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Physiological Regulations of Poly hydroxyalkanoates Production by *Ralstonia eutropha*

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Polyhydroxyalkanoates is a type of biopolymer that received particular attention due to their nature of biodegradability and biocompatibility which promotes environment friendly condition. Polyhydroxyalkanoates contributed a huge impact in several industrial sectors by reducing the cost of their plastic waste management as its bioplastic characteristics promotes faster degradation process compared to conventional process (incineration, recycling and landfill disposal methods). Therefore, abundant of research was carried out regarding polyhydroxyalkanoates produced from renewable resources by using microorganisms. It is well known that *Ralstonia eutropha,* a Gram-negative soil bacterium have the ability in producing this biopolymer in large quantity. In this review, we demonstrate the critical aspects of *Ralstonia eutropha*, contributing for its role in producing high amount of polyhydroxyalkanoate, its characteristics and properties, biosynthesis, production process and applications in industry.

Keywords: Polyhydroxyalkanoates, PHA, PHB, *Ralstonia eutropha, Wautersia eutropha,* Industrial applications

INTRODUCTION

Ralstonia eutropha bacteria (also known as *Wautersia eutropha* and *Alcaligenes eutrophus*) is one of the most important species as a microbial factory to produce various chemical metabolites, due to its highly deversified metabolism and biotechnological capabilities (Huschner et al. 2015; Pearcy et al. 2022). *R. eutropha* species has been extensively studied among the bacteria that have an ability to accumulate a production of a large quantity of polyhydroxyalkanoate (PHA) which is consumed various carbon and energy source for growth. PHA is a bio-based, biosynthesized, biocompatible, non-toxicity and biodegradable in nature and has the potential to replace traditional plastics in sectors such as plastic market mainly in the packaging field (Mudenur et al. 2019; Muneer et al. 2020). PHA is considered as one of the most promising bioplastics. Strains of *R. eutropha* were engineered so that they can express and accumulate high levels of PHA copolymers of 3-hydroxybutyrate (PHB) and 3-hydroxyhexanoate (PHV) (Priyadarshi et al. 2014).

Usage of PHA has increased significantly around the world because of its advantages in producing sustainable bioplastics that can degrade rapidly. As times goes its usage has been expanded to another field like the packaging industry. This is due to their biodegradability and biocompatibility nature. PHA is used as a packaging film in containers, paper coatings and bags (Koller, 2017). Other than that, PHA is used in medical implant biomaterials such as sutures, cardiovascular patches, artificial oesophagus and wound dressing. One of the greatest achievements of the usage is as the drug delivery carrier, the study has been carried out that only PHB can be as drug delivery carrier due to its potential carriers for drug that have a faster reaction. (Chen, 2009).

The bacteria can be divided into two groups with focuses on PHA production. For the first group, bacteria requiring limitation of a nutrient such as phosphorous, nitrogen, oxygen or magnesium to accumulate PHAs and they do not accumulate PHAs during the growth phase. The second group accumulates PHAs during the growth phase and do not require any nutrient limitation (Muhammadi et al. 2015). PHA can be obtained from renewable resources like carbon dioxide, sugar, and plant oil and also easy to get renewable resources such as palm oil mill effluent, waste activated sludge and organic waste that are mainly produced from industry waste. The *R. eutropha* will let growth on the media of renewable resource and later will be extracted with an organic solvent to obtain PHA. Once pure PHA is obtained, it will undergo filtration to get dried PHA which is easier form to weighed and get the purity of PHA products (Yan and Chen, 2003; Sayyed et al. 2021).

Ralstonia eutrophaBacteria

R. eutropha is a prokaryote bacterium that are gram-negative soil bacterium found in the soil. It is from the kingdom of *Bacteria*; phylum of *Proteobacteria*; class of *Betaproteobacteria*; order of *Burkholderiales*; family of *Burkholderiaceae*. The bacterial genus *Ralstonia* was established in 1995 and named after E. Ralston, who first found about *Ralstonia*. There are five species which was categorized under this genus which are namely, *R. mannitolilytica, R. pickettii, R. solanacearum, R. pseudosolanacearum, and R. syzygii*, representing diverse lifestyles (Yabuuchi et al. 1992; Vaneechoutte et al. 2004). *R. pickettii* can be found in moist environments including soils, freshwater rivers, and lakes, but also as a biofilm on plastic (water) pipes. *R. mannitolilytica* is closely related to *R. pickettii*, and is previously been called ‘‘*Pseudomonas thomasii*’’ (De Baere *et al.* 2001). *R. syzygii*, the causal agent of Sumatra disease on clove trees in Indonesia. *R. solanacearum* and *R. pseudosolanacearum*is an important phytopathogen that has an unusually broad host range and causes bacterial wilt on a variety of economically important crops affected to include potato and tomato, along with banana, pepper, eggplant, tobacco, and geranium (Safni, 2018). *R. solanacearum* has been classified as one of the world’s most important phytopathogenic bacteria due to its wide geographic distribution, lethality, and persistence (Wicker et al. 2012). Phylotypes I, II and III are composed of strains mainly from Asia, America and Africa, respectively, and surrounding islands, while phylotype IV is primarily composed of strains from Indonesia and some isolates from Japan, Australia and the Philippines. Phylotype IV is the most diverse group, as it consists of strains assigned to *R. solanacearum, Ralstonia syzygii* and the blood disease bacterium (BDB). *R. syzygii* is the causal agent of the Sumatra disease of clove trees in Indonesia and it is also a member of the *R. solanacearum* species complex. BDB is the causal agent of banana blood disease, which is one of the most destructive bacterial wilt diseases affecting banana (Safni et al. 2014).

R. eutropha is the most common bacteria that has the ability to produce PHB with the use of simple carbon substances like glucose and produces significant quantities of PHB within the cells. We can also use olive oil, corn oil, and palm oil as the substrate for PHB synthesis in this strain, which could produce a greater amount of PHB on dry cell weight. Common PHB molecules produced by such kind of bacteria are very brittle thermoplastic. Here the main focus is on producing bioplastic or even biofilm in *R. eutropha* and other bacteria to enhance the properties of the PHAs produced (Priyadarshi et al. 2014). *R. eutropha* is an industrially significant organism and some of its advantageous traits are discussed in Table 1. Due to its similar physical properties to synthetic plastics and features like bio-based, biodegradable, and biocompatible, PHA which is a biologically synthesized plastic is attracting major interests in green material industries. Thus, being more environment friendly, PHA is considered as an alternative to chemically synthesized plastics and has many applications in industry, household, and medical. (Priyadarshi et al. 2014). Table 1 shows the characteristics of *R. eutropha* strain that make it useful for the production of Bio-based products.

Polyhydroxyalkanoates (PHA)

Polyhydroxyalkanoates are a class of polyesters that were produced by prokaryotic microorganisms that are accumulated inside cells as carbon and energy reserves. These biopolymers have given great interest due to their biodegradability, biocompatibility, the possibility of biosynthesis from renewable resources, and similar physical and chemical characteristics to the main bio-based polymer (Sudesh et al. 2000).

The PHA can be roughly divided into three different groups that are short-chain-length polyhydroxyalkanoates (scl-PHA) of 3 to 5 carbons, medium-chain-length polyhydroxyalkanoates (mcl-PHA) of 6 to 14 carbons and long-chain-length polyhydroxyalkanoates (lcl-PHA) have more than 14 carbon atoms but are uncommon and less studied. The size of the side chain (R) greatly affects the material properties of PHAs. The small side chains, such as methyl and ethyl groups, of scl-PHAs result in a stiff material with high crystallinity, high tensile modulus, and low elongation at break, while the large side chains (C6 to C14) of mcl-PHAs make the material elastic with relatively low crystallinity and melting temperature, but improved elongation at break. Manipulating the side chains and compositions of PHA copolymers, therefore, can create new polymers with desired material properties. Manipulating the side chains and compositions of PHA copolymers, therefore, can cre*ate new polymers with desired material properties (*Volova et al. 2001).

Due to the structural variations in monomers constituting PHAs, they have different in properties and chemical composition as homo or copolymers. PHB is comparable to polypropylene and shows good resistance to moisture and acquire excellent barrier properties to gases. According to (Bugnicourt et al. 2014) PHAs are insoluble in water, have good resistance to hydrolytic attack, well resistant to UV, sink in water which facilitates anaerobic biodegradation in sediments. In addition, they are biocompatible and biodegradable for example can undergo degradation in soils. Moreover, PHAs also have chiral molecules and the degradation of PHAs depends mainly on their type and composition (Chen, 2009; Dailin et al., 2022). PHAs are soluble in chloroform and other chlorinated solvents. Their glass transition temperature varies from −50 to 4 °C, melting temperature from 40 to 180 °C. Thermodegradation temperature, tensile strength, Young's modulus, water vapor, and oxygen transmission rate vary according to the type of polymer produced and the composition of the monomeric unit (Bugnicourt et al. 2014), (Vega-Castro et al. 2016).

Polyhydroxyalkanoates have attracted much attention in recent years because of their similarities in properties with petrochemical polymers like polypropylene or polystyrene. The properties of mcl-PHAs are quite different compared to scl-PHAs, refer to Table 2. However, the features of PHAs are dependent on a bacterial host of this biopolymer and the fermentation conditions used towards their production. Scl-PHAs are highly crystalline (typically 55–80%) of polyester with high melting and low glass transition temperatures. The high crystallinity makes them relatively stiff and brittle. The melting point (Tm) ranges from 173 to 180 °C whereas the glass transition temperature (Tg) ranges between 5 and 9 °C. Glass and melting transition temperatures are important parameters relative to in-service applications of PHAs. They define lower and upper-temperature limits for numerous applications. The scl-copolymers were reported to have better properties. Short-chain-length copolymers such as P(3HB-co-3HV) are known as more desirable than scl-homopolymers because their melting point is much lower, and they are less crystalline, easier to mold and tougher. Moreover, these thermomechanical properties can be widely varied by the percentage composition of P(3HB-co-3HV) (Możejko-Ciesielskaand Kiewisz, 2016).

Polyhydroxyalkanoate production by pure bacteria cultures

A wide variety of bacteria are able to accumulate PHAs in the form of intracellular granules, as carbon and energy reserves. PHA accumulation is usually promoted when an essential nutrient for growth is present in limited amount in the cultivation medium such as nitrogen and phosphorus, whereas carbon is in excess. Although, several bacteria are able to produce PHAs during growth and do not require growth-limiting conditions. This carbon storage is used by bacteria as an alternate source of fatty acids, metabolized under stress conditions, and is the key mechanism for their survival (Singh et al. 2014). Pure culture biotechnology is implemented on an industrial scale since a wide variety of food, pharmaceutical, and cosmetic agents derive as metabolic compounds from certain bacterial strains. Within the last few decades, research has been focused on finding ways to decrease the high production cost of PHAs. One of the main contributors to their high cost is the use of high purity substrates, which can account for 45% of the total production cost (Kourmentza et al*.* 2015) this is supported by (Bhatia et al. 2019).

A well-known species involved in industrial PHA production such as *Alcaligenes latus, Cupriavidus necator*, and *Pseudomonas putida*, bacteria need to combine several features in order be selected and regarded as promising PHA producers (Tan et al. 2015). Such features include their performance utilizing renewable feedstocks and/or environmental pollutants, seawater instead of fresh water, the possibility of PHA production under open, non-sterile conditions, and their potential to develop contamination-free continuous bioprocesses. The use of agricultural by-products and forest residues as an abundant and renewable source of lingo cellulosic material for PHA production is mainly considered after its physicochemical or biological hydrolysis (Ward et al. 2005). R. eutropha is a well-reported microbe known for its PHA production potential (Soto et al. 2019). Various research groups are working on different strategies to reduce the PHA production cost by exploring inexpensive and readily available alternative carbon sources and engineering microbes to improve carbon source utilization for enhanced PHA production.

Effect of different carbon source on PHA production

R. eutropha is known for its ability to metabolize a wide range of carbon sources. These include glucose, sucrose, lactose, starch, organic acid, agricultural waste, and waste oil. Research by (Khanna and Srivastava, 2005) state that based on the experiment that had been done, a comparative study was carried out by growing the culture in different carbon sources. It was thought that this would not only help in finding a cheaper carbon source but would also help in accessing the carbon source utilization capacity of the culture (Mohandas *et al.* 2017). There is, twelve different carbon sources were selected based on easy availability or cheaper cost. Table3, shows the biomass (g/L) and PHA (g/L) content obtained with the different substrates tested.

In order to increase the yield and productivity, their high concentrations would be required which would again add to the cost. Thus, it was seen that maximum biomass and PHA were produced when fructose is taken as the carbon source. Naturally occurring strains of *R. eutropha* has been reported to utilize only fructose as carbon source (Table 3)

Table 1: Characteristics of *R. eutropha* that is useful for production of Bio-based products

Characteristics	Production of biological materials	Reference
Adjustable polymer material properties	Produces distinct type of polymers having medium and longer length monomers through fermentation process control	Riedel et al. 2012
Autotrophic growth	Utilizes carbon dioxide for the production of biopolymers and other products	Volova et al. 2001
Carbon source utilization range	Produces value-added products like agricultural and food processing waste stream using plant oils or other inexpensive carbon sources	Yang et al. 2010
Genetically manipulate	Production of different types of a bio-based compound of many types of PHA	Budde et al. 2011
Non-pathogenic / biocompatible	Produce biopolymer for medical materials, devices, and medical compound	Yan and Chen, 2003
Resistance to some toxic compounds	Can produce biopolymer from phenol and from toxic mixtures like syngas.	Volova et al. 2001
Robust carbon storage pathway	Produce intracellular biopolymers with a high productivity and purity	Reinecke and Steinbüchel, 2009

Table 2: Comparison of properties of scl-PHAs, mcl-PHAs, and their copolymer (adapted from Valappilet al. 2006 ; Możejko-Ciesielska and Kiewisz, 2016).

	Homopolymer scl-PHAs	Homopolymer mcl-PHAs	copolymer P(3HB-co-3HV)	Copolymer P(3HB-co 3HD)
Melting temperature (°C)	179	80	137–170	130
Glass transition temperature (°C)	4	−40	10 to −6	−8
Young’s modulus (GPa)	3.5	–	0.7–2.9	–
Elongation to break (%)	40	300	30–38	680
Tensile strength (Mpa)	5	20	up to 690	17

Table 3: Biomass and PHA produced by growing the culture in different carbon sources

Carbon source	Biomass (g/L)	PHB (g/L)	References
Fructose	3.5	1.4	Khanna & Srivastava, 2005
Lactic acid	1.188	0.089	Khanna & Srivastava, 2005; Koller et al. 2009
Sucrose	2.179	0.042	Khanna & Srivastava, 2005; Favaro et al. 2018;
Molasses	1.960	0.039	Khanna & Srivastava, 2005; Panda, 2011
Glycerol	2.879	0.034	Khanna & Srivastava, 2005; Mohandas *et al.* 2017
Glucose	2.332	0.031	Khanna & Srivastava, 2005
Sorbose	0.302	0.007	Khanna & Srivastava, 2005; Singh et al. 2014
Acetic acid	0.151	0.023	Khanna & Srivastava, 2005; Kedia et al. 2014
Xylose	0.263	0.003	Khanna & Srivastava, 2005; Jiang et al. 2016
Sodium acetate	0.095	0.001	Khanna & Srivastava, 2005; Myshkina et al. 2008
Propionic acid	0.055	0.001	Khanna & Srivastava, 2005; Munir & Jamil, 2018
Starch	0.117	0.0	Khanna & Srivastava, 2005; Koller et al. 2009
Lactose	2.251	0.0	Khanna & Srivastava, 2005; Berwig et al. 2016; Colombo et al. 2016

Effects of different nitrogen source PHA production

In PHA production, yeast extract, peptone, tryptone, and beef extract are usually used as a source of vitamin and growth factor. But it can only be used in small scale reactor as it consumed a lot of costs. Nitrogen can be obtained from ammonium salt is widely used for growth and production of PHA on a variety of carbon sources by microorganisms. Meanwhile, for complex nitrogen source which is yeast extract, peptone and beef extract have been shown to improve the production of copolymer scl-PHA production such as poly-3-hydroxybutyrate (PHB) (Annuar et al*.* 2008).

Since nitrogen source in one of important factor for accumulation of PHA, different ammonium salts were tested to get the highest yield. The highest yield of PHA production was obtained by urea as nitrogen source followed by ammonium chloride and ammonium sulphate. However, the addition of ammonium nitrate and ammonium acetate leads to fewer accumulation of PHA (Alsafadi, et al. 2020). Table 4 shows the different nitrogen sources and their yield of PHA.

Table 4: Biomass and copolymer PHB obtained with different nitrogen sources.

Nitrogen source	Biomass (g/L)	PHB (g/L)	Reference
Ammonium sulphate	5.80	2.26	Khanna and Srivastava,2005 ; Aghjeh & Aramvash, 2015
Urea	7.92	3.84	
Ammonium chloride	6.73	2.05	
Ammonium nitrate	2.54	0.66	
Ammonium acetate	1.80	0.192	Sreekanth *et al.* 2013; Portugal-Nunes et al. 2017; Singh et al. 2014

In other experiment (Table 5), it was found that adding mineral source such as corn steep liquor will result in increasing of PHB content due to the high amount of growth factor present in corn steep liquor.

Table 5: Biomass and copolymer PHB obtained with different enhance mineral sources.

Mineral source	Biomass (g/L)	PHB (g/L)	Reference
Corn steep liquor	6.23	2.70	Khanna and Srivastava, 2005; Wei et al. 2011; Hoseinabadi et al. 2015
Yeast extract	5.80	2.26	

Effects of trace elements on PHA production

Trace elements are metals that bacteria need in small amounts. The most common important ones are iron, nickel, cobalt, copper, chromium, zinc and manganese. If there is not enough of these in the food that is fed into the digesters, the bacteria would not be able to efficiently break it down and form biogas (Banks et al. 2012). Table 6 shows the function trace element in bacteria cultivation.

Applications of Polyhydroxyalkanoate

In recent years most companies have been interested in the use of PHAs in packaging, biomedical and materials industry of applications. PHAs is well known for its advantages in manufacturing cosmetic containers such as shampoo bottles, moisture barriers in sanitary products or pure chemicals as raw materials for the production of latex paints (Table 7). The ultrahigh molecular weight of PHAs can be useful to produce strong fiber for the fisheries industry (Bugnicourt et al. 2014) and further study by (Scholz, 2000). However, in view of the properties of PHAs are promising materials especially in a biomedical field. Especially, P(3HB) homopolymer and a copolyester of P(3HB-co-3HV) are the most studied PHAs for medical applications.

Table 6: Function of trace element in the cultivation of bacteria

Trace element	Function	Reference
Zinc	Preventing free radical formation, in protecting biological structures from damage and in correcting the immune functions.	Stefanidou et al. 2006; Gomaa, 2014; Foong et al. 2019
Iron	Cofactor for some enzymatic reaction.	
Calcium	Inorganic cellular cation, a cofactor for certain enzymes and a component of endospores.	Kahar et al. 2004
Cobalt and nickel	Can modify metabolic pathway	Kalantari and Ghaffari, 2008; Winnacker, 2019

Table 7: Application of PHA in various fields

Application	References
Packaging industry	Darani & Bucci, 2015
Printing & photographic industry	Chen, 2009
Other bulk chemicals	Chen, 2009
Block copolymerization	Pederson et al. 2006
Plastic processing	Darani & Bucci, 2015
Textile industry	Chen, 2009
Fine chemical industry	Chen, 2009
Medical implant biomaterials	Chen & Zhang, 2017
Medical	Ray & Kalia, 2017
Healthy food additives	Ali & Jamil, 2016
Industrial Microbiology	Bugnicourt et al. 2014
Biofuels of fuel additives	Zhang et al. 2009
Protein purification	Maestro & Sanz, 2017
Specific drug delivery	Elmowafy et al. 2019

Today, they are considered as materials in the fabrication of cardiovascular products (heart valves, stents, vascular grafts), in drug delivery system (tablets, micro-carriers for anticancer therapy), in wound management (sutures, nerve cuffs, swabs, strapless), in orthopedic (bone plates, spinal cages) (Gregory, et al. 2022 ; Valappil et al. 2006).

CONCLUSION

Polyhydroxyalkanoates (PHAs) are a promising biodegradable polymeric material; an environmental-friendly material which helps reduce the production cost in several industrial sectors; mainly plastic-based industries. Its bioplastic characteristics were believed to have continuous novel applications in packaging, biomedical and materials industry. PHA is advantageous over the synthetic plastics by improving the efficacy towards degradation process; time consuming and safer to the environment. Thus, in the years to come, there will be continued interest in natural biopolymers generated from renewable sources (microorganisms) and their modifications aimed at the media optimization of microorganisms for high amount of PHA production.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

RM, NA, DJ, HE, NZ, RP, HA involved in data collection, writing the manuscript and designed the work. AZ, DS, RW, HA reviewed the manuscript. All authors read and approved the final version.

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