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A Comparative Review on Polyhydroxybutyrate (PHB) Production by *Bacillus megaterium* Using Different Substrates under Batch Fermentation

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Abstract

The use of non-degradable plastics as packaging has led to significant environmental issues because they are resistant to degradation and contributes to environmental pollution. The application of biodegradable plastics offers a potential solution to this problem. Polyhydroxybutyrate (PHB) is an environmentally friendly thermoplastic polyester polymer that exhibits advantageous properties compared to conventional plastics, especially its biodegradability. The synthesis of PHB can be achieved through three main routes: synthetic polymerization, genetic engineering, and microbial fermentation. This paper aims to explore most suitable substrate used for PHB biosynthesis via batch microbial fermentation based on the highest yield. The microorganism employed in PHB biosynthesis was Bacillus megaterium, which utilized substrates, such as cheese whey, sugarcane molasses, glucose, and glycerol. The highest PHB yield, 8.29 g/L, was obtained when cheese whey was used as the substrate. These finding indicates that cheese whey provides an excellent nutrient source for PHB production.

Keywords: *Bacillus megaterium*; biodegradable; fermentation; polyhydroxybutyrate; substrate

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INTRODUCTION

Plastics are petroleum-based polymers that have been widely used as alternatives to paper, glass, and other materials. Plastics are highly beneficial due to their durability and their ability to reduce CO_2 emissions as lightweight materials. Each year, more than 500 million tons of plastics are produced. For several decades, plastics derived from petroleum and fossil fuels have been employed for various purposes. Initially, plastic use was considered harmless. However, its uncontrolled application and high resistance to chemical and thermal degradation have led to plastic pollution, posing threats to both living organisms and inanimate systems (Thushari & Seneviratha, 2020). Plastics represent a persistent source of air, soil, and water pollution (Rochman *et al.*,

2013) and have detrimental effects on wildlife (Wilcox *et al*., 2015). Moreover, the carbon footprint associated with petroleum-based plastic production is considerably high. Similar to other contaminants, plastic pollutants, can disperse to distant locations through water bodies, thereby endangering the life of humans and other earth inhabitants (Teles *et al*., 2020). To address these challenges, environmentally friendly bioplastics with lower carbon footprints have been developed. One of the most widely utilized bioplastics is polyhydroxybutyrate (PHB).

Polyhydroxybutyrate (PHB) was first discovered by the French researcher Maurice Lemoigne in 1925 while working with the bacterium *Bacillus megaterium*. PHB is synthesized by microorganisms using a variety of substrates, including renewable sources such as sucrose, glucose, and starch, under aerobic or anaerobic conditions with varying temperatures, pH levels, and fermentation modes. The production cost of PHB remains the primary barrier to replacing conventional plastics with biodegradable alternatives. To make commercial production economically feasible, the use of low-cost substrates is strongly recommended.

The selection of raw materials depends on several factors, including availability, productivity, applicability, cost, regulatory requirements, and others. Substrate sources can be categorized into agricultural residues (e.g., molasses, sugarcane bagasse), food waste (e.g., fruit and vegetable peels), dairy waste (e.g., cheese whey, buttermilk, skim milk), forestry waste (e.g., lignocellulosic materials, leaves), and various types of industrial waste (Sumaiya *et al*., 2023).

The biosynthesis of PHB in microorganisms proceeds through the accumulation of acetyl-CoA molecules, which first form acetoacetyl-CoA and subsequently condense into hydroxybutyryl-CoA. This final complex serves as the building block for PHB synthesis. PHB synthesis relies on the central carbon metabolite acetyl-CoA through a series of three enzymatic reactions:

1. **Reversible condensation** of two acetyl-CoA groups to form acetoacetyl-CoA, catalyzed by β-ketothiolase (PhaA);
2. **Reduction** of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB);
3. **Polymerization** of (R)-3-hydroxybutyryl-CoA, catalyzed by PHB synthase (encoded by the *phaC* gene), resulting in PHB formation.

A schematic diagram illustrating the three-step PHB biosynthesis pathway involving PhaA, PhaB, PhaC is presented in Figure 1.

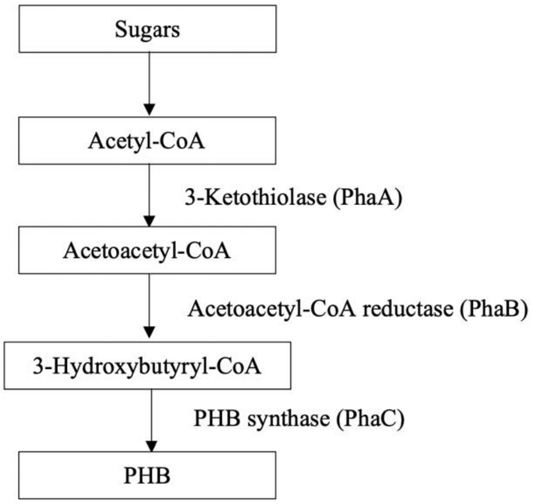

Figure 1. Three-step PHB biosynthesis pathway

Through this biosynthetic pathway, acetyl-CoA is converted into PHB, a partially crystalline polymer with properties similar to polypropylene and polyethylene. Consequently, PHB has been recognized as one of the most promising biodegradable plastics and a potential substitute for petrochemical-derived synthetic plastics. Its properties, namely biocompatibility, biodegradability, and versatile applicability, make it a valuable bio-based additive to synthetic polymers. PHB also offers advantages over polyethylene and polypropylene due to its lower permeability and reduced leakage, and it is regarded as a superior material for food packaging because it does not require antioxidant additives. However, the industrial utilization of PHB remains limited due to its relatively low thermal stability and susceptibility to degradation during storage.

WRITING METHOD

The method employed in this study was a literature review, drawing upon several scientific articles from peer-reviewed journals. The primary focus was on the substrates used as carbon sources for the growth of *Bacillus megaterium* in the synthesis of polyhydroxybutyrate (PHB).

In general, the production of PHB begins with the preparation of growth media and the inoculum of *Bacillus megaterium*. The composition of the medium depends on the type of substrate employed. The inoculum of *Bacillus megaterium* is then introduced into the respective media. When metabolized by the microorganism, the substrate is stored intracellularly as an energy and carbon reserve (Ghosh *et al*., 2013). Following the initial stage of PHB accumulation within the bacterial cells, PHB is extracted and purified to obtain a dry, solid final product ready for downstream processing (Reddy *et al*., 2003).

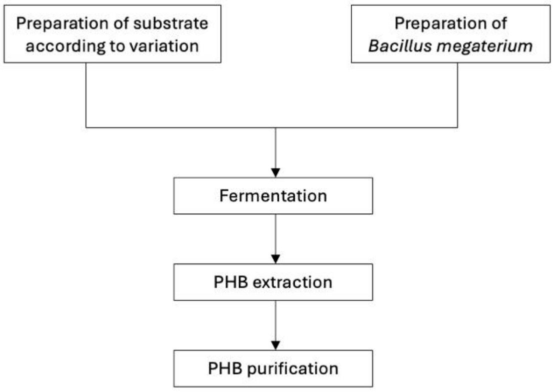

Figure 2. General procedure for PHB production by *Bacillus megaterium*

This review specifically focuses on the use of different substrates, while employing the same bacterium, *Bacillus megaterium*, for PHB synthesis. According to López *et al*. (2012), glucose and glycerol were used as substrates. In the study by Gouda *et al.* (2001), sugarcane molasses served as the substrate, whereas Das *et al*. (2018) employed cheese whey. A summary of these variations is presented in Table 1.

Tabel 1. Utilization of Different Substrates for PHB Synthesis

Microorganisms	Substrate	Fermentation Time (h)	pH	Reference
Bacillus megaterium	Glycerol	32	7	(López *et al*., 2012)
Bacillus megaterium	Glucose	32	7	(López *et al*., 2012)
Bacillus megaterium	Sugarcane molasses	48	7	(Gouda *et al*., 2001)
Bacillus megaterium NCIM 5472	Cheese whey	48	7	(Das *et al*., 2018)

Each type of substrate was subsequently fermented using the batch fermentation method at pH 7, and the PHB yield produced was observed. The data obtained were then reviewed, analyzed, and discussed based on the findings of the literature.

RESULTS AND DISCUSSION

The findings from the reviewed articles indicate that *Bacillus megaterium* can utilize various types of substrates to produce PHB. The PHB yields reported in each of the reviewed studies are presented in Table 2. The selection of substrates for PHB production depends on various factors, including availability, productivity, applicability (either directly or after pretreatment), cost, regulatory considerations, and other relevant aspects. Table 2 highlights several substrates that have been explored for PHB production.

Table 2. PHB Yield Based on Substrate Type

Microorganisms	Substrate	Yield (g/L)	Reference
Bacillus megaterium	Glycerol	2,4	(López *et al*., 2012)
Bacillus megaterium	Glucose	3,3	(López *et al*., 2012)
Bacillus megaterium	Sugarcane molasses	4,8	(Gouda *et al*., 2001)
Bacillus megaterium NCIM 5472	Cheese whey	8,29	(Das *et al*., 2018)

The studies conducted by Gouda *et al*. (2001), López *et al*. (2012), and Das *et al*. (2018) reported variations in the PHB yield obtained using different substrates. López *et al*. (2012) achieved a PHB yield of 2.4 g/L when utilizing glycerol as the substrate and 3.3 g/L when using glucose. Gouda *et al*. (2001) reported a PHB yield of 4.8 g/L with sugarcane molasses as the substrate, while Das *et al*. (2018) obtained the highest PHB yield of 8.29 g/L using cheese whey as the substrate.

As illustrated in Table 2, *Bacillus megaterium* accumulated the maximum PHB when cheese whey was used as the carbon source, followed by glycerol, glucose, and sugarcane molasses. This review highlights that *Bacillus megaterium* efficiently metabolizes cheese whey, resulting in higher PHB production compared to other carbon sources.

The use of different substrates for PHB production by *Bacillus megaterium* has been shown to yield varying PHB outputs. This variation arises from differences in substrate composition, nutrient content, metabolic pathways, and genetic regulation involved in PHB biosynthesis. Among the four substrates examined, namely glycerol, glucose, sugarcane molasses, and cheese whey, the highest PHB yield was obtained with cheese whey. This superior performance is attributed to the more favorable carbon-to-nitrogen (C:N) ratio in cheese whey compared to the other substrates. Environmental conditions with high carbon and limited nitrogen availability enhance the ability of *Bacillus megaterium* to efficiently utilize carbon and energy sources, thereby maximizing PHB synthesis. Furthermore, cheese whey contains lactose and other nutrients that provide a balanced ratio conducive to bacterial growth and efficient PHB production. The composition of cheese whey can also stimulate genetic regulation and activate metabolic pathways that support optimal PHB accumulation. The diverse components of cheese whey create a synergistic effect in meeting the nutritional requirements of *Bacillus megaterium*, which cannot be as effectively provided by the other substrates.

Beyond substrate availability, PHB accumulation is strongly influenced by how efficiently each carbon source is channeled toward acetyl-CoA formation. Cheese whey provides lactose, which is hydrolyzed into glucose and galactose, allowing dual carbon flux into central metabolism, and enhancing acetyl-CoA availability for PHB biosynthesis.

The second highest PHB yield was obtained using sugarcane molasses. The nutrient profile of molasses offers a favorable C:N ratio for *Bacillus megaterium* to synthesize PHB. This ratio is higher than that found in glucose and glycerol, which enhances PHB accumulation. Molasses contains a mixture of sugars (such as sucrose, glucose, and fructose), minerals, and amino acids. These sugars provide an abundant carbon source for polymer chain formation, thereby improving PHB yield. Although both glucose and molasses contain glucose as a carbon source, molasses offers a more complex nutrient composition, including essential minerals and amino acids, which better support bacterial growth and PHB production. In contrast, glucose, being a simpler carbon source, often requires additional nutrient supplementation to achieve comparable yields. Moreover, molasses is more economically advantageous than pure glucose because it is a by-product of the sugarcane industry and is readily available, whereas glucose often requires pretreatment for use as a substrate.

The lowest PHB yield was obtained using glycerol. Several factors contributed to this outcome. *Bacillus megaterium* exhibits relatively low biosynthetic efficiency when utilizing glycerol as a substrate, resulting in less effective conversion of glycerol into PHB monomers. The C:N ratio in glycerol is also lower than in the other substrates, reducing the energy and carbon available for PHB biosynthesis. In addition, glycerol can enter alternative metabolic pathways within microbial cells that compete with PHB biosynthesis, diverting carbon toward cellular respiration rather than polymer synthesis. Glycerol and its intermediate metabolites may also act as inhibitors of PHB biosynthetic pathways or hinder microbial growth. These factors collectively contribute to the reduced PHB yield observed when glycerol is used as the substrate.

Although cheese whey resulted in the highest PHB yield (8.29 g/L), its application in Indonesia is limited by regional availability and processing requirements. Cheese whey is primarily generated by the dairy industry, which is less dominant and geographically concentrated in Indonesia. In addition, whey often requires pre-treatment and careful handling due to its high organic load, increasing processing complexity and operational cost.

On the other hand, sugarcane molasses is a widely available by-product of the sugar industry in Indonesia that makes it a reliable and low-cost substrate. According to Gouda *et al*., (2001), molasses supported relatively high PHB production (4.8 g/L) under conventional batch fermentation conditions at neutral pH and aerobic operation, without the need for extensive substrate pre-treatment. Its complex composition, consisting of mixed sugars, minerals, and amino acids, provides a favorable carbon-to-nitrogen ratio that enhances PHB accumulation.

CONCLUSION

Research on the use of various substrates by *Bacillus megaterium* for polyhydroxybutyrate (PHB) synthesis demonstrates that PHB yield varies depending on the type of substrate utilized. PHB is typically synthesized under conditions of high carbon availability and limited nitrogen supply. Cheese whey produced the highest PHB yield compared to sugarcane molasses, glucose, and glycerol because it provides a more favorable carbon-to-nitrogen (C:N) ratio than the other substrates. The C:N ratio in sugarcane molasses is higher than that in glucose and glycerol, as molasses contains a variety of components, including sugars (such as sucrose, glucose, and fructose), minerals, and amino acids, which supply abundant carbon for polymer chain formation and thus enhance PHB yield. The yield obtained using glucose as the substrate was lower than that achieved with molasses, due to the simpler nutrient composition of glucose compared to the more complex and nutrient-rich composition of molasses. The lowest PHB yield was observed when glycerol was used as the substrate. Glycerol can enter alternative metabolic pathways in microbial cells that compete with the PHB biosynthetic pathway. As a result, glycerol is often directed toward cellular respiration as an energy source rather than PHB synthesis, thereby reducing the overall PHB yield.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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