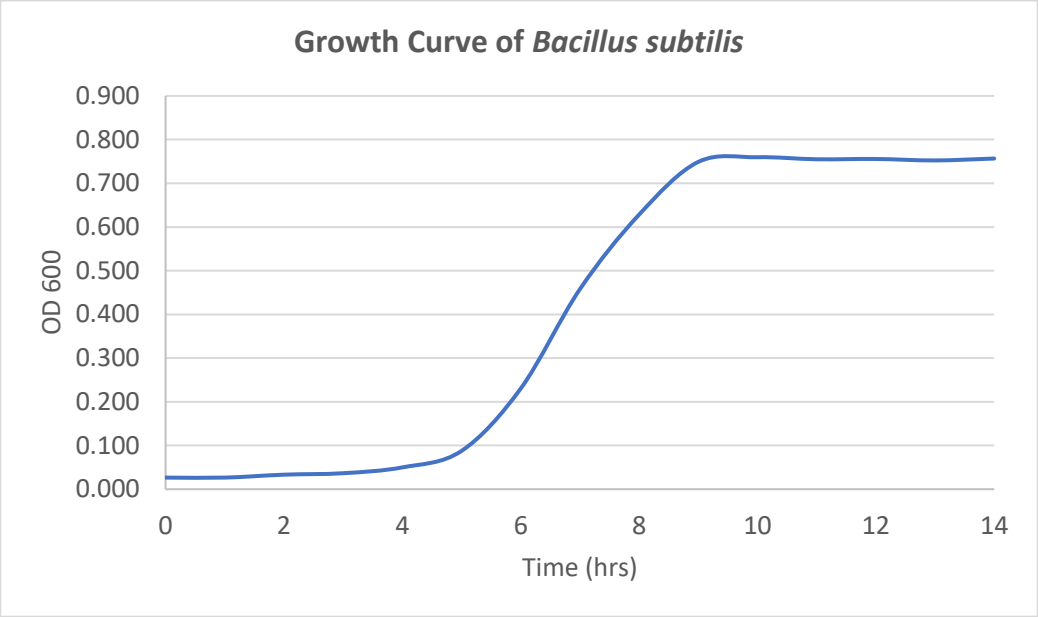

Figure 4.5: Growth curve of *Bacillus subtilis* pure strain in LB liquid media

A typical bacterial growth curve was observed by the growth curve experiment for the selected bacterium *Bacillus subtilis*, which exhibited the lag phase, exponential phase, and stationary phase in 14 hrs. It is important to observe the bacterial growth in the ambient environment for biomass production as it is analogous to the accumulation of PHA (Mohapatra, Mohanta, et al., 2017).

4.5. Estimation of Colony Forming Units of *Bacillus subtilis* (CFU)

SP-SDS approach with series of decimal dilutions worked successfully, yielding well-delineated colonies for *Bacillus subtilis* pure culture. The experiment showed identical colony forming units in plates with 30 mL of fresh medium at 10^5 dilutions. With reduction in the amount of medium per plate, the drying time of the droplets was significantly shortened (6–10 minutes) in the laminar flow cabinet. This experiment resulted in 7.5×10^8 CFU per mL as an average of two separate samples, which were carried out in triplicates (Table 08).

Table 08. Colony Forming Units (CFU) observed in sample 1 and sample 2, using SP-SDS approach

Sample No.	Average No. of colonies in 10µl aliquot	Dilution level yielding acceptable colonies	CFU ml^{-1}
Sample 1	8 ± 1	10^5	8 x 10^8
Sample 2	7 ± 2	10^5	7 x 10^8
Average No. of colonies in Sample 1 & 2	8 ± 1	10^5	7.5 x 10^8

4.6. Moisture Content in Fruit Waste

The procedure was done until the fruit residues were dried well, and then moisture content levels were obtained as 86.1% for papaya (peel and seed), 82.1% for mango (peel and seed), 83.8% for pineapple (peel and core) and 93.2% for watermelon (peel and seed).

4.7. Dry Cell Weight (DCM)

The comparative biomass production in nutrient-limited fermentation media was evaluated by measuring the dry cell weight. Under natural conditions, PHA polymers can be constituted nearly 90% of the dry weight of a cell. (Shah & Kumar, 2020).

Among the four types of fruit waste, maximum dry cell weight (2.15 mg/mL) was observed in 60h by *Bacillus subtilis* when papaya waste (peel and seed) was the carbon source for the nutrient-limited production media but degraded by 72h to 1.22mg/ml. (Table 09).

Table 09. Production of biomass using B. subtilis in different carbon sources at different time intervals

Fruit waste Type / Carbon Source	Incubation Time			
	24 h	48 h	60 h	72 h
	Average Dry Cell Weight (gL^{-1})			
Papaya	1.41±0.24	1.99±0.06	2.15±0.15	1.22±0.10
Mango	1.06±0.24	1.33±0.25	1.53±0.42	1.79±0.12
Pineapple	0.63±0.12	1.44±0.32	1.72±0.04	1.82±0.35
Watermelon	0.51±0.06	0.65±0.1	0.88±0.10	1.23±0.06

Fermentation of pineapple, mango, and watermelon fruit wastes resulted in the highest dry cell weight at 72 h, 1.82 mg/mL, 1.79 mg/mL, and 1.23 mg/mL, respectively. The results suggested that although the strain had the ability to produce biomass using different types of carbon sources with different efficiency, Papaya has proven to be the comparatively best sugar supplement, followed by pineapple, then mango, and watermelon, respectively.

4.8. SEM Images of PHA Polymer Production

The SEM images obtained from the present study confirmed the potential of PHA polymer production. Figure 4.6 (a) and (b) (Petrovova et al., 2014; Kumari, 2014) shows the regular rod-shaped *Bacillus subtilis* (without PHA production), whereas Figure 4.6 (c) indicates the PHA granules produced in nutrient deficient media in the present study. Similar results were observed in (Balakrishna Pillai et al., 2017) and (Mohapatra et al., 2020).

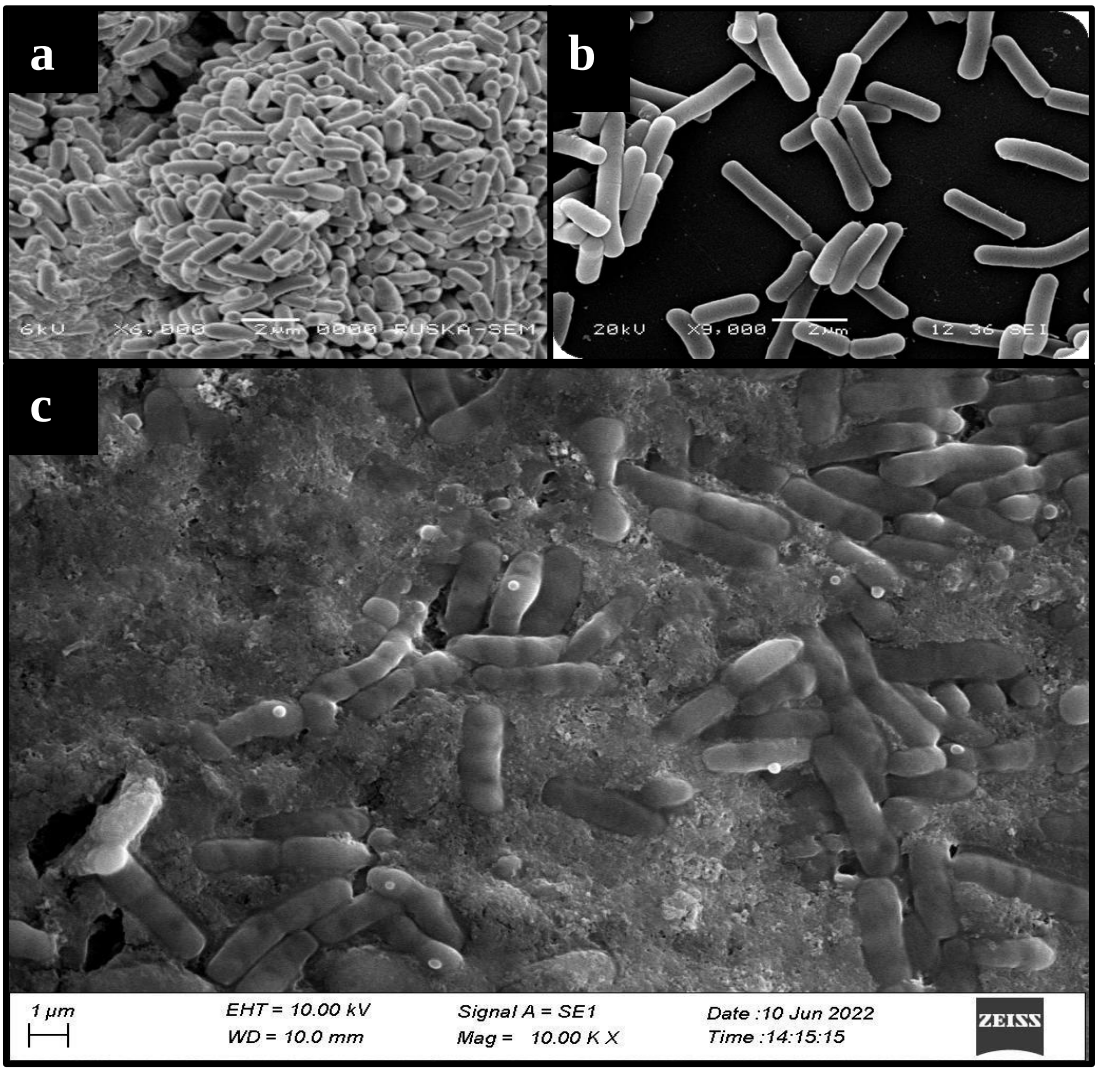

Figure 4.6: Scanning Electron Micrographs of *Bacillus subtilis*; (a) and (b): General morphology of rod-shaped *Bacillus subtilis*; (Petrovova et al., 2014), (Kumari, 2014); (c): Morphological modification of *Bacillus subtilis* after PHA accumulation (present study)

4.9. Fourier Transform Infrared (FTIR) Analysis

The polymer samples obtained in the present work (from each fruit waste type) were applied to record IR spectra (Fourier Transform Infrared analysis) for the PHA production analysis. The IR spectra indicated the prominent bands at 1722–1727, 1370–1398, 2850–2960, and 1000–1300 and 1637–1639 cm^{-1} corresponding to the carbonyl group, the methyl group, methyl and methylene groups, the ester group, and thioester group respectively (Mahato et al., 2021). A series

of intense bands at wavenumber 1000–1300 cm^{-1} corresponded to the stretching of the carbonyl (C–O) bond of the ester group (Kemavongse et al., 2008; Shah K R, 2011).

The hydroxyl group band of PHA polymer was broader because of the moisture content in the extracted polymer, as the lack of water molecules performs a narrower band. Therefore, the intensity of these bands is proportional to the sulphur and moisture content. The FTIR spectroscopic analysis delivered further insights into the chemical structure of the commercial PHBV polymer mentioned in the literature as 1458.96, 978.79, 934.14, 827.63, 726.10, 678.12, 625.03 cm^{-1} (Mahato et al., 2021b). The results obtained by this study suggests precisely similar to that of other researchers (de Smet et al., 1983; Shah K R, 2011).

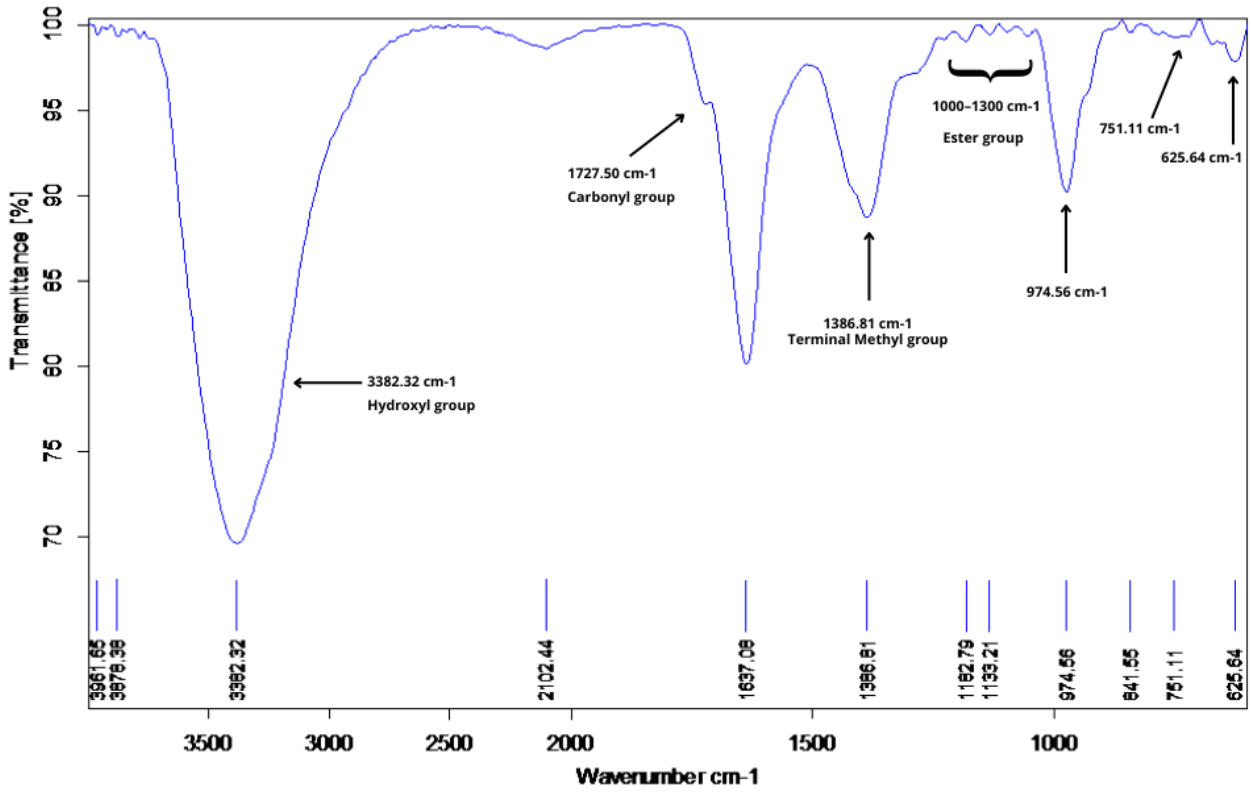

Figure 4.7: Fourier transform infrared spectroscopy (FTIR) spectra of extracted polymer from *B. subtilis* using watermelon fruit waste as the carbon source showing the functional groups of Polyhydroxyalkanoates

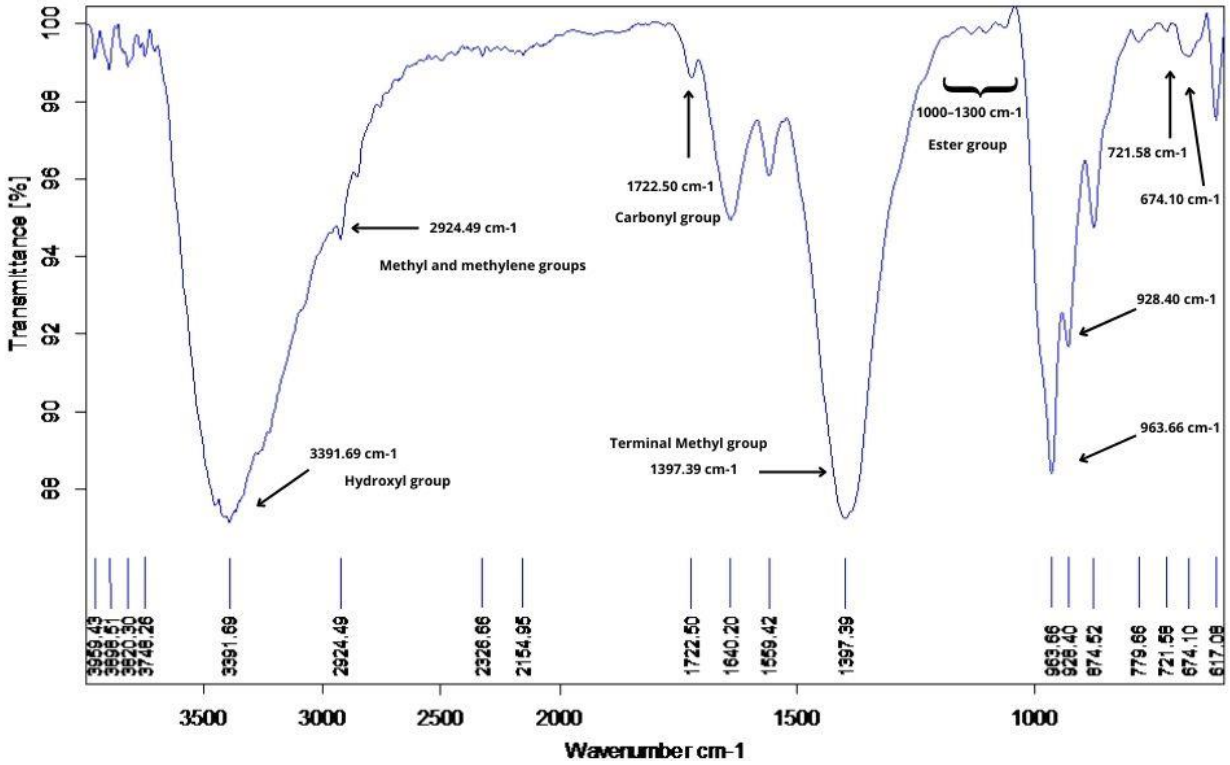

Figure 4.8: Fourier transform infrared spectroscopy (FTIR) spectra of extracted polymer from *B.subtilis* using papaya fruit waste as the carbon source showing the functional groups of Polyhydroxyalkanoates

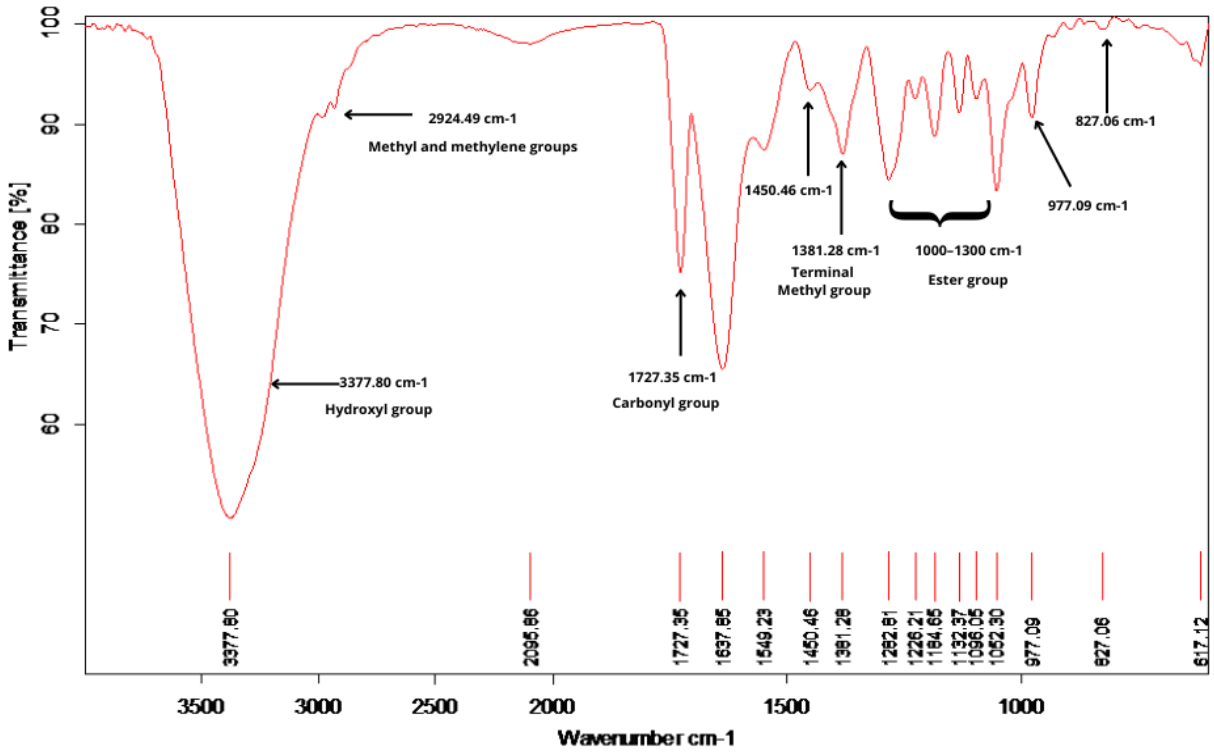

Figure 4.9: Fourier transform infrared spectroscopy (FTIR) spectra of extracted polymer from *B.subtilis* using pineapple fruit waste as only carbon source showing the functional groups of Polyhydroxyalkanoates

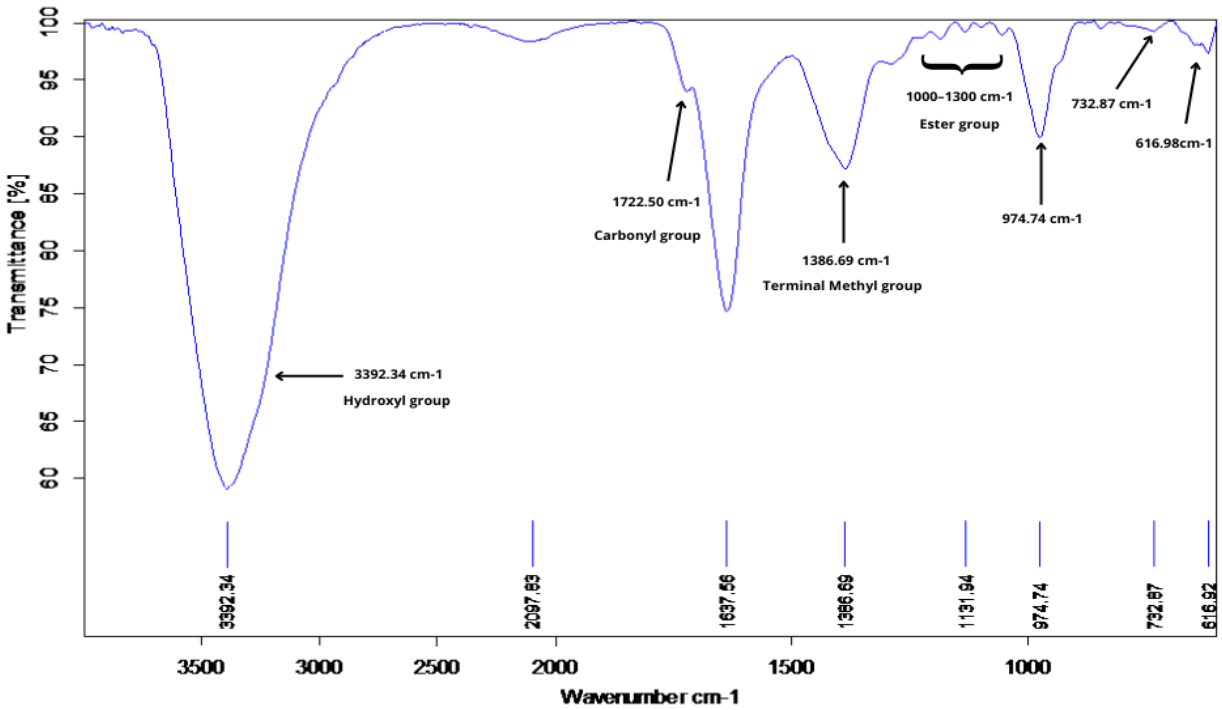

Figure 4.10: Fourier transform infrared spectroscopy (FTIR) spectra of extracted polymer from *B.subtilis* using mango fruit waste as the carbon source showing the functional groups of Polyhydroxyalkanoates.

4.10. Recommendations for Future Aspects

Continuous fermentation is recommended under non-sterilized conditions which should be developed using mix cultures and halophilic bacteria. It is also recommended to development of high cell density bacterial growth within a shorter period. Eco-friendly extraction and purification methods for controllable lysis of cells containing PHA should be developed, especially using non-halogenated inorganic solvents and novel sustainable methods such as using meal worms for PHA recovery (Zainab-L et al., 2022). Procedures should be developed to increase the reducing sugar concentration of the substrates, which will enhance the efficiency of PHA transformation. In tropical countries such as Sri Lanka, used scraped coconut (left over of coconut milk pulp) also can be used as an abundant feed stick since its enriched with lipids and carbohydrates. However, present worldwide research efforts are focusing on decreasing the cost of PHA production, thus, more cost-effective processing of PHA should be developed as synthetic packaging materials.

CHAPTER 5

CONCLUSIONS

Plastics are now all-pervading in our lives within the modern society and has become a planetary boundary threat because of leaking large amount of plastic to the environment. Global efforts are being taken in order to address the plastic pollution and bio-plastics play an increasingly significant role in this regard. Bioplastics can be bio-derived and/or biodegradable. However biodegradable and bio-derived are different and independent classifications. There are bio-derived plastics (i.e. BioPE), which are non-biodegradable, while plastics such as PHA, TPS and PLA are biodegradable. Even though plastics such as PLA are biodegradable, they require specific synthetic conditions for biodegradation. In terms of synthesis, PLA also requires synthetic polymerization process as only the monomer is being produced by the microorganisms.

PHA is a bioplastic which is both bio-derived and readily biodegradable under ambient environment conditions, thus has been recognized as a truly "eco-friendly" plastic. Unlike PLA and TPS, PHA is inherits significant plastic properties such as being resistant to both water and O_2. The polymerization of PHA is completed by the microorganism itself, thus synthetic polymerization and chemical synthesis is not involved. Hence, PHA paves the way to the prospect of manufacturing a plastic that is commercially available, which does not have the legacy of accumulation in the environment.

PHAs perform similar characteristics to those of currently widely applied petrochemical based plastics, and therefore, are expected to compete with petrochemically-derived plastics. The range of applications and products of PHAs are extensive, and have been approved for food contact single-use plastics such as cutlery, straws while they have been also approved for molded toys, eyewear frames because of its biocompatibility. The biocompatibility of PHA makes it suitable for biomedical, agricultural and packaging and textile industry applications.

Addressing few key challenges for commercial production of PHAs, such as the increased cost of feedstock, energy cost which is associated with high temperature bioprocesses, the current study was initially carried on to realize the proof-of-concept to utilize abundant fruit-residues as the feedstock to bacterial fermentation for PHA production.

This study demonstrated the feasibility of utilizing four types of fruit wastes available in the fruit-based industry in Sri Lanka as lignocellulosic feedstocks for PHA production. Sudan black B stain screening for PHA production was successfully confirmed using the modified LB agar media with limited nutrients by *B. subtilis* bacterium.

The proof-of-concept stage was successfully realized in order to promote PHA synthesis using the four fruit residues mentioned in the study, papaya, mango, pineapple, and watermelon by *B. subtilis* bacterium (Table 10). A comparison of two pre-treatment methods (Crude aqueous extract and Sulphuric acid treatment) was carried out using the DNS method to assess the fermentable reducing sugar concentrations contained in fruit wastes. A higher concentration of reducing sugars was observed in all four fruit residues pre-treated with sulphuric acid compared to the crude aqueous extract pre-treatment method. Since the non-reducing sugar was oxidized by sulphuric acid, increasing the reducing sugar concentration compared to the crude extract. Consequently, using sulphuric acid pre-treated fruit residue extracts can be adjusted as an efficient carbon source for PHA production by fermentation of fruit residues by *B. subtilis* with higher concentrations of reducing sugar. The four fruit residues, papaya, mango, pineapple, and watermelon, have been successfully used by the pure culture of *Bacillus subtilis* as an inexpensive raw material for producing PHA.

Thus, the potential of the fruit wastes mentioned above to be applied separately as the only carbon source for PHA production was identified. In terms of dry cell weight (g/L) obtained, higher biomass production was observed in papaya fermented media, followed by pineapple, mango, and watermelon. Higher biomass production was delivered in 60hr fermentation in papaya fermented media, which is subsequently decreased due to bacterial utilization of intracellular PHA storage.

FTIR analysis successfully confirmed that the extracted samples of PHA from papaya, mango, pineapple, and watermelon fermented media, by plotting the corresponding bands for the PHA functional groups.

Based on the findings of this study, and previous literature it can also be concluded to the following assumptions, (Table 11), considering the papaya fruit waste.

Table 10. Table of summary for the objectives and corresponding results obtained

Objective	Obtained Results
1. Screening for PHA production from the selected pure strain bacteria in modified LB agar medium.	Successfully confirmed, by staining the colonies in black-dark blue colour, indicating PHA accumulation using the modified LB agar media with limited nutrients by *B. subtilis* bacterium
2. Comparison of two pretreatment methods (Crude aqueous extract and Sulphuric acid treatment) that results in a higher concentration of fermentable reducing sugar	Comparatively, a higher concentration of reducing sugars was observed in all four fruit residues pre-treated with sulphuric acid compared to the crude aqueous extract pre-treatment method
3. Identifying the potential of fruit wastes (mango, watermelon, pineapple, papaya) to be applied separately as the only carbon source for synthesis of PHA	The four fruit residues, papaya, mango, pineapple, and watermelon, have been successfully used by the pure culture of *Bacillus subtilis* as an inexpensive raw material for producing PHA. Thus, the potential of the above fruit wastes to be applied separately as the only carbon source for PHA production was identified
4. Identifying the type of fruit residue and appropriate fermentation time which produces more biomass in the selected bacterium	Higher biomass production was delivered in 60hr fermentation in papaya fermented media, (DCW:2.15±0.15 g/L), followed by pineapple, mango, and watermelon respectively, in 72hrs
5. Characterization of PHA produced by selected bacterium using different types of fruit waste by FTIR Spectroscopy.	PHA synthesis using fruit residues fermentation by *B. subtilis* bacterium was successfully confirmed by the corresponding peaks of PHA functional groups

Table 11. Assumptions that can be generated according to the present study findings source (EDB, 2019):

Total weight of fruits/ fruit products exported annually	6,210,135 Kg
Waste generated in fruit processing	10–20%
	625,013 Kg – 1,242,027 Kg
Dry cell weight obtained from the present study	2.15±0.15 g/L
The weight of the PHA polymers that can be produced (Since PHA polymers can be constituted nearly 10 - 90% of the dry weight of a cell)	2,687.56 Kg – 5,375.12 Kg

However, these amounts can be varied and increased by considering the annual local consumption and also by considering the total generation of fruit wastes produced in Sri Lanka (1,019,000 metric tons of fruits annually).

Notably, essential minerals in fruit waste also supported PHA accumulation in synthetic PHA production media. Fruit wastes pre-treated with sulphuric acid have been determined as potential carbon sources for *Bacillus subtilis* bacteria, providing an innovative pathway for cost-effective production of PHA from locally assimilable fruit waste a feedstock.

Despite the fact that PHAs are synthesized in heterotrophic bacteria, archaea, cyanobacteria and plants, plants have the disadvantage of slow growth and are difficult to operate compared to bacteria. Therefore, bacterial fermentation using locally available and inexpensive nutrient-enriched substrates would be an efficient route for PHA production. However, useful products can be developed from the derived biopolymer, which can be used in the packaging industry, which will be one of the most suitable, appropriate and sustainable panaceas. Therefore, PHA has the potential to play a vital role in a sustainable future. The need for wide-scale PHA production by defeating the potential challenges is important, as it will not only benefit the potential eco-friendly environmental outcomes but will also generate significant benefits of novel economic and employment aspects.

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