Biotransformation of Lignocellulosic Biomass Hydrolysate into Polyhydroxybutyrate Biopolymer via Ralstonia Eutropha

Nausheen Jaffur*, Pratima Jeetah, and Gopalakrishnan Kumar

Abstract—Currently, a rampant cultural shift is occurring in the modern world to progressively substitute fossil-derived plastics and shift to novel biomaterials that are benign to the environment owing to increased awareness of environmental sustainability along with the implementation of strict regulations worldwide. Polyhydroxyalkanoates (PHAs) are promising intracellular biodegradable polymers that have attracted considerable focus owing to their biocompatibility, biodegradability, non-toxicity and environment-friendly nature to function in diverse applications notably in the pharmaceutical, medical, textile, materials, fuel, agricultural industries. Nonetheless, despite its huge market potential, the commercial growth of PHA is achieved on a small extent only, since the cost-effectiveness of this product is highly debatable owing to the high production cost of processing the carbon substrate. The goal behind this research study is to explore the possibility of exploiting low-cost carbon substrates from low-value lignocellulosic materials that would have otherwise been discarded as waste and add stress to the landfill to manufacture biopolymer compounds that are used in everyday lives as well as to enhance the functionality and yields of glucose from PHA substrates that can undergo industrial upscaling. One of the major challenges of transforming lignocellulosic biomass into fermentable sugars is the recalcitrant nature of the fibre which renders it very resistant to the release of sugars for fermentation. Since lignocellulosic biomass has a specific attribute such as an extremely coordinated matrix which renders it very resistant to the release of sugars for fermentation owing to biological degradation, a pre-treatment phase is necessary prior to the hydrolysis stage for the transformation of the fermentable sugars. This study focuses on the biosynthesis of biopolymers from lignocellulosic biomass through sustainable approaches such as enzyme and microbial activities in order to examine its viability as a replacement for traditional polymers. Cupriavidus Necator H16 (Ralstonia Eutropha) having 8×10^8 CFU/ml viable colonies were cultured at 30 °C and was inoculated in submerged fermentation of M9 minimal salt medium using 1% reducing sugar from Furcraea Foetida as carbon source. Batch fermentation of PHB in submerged cultivation conducted for a residence time from 0 to 48h resulted in a dry cell weight from 0.32±0.05% to 1.62±0.05%. The nitrogen limiting phase was achieved after 48h and 17.05±0.35% of PHB was extracted from 3ml of the fermentation broth. The PHB yield was dramatically lower than reported optimal yields of 37.55 to 97.80% from works of literature. Nonetheless, Fourier-transform infrared spectroscopy (FTIR) spectroscopy revealed characteristics bands for carbonyl, methine and ester groups along with intermolecular hydrogen bonds in the biopolymer. Sudan Black B and FTIR spectrum demonstrate that PHB biosynthesis successfully bioaccumulates inside the cells of *Ralstonia Eutropha* using cellulose from Lignocellulosic biomass (LCB) as carbon source. Hence, the process needs to be optimized in terms of variables such as inoculum size, inoculum concentration, incubation time and salt medium conditions in order to maximise the production of PHB from *Furcraea Foetida* in *Ralstonia Eutropha* cultivation.

Index Terms—Sustainability, lignocellulosic biomass, polyhydroxyalkanoates, sustainable waste management, biocompatibility, biodegradability

I. INTRODUCTION

Plastics are becoming widely prevalent in a broad array of applications, both in the household and in industry. About 368 million metric tonnes of plastics were produced worldwide in 2019, of which 98 percent were fossil fuel-based polymers derived from petroleum [1]. Humanity has benefited from the advancement of contemporary science, technology, and markets, which has resulted in increased economic success and comfort as a result of the employment of fossil-derived materials to make a wide range of useful fuels, chemical compounds, and polymers [2, 3]. There are, nonetheless, several drawbacks and downsides to using petroleum as a fuel source. The overuse of petroleum has resulted in a crisis in energy, natural resources, and the environment. When confronted with these circumstances, environmental sustainability becomes an imperative requirement. The non-biodegradability of petroleum-based plastics poses a serious threat to the environment in terms of disposal and recycling, culminating in health and environmental system concerns such as soil infertility, greenhouse gas emissions, and carcinogenic compounds generated during the disposal process, landfilling and incineration. Non-biodegradable plastics presently account for 25 million tonnes of waste each year, making them a major source of pollution. Additionally, when fossil fuel reserves diminish, this predicament will only get worse. Now that climate crisis and depleting fossil carbon resources are in the spotlight, researchers from across the globe are exploring environmentally benign substitutes to fossil-derived polymers. Biopolymers are a feasible alternate, and one class getting loads of attention is the polyhydroxyalkanoates, or PHAs [4]. Industrial biotechnology is currently facilitating the development of bio-based chemicals and polymers sustainably. Biomass is a renewable, environmentally friendly, and sustainable substrate that may be utilized to manufacture items made entirely of recyclable, ecologically responsible, and sustainable materials [5].

In recent decades, there has been an increase in interest in developing products with properties and uses similar to those of fossil-derived synthetic polymers. Polyhydroxybutyrate (PHB), a member of the Polyhydroxyalkanoate (PHA) family, is a material with thermomechanical properties comparable to

Nausheen Jaffur and Pratima Jeetah are with the Chemical and Environmental Engineering Department, University of Mauritius, Reduit, CO 80837 Mauritius.

Gopalakrishnan Kumar is with the Institute of Chemistry, Bioscience and Environmental, Engineering, University of Stavanger, Stavanger, CO 4036 Norway.

*Correspondence: nausheen.jaffur@gmail.com (N.J.)

polypropylene that may be used instead of conventional polymers. Bioplastics are manufactured from renewable biomass, which may be first-generation feedstocks such as maize or sugar beet or second-generation feedstocks such as lignocellulosic biomass. Third-generation feedstocks, on the other hand, such as microorganisms that do not compete with feedstuffs or animal feed, farmed land, or waterways, have recently gained more focus. Insoluble inclusion bodies produce polymers in the cytoplasm of some cells, which are utilized to make microbial biopolymers. These inclusion bodies, according to scientists, are both natural and biodegradable. They operate as carbon storage for the cells during times of stress. The fact that they are storage materials does not alter the concept that they aid microbial cells in maintaining their integrity by protecting them from abrupt osmotic shifts. The most investigated polymer in the PHA family is Poly-3-hydroxybutyrate (P3HB). PHA is an evolved version of naturally occurring polymers. Due to their similarity to traditional plastics, microbial polymers possess material properties that make them suitable for a wide variety of industrial applications [6]. For example, their superior barrier properties have enabled their use in food packaging in the industry. It has a melting point of 175 $^{\circ}$C and a glass transition temperature of 15 $^{\circ}$C. Additionally, it has a molecular mass of 5×10^5 Daltons and a density of 1.25 g/cm^3 (3.5 GPa) [6].

The manufacturing costs of PHB are projected to be four to nine times more than traditional polymers such as polyethylene. Compounding the issue of high production costs are factors such as a demand for pure culture fermentation, substrate needs and different culture conditions. To maximize yields, culture variables like temperature, pH, light requirements, nutrients, and cycle duration must be optimised whilst keeping in mind the critical function carbon sources present in the bioprocess. When it comes to the organic substrates used in traditional PHB manufacturing, the vast majority are derived from raw materials that are exclusively composed of carbohydrates like sucrose and maltose. Other common raw materials include glucose, starch, and other carbohydrate derivatives. The current focus of the study has been on integrating the usage of lignocellulosic materials with PHB synthesis in order to solve the difficulty of obtaining adequate non-competitive carbon sources for PHB synthesis.

Cupriavidus necator is remarkable among biopolymer-producing microorganisms since it can accumulate up to 90% of its cellular mass in PHB and utilize carbon from a variety of substrates. C. necator produces PHB in two stages: the first, which is distinguished by balanced culture conditions, and the second, which is produced by limiting one constituent, such as nitrogen or phosphorus. It developed the ability to metabolize glucose through spontaneous mutation, and it can also consume fructose, glycerol, and organic fatty acids [7]. Nevertheless, as compared to traditional plastics, the price of PHB remains high. Using low-cost feedstocks such as municipal waste (food waste, waste cooking oil, sewage and plastics), animal waste (fats, manure, and lard), forestry waste (bark, sawdust, leaves, straws), agricultural waste (crop waste, wood chips, rice straw) and industrial waste (spent coffee beans, olive pulp) as substrate is one approach to minimize manufacturing costs.

This study aims to do a critical review to assess the different pretreatment techniques for lignocellulosic biomass to be used as carbon source during microbial fermentation of polyhydroxybutyrate which is a medium-chained-length (mcl) PHA. The objectives are to identify potential microorganisms and enzymes capable of synthesizing PHB and to highlight the different challenges during PHB bioprocessing.

A. Metabolic Pathways

Catabolization of the carbon sources required to synthesize PHA is achievable through a variety of metabolic processes [8]. This section focuses on P(3HB), the most synthesized PHA in the entire globe, as a concise overview of the PHA class. There are three distinct enzymes involved in the ultimate stages of creating this biodegradable polymer: PhaA, PhaB, and PhaC. The carbon supply is transformed to acetyl-coenzyme A (CoA), and two molecules of this are turned into acetoacetic acid by a thiolase (-ketothiolase, PhaA). The NADPH-dependent acetoacetyl-CoA reductase (PhaB) generates the synthesis for the PHA, (R)-3-Hydroxybutyryl-CoA, which is subsequently polymerized by the PHA synthase (PhaC) [8].
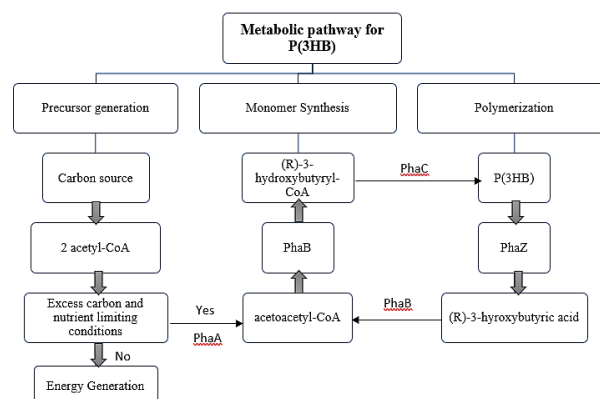

Fig. 1. Metabolic pathway for the production of P(3HB) (PhaA: β-ketothiolase, PhaB: NADPH-dependent acetoacetylCoA reductase, PhaC: PHA synthase and PhaZ: PHA depolymerase).

The metabolic route shown in Fig. 1 is intrinsically associated with the primary metabolic processes of the microorganisms. Additionally, glycolysis, Krebs cycle, de novo fatty acid synthesis, -oxidation, the amino acid metabolic route, Calvin cycle, and serine pathway are all involved in the manufacture of P(3HB). Both of these processes require the use of acetyl-CoA as an intermediary. Essentially, the production of PHA under certain environmental circumstances is dependent on the metabolic flow of acetyl-CoA. When there is an excess of carbon and no other growth restriction, the Krebs cycle, which is responsible for CoA synthesis, is a hindrance to PHA formation. Acetyl-CoA is a metabolite that is integrated into the Krebs cycle, which is responsible for energy production and cell development. It is possible to achieve non-inhibitory CoA concentrations when carbon surplus circumstances are maintained and an important nutrient is limited. This allows the delivery of acetyl-CoA to the PHA synthesis pathways. The metabolic causality framework detailed above is used to mimic these circumstances in bioreactors in order to generate PHA for use in pharmaceutical production. The majority of PHA-generating actions are separated into two phases, in line

with the traditional technique for the synthesis of secondary metabolites that are not associated with the stage of cellular development in which the activity is performed. The main phase is the phase in which the goal is to increase the number of viable cells in the reactor's unrestricted nutrient supply, which is accomplished via the use of a variety of techniques. In this case, a limitation is applied (for instance, a lack of a nitrogen supply), diverting metabolic processes toward the accumulation of PHA, which inhibits cell growth and multiplication. After an accumulation of PHA has occurred, removing these limitations allows the PHA to depolymerize, re-establishing the normal metabolism of the microbe for energy production and growth. An enzyme known as PHA depolymerase (PhaZ) converts PHA to readily absorbed energy elements that the organism may use when a change there is an alteration in environmental circumstances [6, 8].

II. BACKGROUND STUDY

A. Polyhydroxybutyrate (PHB)

PHAs are categorized into short chain length (scl) and medium chain length (mcl) polyhydroxyalkanoates as per their respective monomeric components and carbon atoms. The scl-PHAs are composed of 3 to 5 carbon atoms and can be synthesized by bacterial strains such as Cupriavidus Necator while the mcl-PHAs comprising of 6 to 14 carbohydrate atoms can be orchestrated via pseudomonas species [9, 10]. In addition, the foundation of long-chain PHAs (lcl-PHAs) consisting of over 14 carbon atoms has recently been discovered via in vitro processes [11]. Polyhydroxybutyrate (PHB) is the predominant scl-PHA homopolymer which is identified by its stiffness, brittleness and the challenge to be transformed owing to its highly crystalline framework. Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) also known as PHBV is a copolymer which can be developed via the integration of 3-hydroxyvalerate (HV) units in PHB. The resulting biogenic polyester is relatively more durable, resilient, stable as well as easily processed by escalating the molar mixing ratios of the different species of the copolymer [9, 12, 13]. Moreover, scl-PHAS are employed in the manufacture of disposable polymeric commodities as well as for food packaging products while mcl-PHAS being more elastic and flexible (elastomers) are ideal for the production of drug carriers, medical devices, biocontrol agents, and biodegradable implants which may include dental prosthesis or any other structure to aid in medical treatments [9, 13, 14]. Other medical applications of PHA include tissue engineering, anti-cancer agents and memory enhancers [15]. Also, in the cosmetic sector, PHA and starch blends having a high compatibility to the skin are promising materials to function as bio-based beauty masks [16]

On the basis of value and quantity, the shorter PHA chain form, PHB is expected to dominate the PHA sector in the near future [12, 13]. Greater understanding and knowledge of risks and hazards relating to environmental disequilibrium emanating from plastic waste materials has significantly influenced consumer perceptions and behaviours about bio-based polymers [17, 18]. This element is fundamental in boosting the market and supply demand for scl-PHA as well as mcl-PHA products. Also, packaging and food services are expected to take a drastic lead with reference to product value on the PHA industry [13, 19]. The increasing demand for biocompostable and biodegradable biopolymers to be used for diverse uses like carrying bags, disposable wares, cling films, wrapping materials in the packaging and food market is provoking an increase in production of such eco-friendly commodities [20–22].

There is currently a growing industrial concern towards biotechnological development of polyhydoxybutyrate (PHB) polyester using sustainable raw materials to produce polymer-based commodities as a substitution for traditional petrochemical-based plastics [20, 23, 24]. PHBs may be categorized as thermoplasts or elastomers based upon their specific structure. Traditionally, several prokaryotic strains of sustainable sources of materials such as sugars, lipids, alcohols and organic acids particularly lactic acid are generated in growth limiting conditions leading to a disequilibrium in the availability of nutrient. On a commercial scale, the shift from controlled microorganism growth to PHB build-up is generally achieved via a minimization in phosphates or nitrogen [20, 22, 25]. It is unequivocal that any cost effective large-scale industrial production reckons on a number of considerations particularly the possibility of the microorganisms to make use of a low-cost carbon resource, price for microbial culture, development rate of microorganisms, speed of biopolymer synthesis, cost associated to corresponding methods for the manufacture of plastics, attributes and volume of PHB required [22, 26, 27]. In addition, significant measures need to be being taken to identify safe and affordable raw resources, to test different transgenic species and to use microbiological regulatory bodies to enhance the recovery process in the innovative production of genetically engineered organisms [25, 27].

Nonetheless, owing to elevated manufacturing costs associated with biopolymers, the large-scale commercialisation and widespread industrial growth of PHBs remain challenging leading to relatively greater costs than traditional polymeric materials. The price of PHBs are expected to vary in the range of 2.25 to 2.75 USD per pound which is around fourfold the price of traditional polymers such as polypropylene and polyethylene [13, 28, 29]. The elevated pricing can be attributed to the high purity raw materials (sugars), enormous quantities of solvents, energy and the different types of batch processing required [24, 28, 30]. The supply of sustainable raw materials as well as the growing market demand and usage of bio-based polymers along with the adoption of Green Public Procurement (GPP) coupled with circular economy polices are anticipated to boost the emerging PHA business in the packaging, food, cosmetic, automobile and biomedical sectors. As per market research reports, currently the estimated value of the global PHB market stands at USD 57 million and is expected to experience a growth of 11.20% by 2024 accounting to a projected value of USD 98 million. The fast-growing market is connected to socio-economic and political activism in opposition to traditional plastic products and government policies as well as consumer behaviours [19, 28, 31].

B. Potential Sources of Sugar from Biomass

Sugars can be monosaccharides involving aldehyde groups (aldose) or monosaccharides involving ketone groups (ketose). Aldose is further sub-divide into six carbon sugars such as glucose, fructose, mannose, galactose) and five carbon sugars notably, xylose, arabinose, xylulose [32]. These simple sugars can be extracted from lignocellulosic biomass and plant-derived edible biomass via hydrolysis and each monosaccharide has a variety of applications in the production of chemicals, biopolymers, pharmaceuticals, agriculture as well as cosmetics as mentioned in Table I.

TABLE I: APPLICATIONS OF SIMPLE SUGARS EXTRACTED FROM LCM

Sugar	Applications	References
Glucose	Energy source, PHB and ethanol production	[33–35]
Sucrose	Biopolymer production, chemical carrier for emulsifier and detergent	[36, 37]
Xylose	Low-calorie sweetener, production of fuel and chemicals	[38, 39]
Mannose	Used as dietary supplements in the food, pharmaceutical, and poultry industries	[40, 41]
Galactose	Ethanol production or used for energy	[42]
Fructose	Low-calorie products as taste enhancer	[43, 44]
5-hydroxymethylfurufral and furfural	Precursors for fuel, resin, plastic, nylon, polyester, fine chemical	[45, 46]
Sugar alcohols (sorbitol, mannitol, xylitol, arabitol)	Low-calorie sweetener, adhesive, cosmetics and energy source	[47, 48]
Sugar acids (gluconic acid, xylonic acid, arabinonic acid)	Chelating agent, cement retardant, cosmetics, medicine	[32, 39]
Acids (succinic acid, itanoic acid, formic acid, glycolic acid)	Food and polymer industry	[32, 50]
Alcohols (ethanol, butanol)	Used as fuels and solvents	[32, 51]
Alkyl ethers of sugars (alkyl glucoside, alkyl xyloside	Used as biomass-derived surfactant	[32]

The most prevalent sugars present in lignocellulosic biomass hydrolysates are glucose and xylose irrespective of the pretreatment technique employed. Around 60 to 70% of glucose and 30 to 40% xylose are typically present in lignocellulosic hydrolysates [52]. Cellulose an unbranched crystalline structure is composed mainly of glucose units while hemicellulose being a branched heteropolymer is composed of hexoses (such as l-fructose d-galactose, l-galactose, d-mannose), pentoses (such as xylose, arabinose) and acetylated sugars [53]. Since lignocellulosic biomass are composed of 75% polysaccharides such as cellulose, hemicellulose, starch and saccharose, it is more favourable to use them for direct synthesis of sugars [32].

Table II. gives an idea of the concentration of different types of sugar in a variety of lignocellulosic material.

TABLE II: CONCENTRATION OF SUGAR IN LIGNOCELLULOSIC BIOMASS

Substrate	Type of hydrolysis	Concentration of sugar (g L^{-1})			
		glucose	xylose	mannose	arabinose
Soft wood	Enzymatic	61.8	3.6	7.2	0.4
Bagasse	Chemical	4.1	24.4	-	2.6
Oil palm empty fruit branch	Enzymatic	48.3	29.6	-	-
Rice straw	Chemical	1.1	22.6	-	0.5
Spent coffee grounds	Enzymatic and chemical	3.9	-	23.6	28
Wheat bran	Enzymatic	12.7	4.1	4.1	3.1
Elephant grass	Chemical	22.2	-	-	-
Acacia	Chemical	12.3	-	-	-
Cane tops and leaves	Chemical	23.3	-	-	-
Cane stalk's peel	Chemical	18.2	-	-	-
Municipal Forestry and Greening Wastes	Steam explosion pretreatment combined with other pretreatments such as dilute acid, organosolv, and metal salts	80.0	-	-	-
Coconut husk	hemical	20.8	-	-	-

Sources: [32, 34, 54, 55]

C. Ralstonia Eutropha

Cupriavidus Necator (strain ATCC 17699/H16/DSM 428/Stanier 337) bearing synonyms *Alcaligenes eutrophus*, *Ralstonia eutropha* (most common name) and *Wautersia eutropha* is a facultatively chemolithoautotrophic and gram-negative betaproteobacterium that thrives in the soil/sludge and is exploited in the development of biodegradable polymer such as PHB. This species has the ability to grow aerobically in the presence of hydrogen and carbon dioxide as the only source of energy and carbon [56, 57]. PHA builds up inside cells when there is an imbalance in nutrients in particular, too much carbon and not enough nitrogen, phosphorus or oxygen which are all vital for the bacterial growth. Excess nutrients are stored intracellularly by microorganisms by the generation of insoluble biopolymers from soluble molecules. The biopolymers stored can be released once normal growth conditions are restored [58, 59]. PHA intracellular biosynthesis in *Ralstonia Eutropha* cells interacts with a limited number of feedstocks having carbon chain lengths of C3 to C5. Nonetheless, the microorganism has an inclination towards C4 substrates. Consequently, PHAs formed through this pathway comprise of short-length chain monomers [60]. When there is a deficiency of oxygen in the medium, the microorganism has the capacity to thrive anaerobically while utilizing nitrogen (denitrification) as the source of energy. The chemolithoautotrophic potential of the bacteria while being cultivated on hydrogen can evolve as a cell factory for the development of various materials including value-added products notably, polymers and metabolites [33, 35, 56]. The metabolic processes of *Cupriavidus Necator* is highly appropriate to microorganisms with transitory absence of oxygen and to diverse type of carbon substrates. The respiratory cycle therefore adapts to the substrate and the

concentration of the carbon sources [61]. The molecular weight of P3HB extracted from Ralstonia Eutropha is in the range of 939,000 to 1,400,000 g/mol with a polydispersity varying between 1.9 and 2.25 while the molecular weight of PHA extracted from other microorganisms such as the gram-negative *Pseudomonas oleovorans* ranges between 178,000 and 330,000 g/mol (polydispersity: 1.8–2.4) and gram-negative *Pseudomonas putida* fluctuates between 56,000 and 112,000 g/mol along with a polydispersity of 1.6–2.3 [62]. Table III gives an insight of the characteristics and the applications of *Ralstonia Eutropha* in the biomaterial sector.

TABLE III: ATTRIBUTES OF *RALSTONIA EUTROPHA*

Attributes	Functions in biomaterials	References
Autotrophic growth	Exploitation of CO_2 for manufacture of biomaterials	[63, 64]
Use of a variety of carbon substrates	Cheap substrates from lignocellulosic materials, crop residues, low-quality waste animal fats or agricultural residues can be employed as carbon sources	[64–66]
Biocompatibility and non-pathogenic	-Applications in pharmaceutical, surgical, therapeutic and tissue engineering functions -Production of medical equipment and implants	[63, 67]
Resistivity to toxic compounds	Ability to generate biopolymers from toxic mixtures such as syngas and from phenol	[64, 68]
Intracellular storage compound	Stores PHB intracellularly and result in high PHB yields with good purity	[69, 70]
Genetically modifiable	Construction of *Ralstonia Eutropha* strains to develop bio-based materials	[64, 65, 71]
Modifiable biopolymer properties	Synthesis pathway can be improved by substituting the PHA synthase gene to develop diverse biopolymers with medium or long length chains	[64, 71]

III. MATERIALS AND METHODS

A. Cultivation Media

R. Eutropha was initially cultivated in shake flask in minimal salt of composition in Table IV. A loop inoculated with cells from a 24-hour old agar slant was transferred to 50ml of M9 minimal salt in a 250 ml Erlenmeyer flask. The flask was initially incubated on a rotary shaker at 30 °C and 200 rpm for 18 h following which, 1ml of the culture was transferred in a glass vial and vortexed. Serial dilution and plating were performed on the microbial culture to determine

the mean number of viable colonies in the inoculum following which shake flasks were inoculated with 8×10^8 CFU/ml inoculum (2%). Table IV presents the composition of the seed culture medium, which is a vital component in the bioprocess for cultivating microorganisms before initiating the main fermentation. This medium contains a precisely balanced blend of nutrients and substrates that provide an ideal environment for the growth and multiplication of the selected microbial strain.

TABLE IV: COMPOSITION OF THE SEED CULTURE MEDIUM

Component	Concentration (gL^{-1})
Disodium Phosphate (anhydrous), Na_2HPO_4	33.9
Monopotassium Phosphate, KH_2PO_4	15.0
Sodium Chloride, $NaCl$	2.5
Ammonium Chloride, NH_4Cl	5.0
Glucose	4.0
Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$	0.492
Calcium chloride, $CaCl_2 \cdot 2H_2O$	0.0147

B. Overall Operation Procedure for PHB Production

Fig. 2 illustrates the overall operation procedure for PHB production. This comprehensive process involves several key steps, including substrate preparation, seed culture inoculation, fermentation, biomass harvesting, and PHB extraction. Each step plays a crucial role in achieving efficient PHB production and is carefully designed to optimize the yield and quality of the biopolymer.
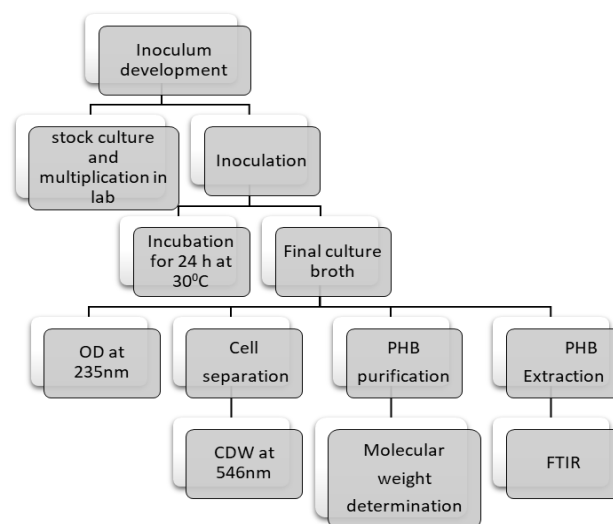

Fig. 2. Overall operation protocol.

C. Quantitative and Qualitative Analysis—Cell Biomass determination of Ralstonia Eutropha Culture

The Cell growth monitoring was achieved via turbidity measurement and the dry cell weight of the biomass pellet.

1) Turbidity measurement

During the course of the fermentation, the Optical Density (OD) of the culture media at 546 nm was measured every 30 mins via a DR 3900 spectrophotometer. OD was plotted against the cultivation time to determine and control proper bacterial growth. Fig. 3 illustrates a schematic diagram for

optical density reading, essential for turbidity measurement during fermentation. Sufficient growth was observed when the desired OD_{546} value [72] was obtained and hence, the inoculum was transferred to the next phase. OD being the logarithm of the transmitted and received light can be calculated as follows:

$$OD = -\log 10 \frac{I}{I_0} \quad (1)$$

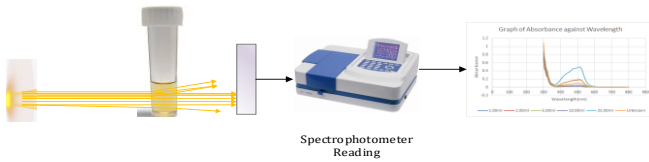where, I is the transmitted light intensity and I_o is the incident light intensity.

Fig. 3. Schematic diagram for optical density reading.

2) Cell Dry Weight (CDW)

The Ralstonia Eutropha cell biomass concentration was determined by its dry cell weight based and adapted on [73] as follows:

i. 3×3ml of culture broth was centrifuged at $3000 \times g$ for 15 mins in pre-weighed glass sterile Eppendorf tubes.

ii. The supernatant was discarded and the biomass pellet was carefully recovered.

iii. The pellet was re-suspended in distilled water and centrifuged again at $3000 \times g$ for 15 mins.

i.   The pellets were then washed in NaCl concentration of 0.98% (w/w) followed by lyophilization for 24 hours.

ii. The cell dry weight was determined as follows:

$$CDW \left(\frac{g}{L}\right) = \left(m_{eppendorf+pellet} - m_{eppendorf}\right) \times 1000 \quad (2)$$

where, $m_{eppendorf+pellet}$ is the combined mass of the Eppendorf and the biomass pellet

3) Cell mass calibration curve

A standard calibration curve was prepared for the estimation of cell mass via spectrophotometry to relate the *Ralstonia Eutropha* cell concentration to its optical density as follows:

i.   six solutions of known concentrations were prepared with cell biomass from a fresh seed culture of *Ralstonia Eutropha*.

ii. The optical densities of each solution were measured by a UV spectrophotometer at the end of 48 h.

iii. The solutions were then centrifugated at $3000 \times g$ for 15 minutes.

iv. The pellets were then washed in NaCl concentration of 0.98% (w/w) followed by lyophilization for 24 hours and their cell dry weights (CDW) were measured.

v.   The calibration curve was established by plotting the optical density against the cell concentration.

IV. RESULTS AND DISCUSSION

A. Colony Characteristics

The colonial characteristics of the bacterial strain was assessed as per Table V:

TABLE V: CHARACTERISTICS OF *RALSTONIA EUTROPHA*

Colony	*Ralstonia Eutropha*	
Colour of bacterial colonies	White	
Reaction to gram-stain	Pink stain: Gram-Negative	
Shape	Rod-shaped	
Motility	Yes	
Opacity	Opaque	
Growth conditions	Aerobes and anaerobes at an optimal temperature of 30 ºC	
Spore formation	No	
Flagellation	Beef extract and peptone medium: Flagellation observed	M9 minimal salt (containing nitrogen): Loss in flagellation

B. Biopolymer Characterization

1) Cell Dry Weight (CDW)

Fig. 4 shows the linear correlation, with R2 of 99.5% between the cell dry weight of *Ralstonia Eutropha* over period of 48 h. It can be observed that the CDW of the cell biomass increased linearly with an increase in residence time. After the 48 h period, a constant CDW was observed.
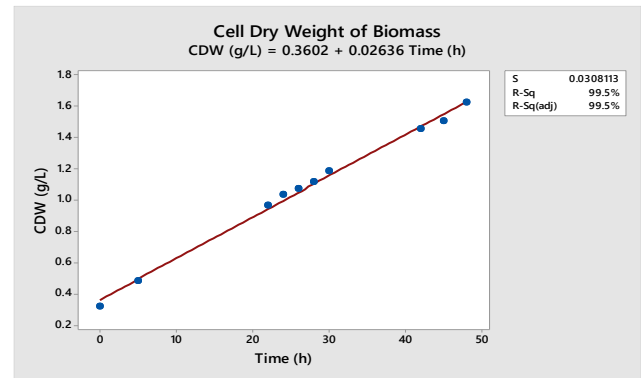

Fig. 4. Chart of cell dry weight of biomass vs time.

2) Cell mass calibration curve

Turbidity measurement by spectrophotometry is a quick and very helpful way to estimate cell counts. A suspension of bacterial cells appears turbid to the naked eye since cells disperse light crossing through the solution. The higher the quantity of cells in the solution, the higher is the extent of cell scattering in the solution resulting in a cloudier suspension. In view of the fact that bacterial cells are fundamentally of uniform size in a population, the extent of dispersion is directly related to the cell biomass and indirectly proportionate to the cell number. Nevertheless, light dispersed from a photocell in a high cell biomass density solution has a high probability of dispersing back to another cell. Thus, the light transmitted back gets unregistered by the photocell. The correlation between the cell number and the turbidity does not follow a linear relationship at high cell

biomass concentrations. Fig. 5 confirms a linear relationship (R^2 of 99.4%) between the six known solutions of cell concentration and their turbidity measured by the optical density at 546nm. Higher cell concentration solutions were revealed out of the linearity range and hence, all subsequent measures were conducted within this range.
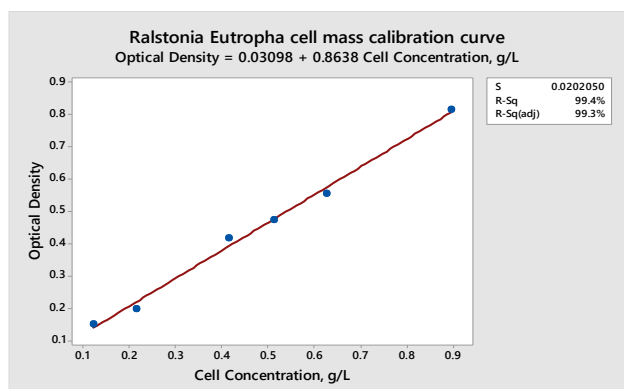

Fig. 5. *Ralstonia Eutropha* cell mass calibration curve.

3) PHB yield

A mean PHB yield of 17.01±0.35% was observed when biomass and PHB were extracted from 3 ml of inoculum of age, 48h as shown in Table VI. When compared to previous studies conducted on *R.Eutropha* from other LCM, the PHB yield is significantly lower than reported optimal yields of 37.55 to 97.80%. However, the observed yield was higher than that observed by microorganism hosts such as *Bacillus Megaterium* and *Saccharomyces cerevisiae* which resulted in a maximum PHB yield of 4.06–19.51% and 2.4–16.4% correspondingly. In view of maximising the yields of PHB recovered from *R.Eutropha*, experiments with varying factors need to be run to determine optimal conditions.

TABLE VI: PHB YIELD

SN	CDW (g/L)	PHB weight (g)	% PHB	Mean PHB Yield (%)	Error
1	1.52	0.26221	17.25		
2	1.60	0.27485	17.18	17.01	0.35
3	1.73	0.28740	16.61		

4) Fourier transfer infrared spectroscopy

The characterization of the PHB was achieved through FTIR analysis. Fig. 6 displays the IR spectrum of the extracted PHB sample with the characteristic peaks produced.
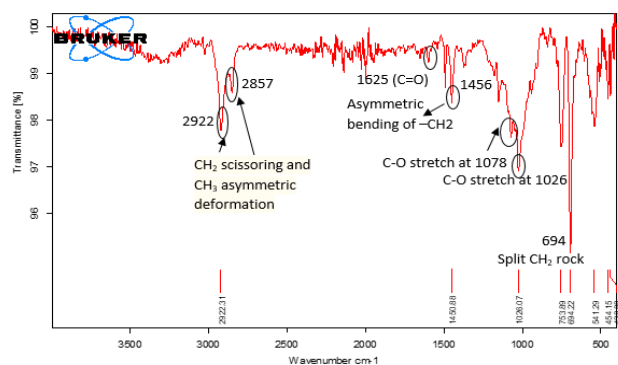

Fig. 6. Transmittance spectrum of PHB sample from 1% reducing sugar.

IR spectrum of the different functional groups in the PHB sample was registered in the range of 400 to 4000 cm^{-1}. Characteristics bands for carbonyl, methine and ester groups could be observed along with intermolecular hydrogen bonds. Bands were detected at 1450.88 and 2922.31 which corresponds to methine grouping such as CH_2 scissoring and CH_3 asymmetric deformation. These values were slightly higher than usual which may be attributed to polymerization. Also, the frequency of carbonyl group at 1625 was lower than the 1673 usually obtained from standard PHB which again, might be due to polymerization. The C-O group revealed a band at 1078 which is more or less close to the frequency value of 1076 observed with standard PHB. Hence, the peaks confirm the presence of PHB in the extracted sample.

V. CONCLUSION

The aim behind this project was to investigate the potential of extracting reducing sugar from locally available lignocellulosic biomass to determine their viability to be used as a substitute bioresource for the production of biopolymers. The main technique involved the hydrolysis of cellulose and hemicellulose into smaller monomer untis that could be employed by microorganisms as carbon source for biosynthesis of polymers. *Ralstonia Eutropha* determined to be gram-negative and rod shaped under the microscope was successfully cultured at 30 ℃ in different mediums notably, M9 minimal salt, nutrient agar along with beef and yeast extract broth. The number of viable colonies in 1ml culture was determined to be 8×10^8 CFU/ml by serial dilution and plating. Batch fermentation of PHB in submerged cultivation conducted for a residence time of 48h resulted in a dry cell weight growing from 0.32±0.05% to 1.62±0.05%. Also, a correlation was found between the turbidity and the quantity of cell biomass where the extent of dispersion was directly related to the cell biomass and indirectly proportionate to the cell number. After 48 hours, the cells appeared to have attained the nitrogen limiting phase and 17.05±0.35% of PHB was extracted when 1% of hydrolysate containing the reducing sugar was fed to the microorganisms as carbon source. The PHB yield was dramatically lower than reported optimal yields of 37.55 to 97.80% from literatures. Nonetheless, FTIR spectroscopy revealed characteristics bands for carbonyl, methine and ester groups along with interamolecular hydrogen bonds in the biopolymer. Sudan Black B and FTIR spectrum demonstrates that PHB biosynthesis successfully bioaccumulates inside the cells of *Ralstonia Eutropha* using cellulose from LCB as carbon source. Hence, the process needs to be optimized in terms of variables such as inoculum size, inoculum concentration, incubation time and salt medium conditions in order to maximise the production of PHB from *Furcraea Foetida* in *Ralstonia Eutropha* cultivation.

COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

N. Jaffur conducted the lab work, while P. Jeetah and G. Kumar provided their expert supervision and guidance throughout the research process. All authors had approved the final version.

REFERENCES

[1] Statista, "Statista—The statistics portal for market data, market research and market studies," *Statista*, 2019.

[2] F. Alshehrei, "Production of polyhydroxybutyrate (PHB) by bacteria isolated from soil of Saudi Arabia," *J. Pure Appl. Microbiol.*, vol. 13, no. 2, 2019. doi: 10.22207/JPAM.13.2.26

[3] Z. A. Raza, S. Riaz, and I. M. Banat, "Polyhydroxyalkanoates: Properties and chemical modification approaches for their functionalization," *Biotechnology Progress*, vol. 34, no. 1. 2018. doi: 10.1002/btpr.2565

[4] R. Rai and I. Roy, "Polyhydroxyalkanoates: The emerging new green polymers of choice," *RSC Green Chem.*, 2011.

[5] Y. Wang and G.-Q. Chen, "Polyhydroxyalkanoates: Sustainability, production, and industrialization," in *Sustain. Polym. from Biomass*, 2017. doi: 10.1002/9783527340200.ch2

[6] A. A. Amadu *et al.*, "A review of biopolymer (Poly-β-hydroxybutyrate) synthesis in microbes cultivated on wastewater," *Science of the Total Environment*, 2021. doi: 10.1016/j.scitotenv.2020.143729

[7] R. R. Dalsasso, F. A. Pavan, S. E. Bordignon, G. M. F. de Aragão, and P. Poletto, "Polyhydroxybutyrate (PHB) production by Cupriavidus necator from sugarcane vinasse and molasses as mixed substrate," *Process Biochem.*, 2019. doi: 10.1016/j.procbio.2019.07.007

[8] J. M. G. Alcântara, F. Distante, G. Storti, D. Moscatelli, M. Morbidelli, and M. Sponchioni, "Current trends in the production of biodegradable bioplastics: The case of polyhydroxyalkanoates," *Biotechnol. Adv.*, 2020. doi: 10.1016/j.biotechadv.2020.107582

[9] C. Rigouin *et al.*, "Production and characterization of two medium-chain-length polydroxyalkanoates by engineered strains of Yarrowia lipolytica," *Microb. Cell Fact.*, 2019. doi: 10.1186/s12934-019-1140-y

[10] M. Koller, "Advances in polyhydroxyalkanoate (PHA) production," *Bioengineering*, 2017. doi: 10.3390/bioengineering4040088

[11] G. Licciardello, A. F. Catara, and V. Catara, "Production of polyhydroxyalkanoates and extracellular products using Pseudomonas corrugata and P. mediterranea: A review," *Bioengineering*, 2019. doi: 10.3390/bioengineering6040105

[12] N. I. López, M. J. Pettinari, P. I. Nikel, and B. S. Méndez, "Polyhydroxyalkanoates: Much more than biodegradable plastics," *Adv. Appl. Microbiol.*, 2015. doi: 10.1016/bs.aambs.2015.06.001

[13] C. Kourmentza *et al.*, "Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production," *Bioengineering*, 2017. doi: 10.3390/bioengineering4020055

[14] T. Suet *et al.*, *Biotechnological Applications of Polyhydroxyalkanoates. Applications of PHA in Agriculture*, 2019.

[15] S. Ray and V. C. Kalia, "Biomedical applications of polyhydroxyalkanoates," *Indian J. Microbiol.*, 2017. doi: 10.1007/s12088-017-0651-7

[16] M. B. Coltelli *et al.*, "Skin-compatible biobased beauty masks prepared by extrusion," *J. Funct. Biomater.*, 2020. doi: 10.3390/jfb11020023

[17] S. Obruca, P. Benesova, L. Marsalek, and I. Marova, "Use of lignocellulosic materials for PHA production," *Chem. Biochem. Eng. Q.*, 2015. doi: 10.15255/CABEQ.2014.2253

[18] J. Możejko-Ciesielska and R. Kiewisz, "Bacterial polyhydroxyalkanoates: Still fabulous?" *Microbiol. Res.*, 2016. doi: 10.1016/j.micres.2016.07.010

[19] E. Bugnicourt, P. Cinelli, A. Lazzeri, and V. Alvarez, "Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging," *Express Polym. Lett.*, 2014. doi: 10.3144/expresspolymlett.2014.82

[20] M. Koller, "Advances in polyhydroxyalkanoate (PHA) production, volume 2," *Bioengineering*, vol. 7, no. 1, 2020. doi: 10.3390/bioengineering7010024

[21] G. Keskin, G. Klzll, M. Bechelany, C. Pochat-Bohatier, and M. Öner, "Potential of polyhydroxyalkanoate (PHA) polymers family as substitutes of petroleum based polymers for packaging applications and solutions brought by their composites to form barrier materials," *Pure Appl. Chem.*, 2017. doi: 10.1515/pac-2017-0401

[22] G.-Q. Chen, X.-Y. Chen, F.-Q. Wu, and J.-C. Chen, "Polyhydroxyalkanoates (PHA) toward cost competitiveness and functionality," *Adv. Ind. Eng. Polym. Res.*, 2020. doi: 10.1016/j.aiepr.2019.11.001

[23] S. Wang, W. Chen, H. Xiang, J. Yang, Z. Zhou, and M. Zhu, "Modification and potential application of short-chain-length polyhydroxyalkanoate (SCL-PHA)," *Polymers (Basel).*, vol. 8, no. 8, 2016. doi: 10.3390/polym8080273

[24] M. Li and M. R. Wilkins, "Recent advances in polyhydroxyalkanoate production: Feedstocks, strains and process developments," *Int. J. Biol. Macromol.*, 2020. doi: 10.1016/j.ijbiomac.2020.04.082

[25] Z. Liu *et al.*, "Optimization of polyhydroxybutyrate (PHB) production by excess activated sludge and microbial community analysis," *J. Hazard. Mater.*, 2011. doi: 10.1016/j.jhazmat.2010.08.003

[26] K. W. Meereboer, M. Misra, and A. K. Mohanty, "Review of recent advances in the biodegradability of polyhydroxyalkanoate (PHA) bioplastics and their composites," *Green Chem.*, 2020. doi: 10.1039/d0gc01647k

[27] J. R. G. Cardozo, A. L. M. Martínez, M. Y. Pérez, and G. A. C. Londoño, "Production and characterization of polyhydroxyalkanoates and native microorganisms synthesized from fatty waste," *Int. J. Polym. Sci.*, 2016. doi: 10.1155/2016/6541718

[28] U. J. Hänggi, "Requirements on bacterial polyesters as future substitute for conventional plastics for consumer goods," *FEMS Microbiol Rev*, 1995. doi: 10.1111/j.1574-6976.1995.tb00167.x

[29] Y. K. Leong, P. L. Show, C. W. Ooi, T. C. Ling, and J. C. W. Lan, "Current trends in polyhydroxyalkanoates (PHAs) biosynthesis: Insights from the recombinant Escherichia coli," *J. Biotechnol.*, 2014. doi: 10.1016/j.jbiotec.2014.03.020

[30] B. S. Saharan, A. Grewal, and P. Kumar, "Biotechnological production of polyhydroxyalkanoates: A review on trends and latest developments," *Chinese J. Biol.*, 2014. doi: 10.1155/2014/802984

[31] M. Markets, "Polyhydroxyalkanoate (PHA) market," *Marketsandmarkets*, 2019.

[32] P. Bhaumik and P. L. Dhepe, "Conversion of biomass into sugars," *RSC Green Chem.*, 2016. doi: 10.1039/9781782622079-00001

[33] S. K. Bhatia *et al.*, "Bioconversion of plant biomass hydrolysate into bioplastic (polyhydroxyalkanoates) using Ralstonia eutropha 5119," *Bioresour. Technol.*, 2019. doi: 10.1016/j.biortech.2018.09.122

[34] P. Khadoo-Jeetah and R. Mohee, "Bio-ethanol production from readily available lignocellulosic biomass in mauritius through enzymatic hydrolysis," *Clim. Chang. Manag.*, 2013. doi: 10.1007/978-3-642-37753-2_43

[35] N. Annamalai and N. Sivakumar, "Production of polyhydroxybutyrate from wheat bran hydrolysate using Ralstonia eutropha through microbial fermentation," *J. Biotechnol.*, 2016. doi: 10.1016/j.jbiotec.2016.09.001

[36] T. Lopez-Arenas, M. González-Contreras, O. Anaya-Reza, and M. Sales-Cruz, "Analysis of the fermentation strategy and its impact on the economics of the production process of PHB (polyhydroxybutyrate)," *Comput Chem Eng*, vol. 107, pp. 140–150, 2017. doi: 10.1016/j.compchemeng.2017.03.009

[37] H. Arikawa, K. Matsumoto, and T. Fujiki, "Polyhydroxyalkanoate production from sucrose by Cupriavidus necator strains harboring csc genes from Escherichia coli W," *Appl. Microbiol. Biotechnol.*, 2017. doi: 10.1007/s00253-017-8470-7

[38] D. Dasgupta, S. Bandhu, D. K. Adhikari, and D. Ghosh, "Challenges and prospects of xylitol production with whole cell bio-catalysis: A review," *Microbiol. Res.*, vol. 197, 2017. doi: 10.1016/j.micres.2016.12.012

[39] S. Kwak and Y. S. Jin, "Production of fuels and chemicals from xylose by engineered Saccharomyces cerevisiae: A review and perspective," *Microb. Cell Fact.*, vol. 16, no. 1, 2017. doi: 10.1186/s12934-017-0694-9

[40] X. Hu, Y. Shi, P. Zhang, M. Miao, T. Zhang, and B. Jiang, "d-Mannose: Properties, production, and applications: An overview," *Compr. Rev. Food Sci. Food Saf.*, vol. 15, no. 4, 2016. doi: 10.1111/1541-4337.12211

[41] Z. Wei, L. Huang, L. Cui, and X. Zhu, "Mannose: Good player and assister in pharmacotherapy," *Biomed. Pharmacother.*, vol. 129, 2020. doi: 10.1016/j.biopha.2020.110420

[42] A. Madhavan, A. Srivastava, A. Kondo, and V. S. Bisaria, "Bioconversion of lignocellulose-derived sugars to ethanol by engineered Saccharomyces cerevisiae," *Crit. Rev. Biotechnol.*, vol. 32, no. 1, 2012. doi: 10.3109/07388551.2010.539551

[43] C. G. Vargas-rechia *et al.*, "A profile of the South African table grape market value chain," *Bioresour. Technol.*, 2015.

[44] A. C. Meyer-Gerspach, B. Wölnerhanssen, and C. Beglinger, "Functional roles of low calorie sweeteners on gut function," *Physiol. Behav.*, vol. 164, 2016. doi: 10.1016/j.physbeh.2016.01.045

[45] E. E. Anthonia and H. S. Philip, "An overview of the applications of furfural and its derivatives," *Int. J. Adv. Chem.*, vol. 3, no. 2, 2015. doi: 10.14419/ijac.v3i2.5048

[46] P. Rachamontree, T. Douzou, K. Cheenkachorn, M. Sriariyanun, and K. Rattanaporn, "Furfural: A sustainable platform chemical and fuel," *Appl. Sci. Eng. Prog.*, vol. 13, no. 1, 2020. doi: 10.14416/j.asep.2020.01.003

[47] S. J. Hong, M. H. Ahn, J. Sangshetti, and R. B. Arote, "Sugar alcohol-based polymeric gene carriers: Synthesis, properties and gene therapy applications," *Acta Biomater.*, vol. 97, 2019. doi: 10.1016/j.actbio.2019.07.029

[48] J. H. Lim, S. H. Song, H. S. Park, J. R. Lee, and S. M. Lee, "Spontaneous detachment of Streptococcus mutans biofilm by synergistic effect between zwitterion and sugar alcohol," *Sci. Rep.*, vol. 7, no. 1, 2017. doi: 10.1038/s41598-017-08558-x

[49] R. Yao, W. Hou, and J. Bao, "Complete oxidative conversion of lignocellulose derived non-glucose sugars to sugar acids by Gluconobacter oxydans," *Bioresour. Technol.*, vol. 244, 2017. doi: 10.1016/j.biortech.2017.08.078

[50] P. Cinelli, M. B. Coltelli, F. Signori, P. Morganti, and A. Lazzeri, "Cosmetic packaging to save the environment: Future perspectives," *Cosmetics*, 2019. doi: 10.3390/COSMETICS6020026

[51] E. Tomás-Pejó, P. Alvira, M. Ballesteros, and M. J. Negro, "Pretreatment technologies for lignocellulose-to-bioethanol conversion," *Biofuels*, 2011. doi: 10.1016/B978-0-12-385099-7.00007-3

[52] Q. Qi and Q. Liang, "Single-cell biorefinery," *Ind. Biorefineries White Biotechnol.*, 2015. doi: 10.1016/B978-0-444-63453-5.00011-2

[53] K. Kucharska, P. Rybarczyk, I. Hołowacz, M. Ł. R. Glinka, and M. Kamiński, "Pretreatment of lignocellulosic materials as substrates for fermentation processes," *Molecules*, 2018. doi: 10.3390/molecules23112937

[54] G. F. Y. Juarez, K. B. C. Pabiloña, K. B. L. Manlangit, and A. W. Go, "Direct dilute acid hydrolysis of spent coffee grounds: A new approach in sugar and lipid recovery," *Waste Biomass Valorization*, vol. 9, no. 2, 2018. doi: 10.1007/s12649-016-9813-9

[55] T. Jooste, M. P. García-Aparicio, M. Brienzo, W. H. Van Zyl, and J. F. Görgens, "Enzymatic hydrolysis of spent coffee ground," *Appl. Biochem. Biotechnol.*, vol. 169, no. 8, 2013. doi: 10.1007/s12010-013-0134-1

[56] M. Xu, P. L. Tremblay, L. Jiang, and T. Zhang, "Stimulating bioplastic production with light energy by coupling: Ralstonia eutropha with the photocatalyst graphitic carbon nitride," *Green Chem.*, vol. 21, no. 9, 2019. doi: 10.1039/c8gc03695k

[57] D. C. Meng *et al.*, "Production of poly(3-hydroxypropionate) and poly(3-hydroxybutyrate-co-3-hydroxypropionate) from glucose by engineering Escherichia coli," *Metab. Eng.*, 2015. doi: 10.1016/j.ymben.2015.03.015

[58] H. E. Valentin and A. Steinbüchel, "Accumulation of poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid-co-4-hydroxyvaleric acid) by mutants and recombinant strains of Alcaligenes eutrophus," *J. Environ. Polym. Degrad.*, 1995. doi: 10.1007/BF02068468

[59] P. Suriyamongkol, R. Weselake, S. Narine, M. Moloney, and S. Shah, "Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants—A review," *Biotechnol. Adv.*, 2007. doi: 10.1016/j.biotechadv.2006.11.007

[60] A. Steinbüchel and H. G. Schlegel, "Physiology and molecular genetics of poly(β-hydroxyalkanoic acid) synthesis in Alcaligenes eutrophus," *Molecular Microbiology*, 1991. doi: 10.1111/j.1365-2958.1991.tb00725.x

[61] R. Cramm, "Genomic view of energy metabolism in Ralstonia eutropha H16," *J. Mol. Microbiol. Biotechnol.* 2008. doi: 10.1159/000142893

[62] Y. G. García, J. C. M. Contsrerass, O. G. Reynoso, and J. A. C. López, "Synthesis and degradation of polyhydroxyalkanoates: Plastic microbial | Síntesis y biodegradación de polihidroxialcanoatos: Plásticos de origen microbiano," *Rev. Int. Contam. Ambient.*, 2013.

[63] M. Sandhya, J. Aravind, and P. Kanmani, "Production of polyhydroxyalkanoates from Ralstonia eutropha using paddy straw as cheap substrate," *Int. J. Environ. Sci. Technol.*, vol. 10, no. 1, 2013, doi: 10.1007/s13762-012-0070-6.

[64] S. Priyadarshi, A. Shukla, and B. B. Borse, "Polyhydroxyalkanoates: Role of Ralstonia eutropha," *International Journal of Biomedical and Advance Research*, 2014. doi: 10.7439/ijbar.v5i2.639

[65] C. J. Brigham and S. L. Riedel, "The potential of polyhydroxyalkanoate production from food wastes," *Appl. Food Biotechnol.*, vol. 6, no. 1, 2019. doi: 10.22037/afb.v6i1.22542

[66] S. L. Riedel *et al.*, "Polyhydroxyalkanoates production with Ralstonia eutropha from low quality waste animal fats," *J Biotechnol*, 2015, doi: 10.1016/j.jbiotec.2015.09.002

[67] M. Koller, "Biodegradable and biocompatible polyhydroxy-alkanoates (PHA): Auspicious microbial macromolecules for pharmaceutical and therapeutic applications," *Molecules*, 2018. doi: 10.3390/molecules23020362

[68] R. A. J. Verlinden, D. J. Hill, M. A. Kenward, C. D. Williams, and I. Radecka, "Bacterial synthesis of biodegradable polyhydroxyalkanoates," *J. Appl. Microbiol.*, 2007. doi: 10.1111/j.1365-2672.2007.03335.x

[69] M. Raberg, B. Voigt, M. Hecker, and A. Steinbüchel, "A closer look on the polyhydroxybutyrate-(PHB-) negative phenotype of Ralstonia eutropha PHB-4," *PLoS One*, 2014, doi: 10.1371/journal.pone.0095907

[70] J. Eggers and A. Steinbüchel, "Impact of Ralstonia eutropha's poly(3-Hydroxybutyrate) (PHB) depolymerases and phasins on PHB storage in recombinant Escherichia coli," *Appl. Environ. Microbiol.*, 2014. doi: 10.1128/AEM.02666-14

[71] N. Lindenkamp, E. Volodina, and A. Steinbüchel, "Genetically modified strains of Ralstonia eutropha H16 with β-ketothiolase gene deletions for production of copolyesters with defined 3-hydroxyvaleric acid contents," *Appl Environ Microbiol*, 2012. doi: 10.1128/AEM.00824-12

[72] C. Laemmli, C. Werlen, and J. R. V. D. Meer, "Mutation analysis of the different tfd genes for degradation of chloroaromatic compounds in Ralstonia eutropha JMP134," *Arch. Microbiol.*, 2004. doi: 10.1007/s00203-003-0634-4

[73] J. Juengert, S. Bresan, and D. Jendrossek, "Determination of Polyhydroxybutyrate (PHB) content in Ralstonia eutropha using gas chromatography and Nile red staining," *Bio Protoc*, 2018. doi: 10.21769/bioprotoc.2748