INTERNATIONAL JOURNAL OF RESEARCH AND INNOVATION IN APPLIED SCIENCE (IJRIAS)
ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume X Issue VII July 2025

Bacterial PHB Synthesis Using Agricultural Waste For Bioplastics

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DOI: https://doi.org/10.51584/IJRIAS.2025.100800017

Received: 24 July 2025; Accepted: 30 July 2025; Published: 29 August 2025

ABSTRACT

Polyhydroxybutyrate (PHB) is a biodegradable and renewable biopolymer with the potential for sustainable plastic production. This study explores the utilization of agricultural waste streams as feedstock for PHB synthesis through bacterial fermentation, addressing both plastic pollution and agricultural waste valorization. Approximately 90 million tons of oil-equivalent agricultural waste remain underutilized, presenting a viable source for biopolymer production. PHB accumulation was optimal with glucose as a carbon source and peptone as a nitrogen source. Pretreated sugarcane bagasse, maize cob, teff straw, and banana peel also supported PHB biosynthesis. Targeting specific bacterial metabolic pathways enhances waste-to-PHB conversion, contributing to economic and environmental sustainability. Fourier Transform Infrared Spectroscopy (FTIR) confirmed the presence of C–H, CH_2, C=O, and C–O functional groups. Biodegradability assessment and UV-Vis spectrophotometric analysis validated PHB's potential for bioplastic applications. This study underscores the role of agricultural waste in sustainable polymer production, aligning with global environmental and economic goals.

Keywords: Polyhydroxybutyrate, biodegradable, polymer, agricultural waste, carbon source.

GRAPHICAL ABSTRACT

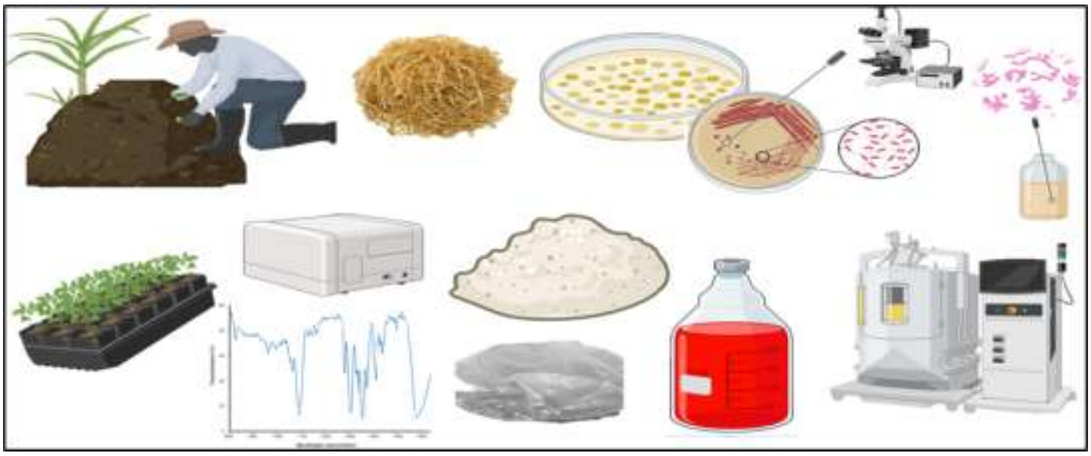

Fig 1. Graphical Abstract of PHB Synthesis and Characterization

Introduction

Plastic materials originating from mega-sized petrochemical industries cause serious environmental problems due to their non-biodegradable nature. Industrial plastics production plays a crucial role in modernizing our daily lives (Getachew et al., 2016). Plastic, despite being a necessary substance for modern life, frequently poses concerns to human health and the environment. Since plastics are an essential component of contemporary society, they often carry risks to both human health and the environment (Canfield S, et al. 2013). Some of the toxic substances and chemicals found in plastics include bisphenol A (BPA), phthalates, antioxidants, brominated flame retardants, and polyfluorinated chemicals. Despite being a necessary substance for modern life, plastic

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frequently poses concerns to both human health and the environment (Ragni & Kumari, Ragni. 2023). However, due to the enormous accumulation of plastic waste materials in the environment, the accumulation of these materials exceeds the biosphere tolerance limit. Hence, the need of the times is to minimize and replace environmentally hazardous petroleum-based plastic substances with eco-friendly biodegradable plastics (Fuller RC et al, 1990). The occurrence of totally biodegradable plastics provides the best solution to protect the environment from hazards caused by conventional petroleum-based plastics (Christopher et al., 2019). Biodegradable plastics refer to plastics that are broken down by natural biological processes into carbon dioxide, water, biomass, and other minerals on their return to the ecosystem and thus do not cause environmental pollution. Among them, polyhydroxyalkanoate (PHAs) have been rapidly developed as natural biodegradable plastics for over three decades by industrial biotechnologists. PHAs have several advantages over these conventionally synthesized plastics: they are biobased, biocompatible, and biodegradable polymers (Sinskey AJ et al, 2012). PHAs are also inclusion bodies accumulated as reserve material inside the bacterial cell. PHAs are typically made in microbial cultures, which frequently use carbon-rich waste streams as feedstocks. At pilot scales or greater, polymers with different monomer compositions have been created to provide bioplastic raw materials for a range of applications, including packaging, cosmetics, medicinal, industrial, agricultural, and home. PHAs possess polymer properties like various synthetic thermoplastics like polypropylene (Christopher et al., 2019; Sridevi N et al., 2011).

PHA

Polyhydroxyalkanoates (PHAs) are linear polyesters formed naturally through bacterial fermentation of sugars or lipids. This family of polymers contains about 150 monomers that can be combined to create materials with a variety of useful qualities (Raposo et al,2014; Li Z et al., 2016). These biodegradable plastics are utilized in the manufacture of bioplastics. They can be thermoplastic or elastomeric materials, with melting temperatures ranging from 40 to 180 °C (Cataldi, P., July 2020). Carbon and energy-storing compounds are synthesized by a variety of microbial bacteria under unbalanced feeding conditions (Lahtinen, Jussi, et al., 2022). Polyhydroxyalkanoates (PHAs) are biologically synthesized polymers that store carbon. Microorganisms create PHAs as a response to stress. Circumstances and protect against nutritional deprivation. in severe settings (McAdam et al, 2020). Polyhydroxyalkanoate (PHA), a plastic that degrades over time made by microorganisms identified by Lemoigne in 1925 (Pandey et al., 2018). Blending, changing the surface, or blending PHA with other polymer chains, enzymes, and inorganic materials can vary its mechanical and biocompatibility properties, allowing for a wide range of applications (Cataldi, P., July 2020).

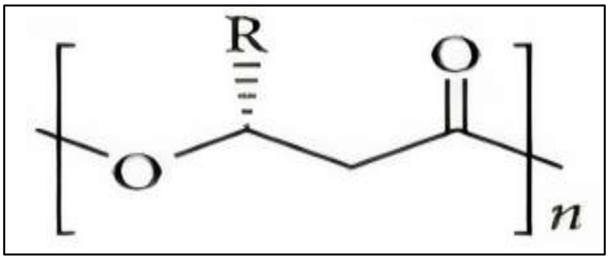

Fig 2. Chemical structure of Polyhydroxyalkanoates (PHA) molecule (Lahtinen, Jussi, et al., 2022)

PHB

In 1925, Maurice Lemoigne discovered phb, which is an intracellular inclusion in bacteria (Raposo et al.,2014). Polyhydroxybutyrate (PHB) is a type of polyhydroxyalkanoate (PHA), which is a polymer within the polyester group known for being environmentally friendly and biodegradable (Aramvash A et al., 2015). The most widely seen form of PHB is poly-3-hydroxybutyrate (P3HB), although other variations like poly-4-hydroxybutyrate (P4HB),

polyhydroxy valerate (PHV), polyhydroxyalkanoate (PHH), and polyhydroxyalkanoate (PHO) are also produced by various organisms along with their copolymers (Ravindra R et al., 2014). Polyhydroxybutyrate polymer is a polyester that is created on an industrial scale by the activity of Lactobacillus acidophilus or another biotechnological technique (Pandey et al., 2018).

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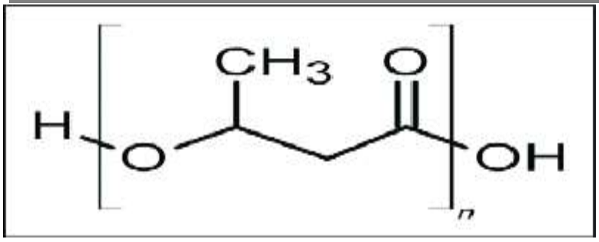

Fig 3. Chemical structure of Polyhydroxybutyrate (PHB) molecule (Lahtinen, Jussi et al., 2022)

The mid-1970s petroleum crisis sparked fresh interest in developing alternatives to petroleum-based products (Gross R.A et al, 2002). Following that, the rise of molecular genetics and RDT fueled research, and by the turn of the twenty-first century, the structures, methods of manufacture, and uses for a wide range of bioplastics had become established (Fridovich-Keil, J. L, 2024). PHB and polyhydroxyalkanoate (PHA), both of which are synthesized by specialized microbes, as well as polylactic acid (PLA), which is polymerized from monomers of lactic acid, produced by microbial fermentation derived from plant sugars and starches, were among the bioplastics in use or being studied (Britannica, 2024).

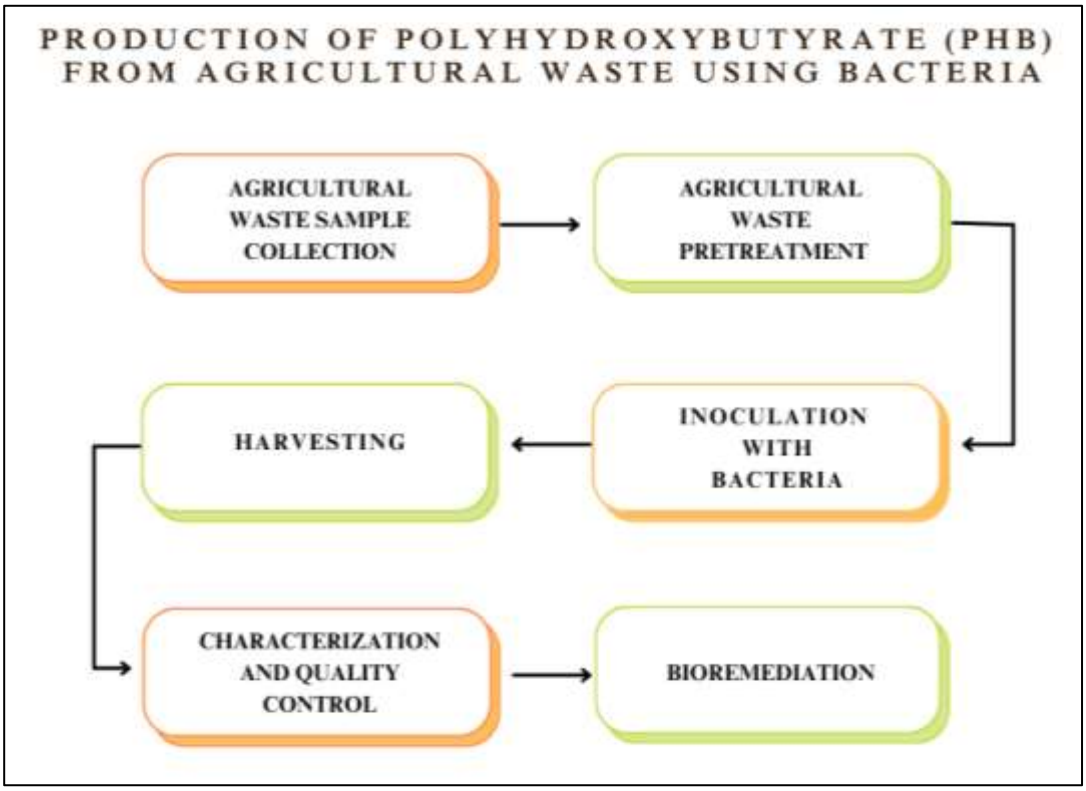

Fig 4. Production of PHB from agricultural waste using bacteria (Mudliar et al., 2008; Getachew et al., 2016).

Production Of Phb From Agricultural Waste

The waste products produced by the agriculture industry are widely available and include a lot of carbs (Zhong Y et al., 2020). Because many bacterial species contain hydrolytic enzymes that can digest these complex residues, they can continue the manufacture of PHB from these affordable, renewable carbon sources in a sustainable manner (Adnan et al., 2022; Sayyed et al., 2021). The production of PHB from agricultural waste is an attractive approach due to the abundance of low-cost carbon sources and the environmental benefits of biodegradable plastics. Several bacterial strains have been identified as effective producers of PHB using various agricultural wastes, such as rice bran, barley bran, corn bran, and wheat bran (Getachew et al., 2016). Many microorganisms that collect PHB intracellularly have been identified since the first publication, including cyanobacteria, archaebacteria, Gram-negative (Bonnin et al., 2014), Gram-positive, and photosynthetic bacteria (Nain L et al., 2022).

Table 1. List the several bacterial strains that are used to produce polyhydroxy butyrate (PHB) from agricultural waste: An overview of the bacterial strains frequently used to produce PHB from agricultural waste is given in this table, collectively with information on their traits and effectiveness.

Bacterial Strain	Key Characteristics	Source of Agricultural Waste	PHB Production Efficiency	References
Cupriavidus necator	High yield of PHB; adaptable use of carbon sources	Various agricultural wastes, such as maize stover and sugarcane bagasse	High	(Park et al., 2021)
Bacillus megaterium	Able to collaborate with a variety of substrates; acceptable PHB yield	Fruit peels and crop leftovers	Moderate to high	(Tang et al., 2013)
Ralstonia eutropha	Productive PHB manufacturer, also called Cupriavidus	Wheat straw and a corn stove	high	(Mohanrasu et al, 2021)
Alcaligenes eutrophus	Good PHB accumulation and flexibility with various waste types	Agricultural waste contains significant amounts of cellulose and byproducts	high	(Park et al., 2021)
Escherichia coli	Produces PHB by converting xylose and glucose from lignocellulosic sources.	Algae biomass residue	less	(Sathish eta l, 2014; (Raposo et al,2014)
Ralstonia eutropha	Numerous carbon sources promote a significant build-up of PHB.	Animal by-products	High	(F. Saad et al, 2021; (Raposo et al,2014)
Pseudomonas canadensis	Several procedures and PHB build-up	Tapioca powder	less	(Preethi Rathna et al., 2023)

PHB SYNTHESIS METHODOLOGY

Sample collection

The initial phase of sample collection involved a thorough screening of agricultural fields to identify potential sources of interest (Mikkili et al., 2014). Samples were collected aseptically to prevent contamination, followed by serial dilution to reduce microbial load and ensure isolation of individual colonies (Singh G et al., 2011). The diluted samples were then inoculated onto sterile nutrient agar plates and incubated at 37°C for 24 hours (Musa, H et al., 2016). After incubation, colonies exhibiting distinct morphological characteristics were carefully selected and subjected to repeated streaking on fresh agar plates to achieve purification. The purified isolates were subsequently preserved on nutrient agar slants and stored at 4°C for future use. Screening of PHB-producing bacteria (Mulamattathil et al., 2014).

Pretreatment of the Sample

Agricultural residues such as sugarcane bagasse, corn cobs, and banana peels were first shredded into small pieces and then oven-dried at 60°C for approximately one week. After drying, the materials were pulverized into fine particles to facilitate further processing (Chen LF et al., 1984). These pulverized residues were then hydrolyzed using the zinc chloride method, as described in the relevant literature, to break down the complex carbohydrates into simpler sugars. The reducing sugar content in the hydrolysates was subsequently quantified using the Di-Nitrosalicylic Acid (DNSA) method, which is a standard procedure for estimating reducing sugars (Wilkins, M, et al., 2019). In addition to the zinc chloride method, another commonly used hydrolysis approach involves acid or enzymatic treatment. For instance, residues can be subjected to dilute sulfuric acid hydrolysis, where the materials are treated with 1-2% H_2SO_4 at elevated temperatures to release fermentable sugars (Taj Keshavarz et al., 2014). Alternatively, enzymatic hydrolysis using cellulase and hemicellulase enzymes can be employed to convert the cellulose and hemicellulose in the residues into simple sugars, which can then be used for microbial fermentation and PHA production (Maitan-Alfenas et al., 2015).

Screening

Screening for PHB-producing bacteria was conducted by subjecting the isolated colonies to Sudan Black staining, which identifies intracellular polyhydroxyalkanoates (PHAs) through the selective staining of lipid inclusions (Narayanan et al., 2020). Additionally, the colonies were tested using Nile Blue staining, a method that enables the detection of fluorescence, indicating the presence of PHAs within the bacterial cells. These combined staining techniques allowed for the effective identification and selection of bacterial strains capable of PHB production (Adebayo Oyewole et al., 2024; Canovas et al., 2020).

Production of PHB

To produce PHA by the selected bacterial isolates, the isolates were cultured in a Mineral Salts Medium (MSM) with the following composition per liter: Urea (1.0 g), Yeast Extract (0.16 g), KH_2PO_4 (1.52 g), Na_2HPO_4 (4.0 g), $MgSO_4·7H_2O$ (0.52 g), $CaCl_2$ (0.02 g), Glucose (40 g), and trace element solution (0.1 ml) (Sangkharak et al, 2007). In addition to MSM, the isolates were also grown in other nutrient media to evaluate their PHA production capabilities under varied conditions. These included Luria-Bertani (LB) broth, which contains Tryptone (10 g/L), Yeast Extract (5 g/L), and NaCl (10 g/L), and Nutrient Broth (NB), composed of Peptone (5 g/L), Beef Extract (3 g/L), and NaCl (5 g/L). The combination of these media provided a comprehensive assessment of the isolates' ability to produce PHA under different nutrient conditions (Luo et al., 2023; Garza Herrera et al., 2023).

Characterization of PHB-producing bacteria

The bacterial isolates identified as PHA producers were further characterized through a series of morphological, physiological, and biochemical tests (Samrot et al., 2021). These tests were conducted to determine specific traits and characteristics of the isolates, enabling accurate identification. The results were then compared with standard references outlined in Bergey's Manual of Determinative Bacteriology, allowing for the classification of the isolates to the genus level (Brenner et al., 2006; Bergey et al., 1993).

Biomass Production

To measure the dry biomass, the bacterial culture was first centrifuged at 10,000 rpm for 15 minutes to separate the cells from the supernatant (Sakai et al., 2023). The resulting cell pellet was then carefully collected and dried in an oven at 55°C until a constant weight was achieved. This method ensured accurate determination of the dry biomass, providing a reliable measure of the cell mass produced during the cultivation process (Mauerhofer et al., 2019). For the extraction and quantification of PHA, 10 mL of the bacterial culture was centrifuged at 10,000 rpm for 15 minutes to collect the cell pellet. After discarding the supernatant, the pellet was treated with 10 mL of sodium hypochlorite, followed by incubation at 30°C for 2 hours to lyse the cells and release the intracellular PHA (Grigary S et al., 2024; Getachew et al., 2016). The mixture was then centrifuged at 5,000 rpm for 15 minutes, and the resulting pellet was washed sequentially with distilled water, acetone, and methanol to remove impurities (Flieger et al., 2021). The cleaned pellet was subsequently dissolved in 5 mL of boiling chloroform. The chloroform solution containing the dissolved PHA was then poured onto a sterile glass tray and allowed to evaporate at 4°C, leaving behind the purified PHA. The dried PHA was weighed to determine the yield, and the relative accumulation of PHB by the different bacterial isolates was compared to identify the most efficient producer (Shah, Kamlesh. Et al, 2014).

PHB Optimization

The production of PHB is highly sensitive to several environmental and nutritional factors, including pH, temperature, glucose concentration, and nitrogen sources (Mostafa et al., 2019). The pH of the medium is critical, with optimal PHB production generally occurring in the neutral to slightly alkaline range of pH 7.0 to 7.5. This range supports optimal enzyme activity and metabolic processes within the bacteria (Mostafa et al., 2020). Temperature is another vital factor, with most PHB-producing bacteria showing maximum productivity at temperatures between 30°C and 37°C; deviations from this range can lead to decreased bacterial growth and PHB accumulation. Glucose, as a primary carbon source, directly influences PHB production, with higher concentrations typically promoting greater PHB synthesis, though excessive glucose can lead to substrate inhibition (Jiang et al., 2011; Rehman et al., 2016). Nitrogen sources also play a crucial role, as limited nitrogen conditions are known to trigger PHB accumulation in many bacteria (Zhou et al., 2022). The choice of nitrogen source, whether organic or inorganic, can significantly affect the overall yield. By carefully optimizing these parameters, it is possible to enhance PHB production, with each factor contributing to the efficiency and scalability of the process (Wang et al., 2024).

Bioplastic film of PHB

A bioplastic film was prepared by dissolving 50 mg of the PHB extract in 10 mL of chloroform. This solution was then cast onto a clean, flat surface and allowed to evaporate the solvent at room temperature or under controlled conditions to form a solid film. This film formation process is crucial for assessing the properties of the bioplastic, including its mechanical strength, flexibility, and potential applications (Mostafa et al., 2019).

Characterization of extracted PHB

The characterization of PHB involves various analytical techniques to determine its chemical structure and properties, with UV spectroscopy and Fourier-transform infrared (FTIR) spectroscopy being particularly important (Samrot et al., 2020). UV spectroscopy is used to evaluate the purity and concentration of PHB by measuring its absorbance at specific wavelengths, which provides insight into the presence of the polymer and any potential contaminants (Schlindwein et al., 2018). FTIR spectroscopy, on the other hand, offers detailed information about the functional groups present in PHB. This technique involves passing infrared light through the sample and analyzing the absorbance at various wavelengths, revealing characteristic peaks associated with functional groups such as carbonyl (C=O) and methylene (–CH2–). These peaks confirm the polymer's identity and provide insights into its chemical structure, molecular interactions, and overall composition. Together, UV and FTIR spectroscopy provide a comprehensive understanding of PHB's structural and chemical characteristics (Trakunjae et al., 2021). PHAs made in this manner have more sustainable characteristics (Christopher et al., 2019). PHBs have shown encouraging results in the food sector, pharmacology, medicine, and general packaging applications (Luckachan G.E et al, 2011). It is also utilized in the production of latex paints, sanitary products, electrical devices, agricultural supplies, and bottles and containers (Gironi F et al., 2011). Additionally, bioplastics have been employed in the production of non-woven materials, tissue engineering, translatology, pharmacology, pharmaceutical goods, sutures, and surgical polymer films (Bugnicourt E et al., 2014). The use of PHAs as a source of organic acids in animal feed is another new use for bioplastics. It is used in surgical supplies, bags, containers, disposable cups, nappies, and packaging materials. Bioplastics cannot completely replace petroleum-based polymers due to their lower resilience and durability (Mahitha G et al., 2016). Just under half of the production costs are accounted for by the carbon source, which is the only significant factor that affects the economics of PHA production. The carbon source is needed in substantial quantities in the PHA production medium. Therefore, choosing affordable and readily available carbon sources is crucial (Gironi F et al., 2011).

PHB Synthesis Pathway

(i) Biosynthesis process

PHB is generated by microbial secondary metabolism in the cells of microorganisms. This process occurs primarily when the cells are under nutritional stress or in an unfavorable environment, such as an excessive carbon environment with limited nutrients, which can occur in both gram-positive and gram-negative bacteria (Rao U et al., 2010; Bharti S.N. et al., 2016). Microorganisms naturally accumulate this substance as a means of storing energy and carbon if vital nutrient supplies become unbalanced or run low (Luckachan G.E et al, 2011). It is noteworthy that a wide range of bacterial species have been shown to accumulate materials, including PHBs as intercellular granules. Reports suggest that this number might exceed 75 distinct genera (Radecka I et al., 2007; Sindhu R et al., 2011).

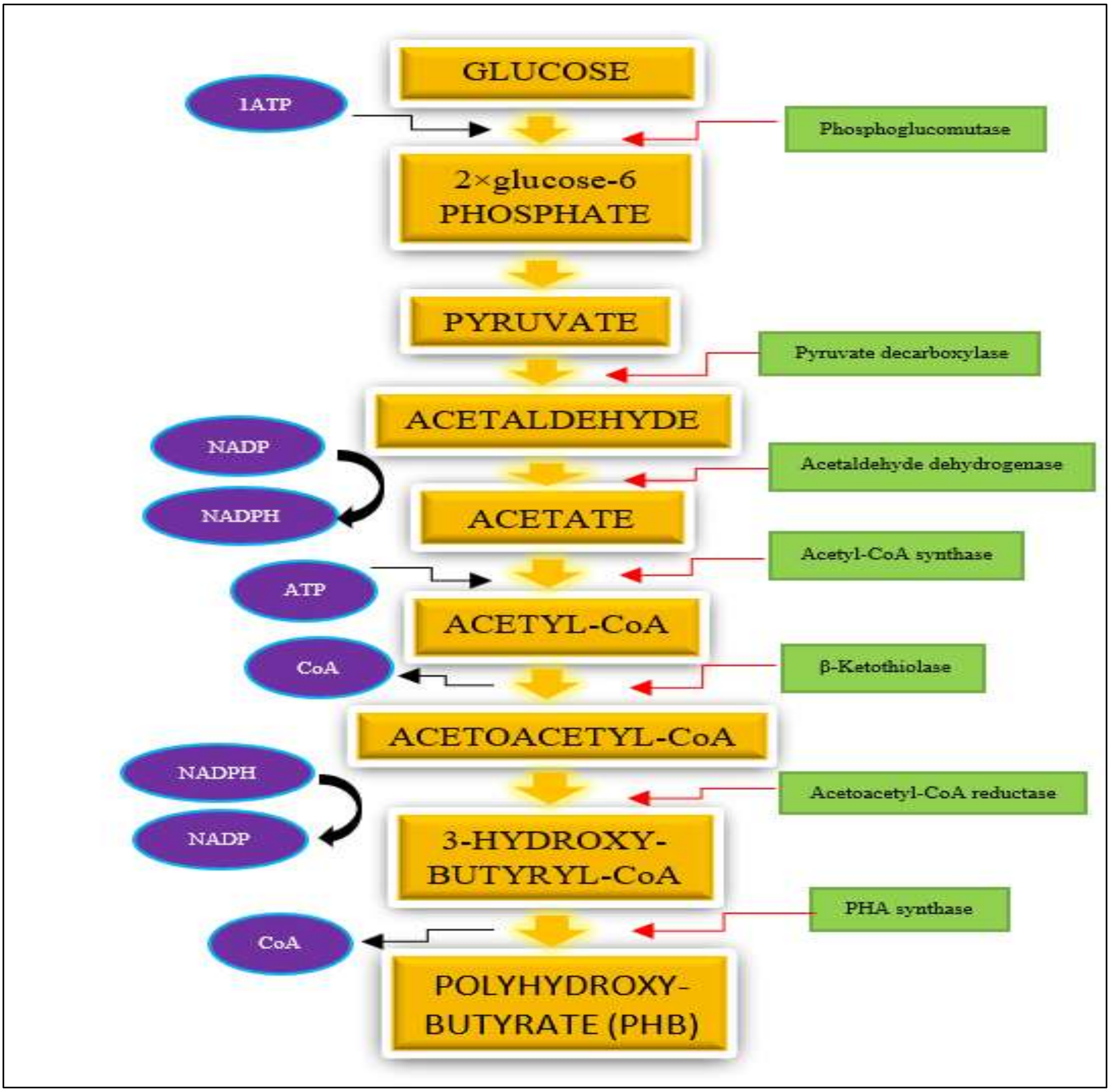

Fig 5. Schematic representation of PHB biosynthesis pathway (Rathna et al., 2023).

Degradation process

PHA breakdown typically doesn't result in any hazardous intermediate compounds. Since many bacteria have intracellular PHB depolymerase to regulate intracellular PHB degradation and utilization, PHB is easily decomposed (A.K. Urbanek et al., 2018). As a result, compared to other bioplastics, PHB-degrading bacteria have been the subject of more investigations. Acetyl-CoA, the final byproduct of PHB breakdown, might join the Krebs cycle or the PHB synthesis pathway (N. Korotkova et al.,2001).

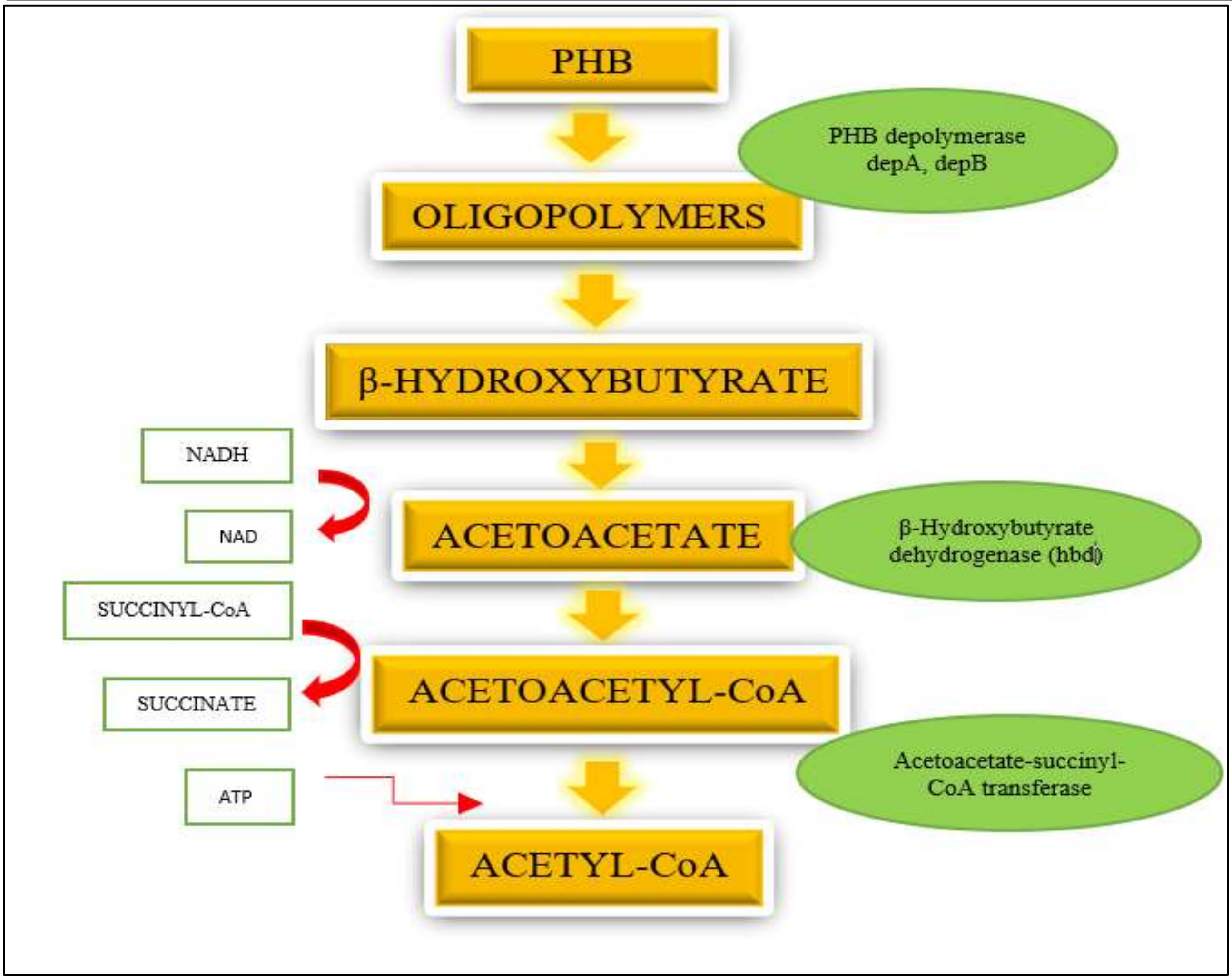

Fig 6. Schematic representation of PHB degradation pathway (Rathna et al., 2023).

CONCLUSION

This study confirmed that inexpensive agro-residues can be utilized to produce PHB, addressing the cost of biodegradable plastics, mitigating environmental pollution from conventional plastics, and solving the disposal issues of agricultural wastes. The selection of carbon feedstocks (e.g., prices) and polymer recovery techniques have always been a major challenge to the large-scale industrial production of PHA. To help with one of these issues, food wastes, food processing wastes, treatment processing wastes, and municipal wastes can all be used as carbon feedstocks. To develop workable ways to lower the cost of producing PHA, numerous experiments have been conducted. Businesses claim to have unique solutions to the cost issue. These claims, meanwhile, still need to be empirically supported. The production of PHB from agricultural waste using bacteria presents a sustainable alternative to conventional plastics. The ability of various bacterial strains to utilize low-cost substrates not only reduces waste but also contributes to the development of biodegradable materials that can mitigate environmental pollution. Further research and optimization are necessary to scale up production processes for commercial viability.

Future Perspective

The future of Polyhydroxybutyrate (PHB) production using agricultural waste holds promising potential as a sustainable and eco-friendly approach to bioplastic manufacturing. Recent studies have highlighted the growing interest in utilizing agricultural by-products, such as rice husks, corn stover, and sugarcane bagasse, as feedstocks for PHB production. These waste materials are abundant, cost-effective, and provide a renewable source of carbon, making them an attractive alternative to traditional petrochemical-based plastics. Advances in biotechnology and microbial engineering have significantly improved the efficiency of PHB production from these feedstocks, with optimized fermentation processes and genetically modified microorganisms showing enhanced yields. Additionally, the integration of PHB production into existing agricultural practices could provide farmers with additional revenue streams while contributing to waste reduction and environmental sustainability. As the demand for biodegradable plastics increases in response to environmental concerns and regulatory pressures, PHB derived from agricultural waste is poised to play a crucial role in the global transition towards a circular economy. Continued research and development, coupled with supportive policies and investments in bioplastic infrastructure, will be essential to scaling up this technology and making it economically viable on a large scale.

Conflicts Of Interest

The authors declare that they have no conflicts of interest.

ACKNOWLEGMENT

The author extends sincere gratitude to Dr. Prawin Nalawade, Head, of the Department of Environmental Science at KTHM College, Nashik, Maharashtra, India, and Dr. Sandip Wagh, Head, of the Department of Biological Science at the School of Science, Sandip University, Nashik, Maharashtra, India, for their invaluable guidance and support throughout the preparation of this review article. Their expertise and insights were instrumental in shaping this work.

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