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Utilization of Waste Carbon source as substrate and Economical Production of Biopolymer Polyhydroxyalkanoate Through Bacterial Consortia

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I. ABSTRACT

Petro-plastic being resistant to biological breakdown results in accumulation in the environment. The objective of this review is to look for an alternative which is easily disposable or say biodegradable and does not cause pollution to the environment. Bacterial polymer named Polyhydroxyalkanoate (PHA) has the property similar to petro plastic such as high tensile strength, water resistant, high melting temperature, superior resistance to various solvents. Polyhydroxyalkanoate (PHA) has characteristics similar to petro plastic but are biodegradable and non toxics to environment. Ralstonia eutropha also known as Cupriavidus necator or Wautersia eutropha can synthesize Polyhydroxyalkanoate (PHA) in nutrient limiting condition by utilizing renewable environment friendly waste carbon source. Utilizing waste carbon source leads to minimization in cost of production of Polyhydroxyalkanoate (PHA). Environment friendly microbial polymers have agricultural, biomedical and industrial applications

KEYWORDS: *Polyhydroxyalkanoate (PHA), Polyhydroxybutyrate (PHB), Carbon Source, Bacteria, Biodegradable.*

II. INTRODUCTION

Petrochemical plastics have become an integral part of our daily lives since their first industrial production in the 1940s. They are durable, lightweight, inexpensive, strong and easy to handle, with properties that make them very useful in agricultural, industrial and domestic applications (Derraik *et al*., 2002, Siddique *et al*., 2008). However, these properties also make them susceptible to excessive use and irresponsible disposal (Thompson *et al*., 2009). Thus, plastic waste accounts for a significant share of municipal solid waste (MSW) in the world. It is estimated that plastics contribute up to 80% of marine debris causing serious environmental and health problems worldwide (Hopewell *et al*., 2009). It is known that Polyethylene (PE) is the most widely manufactured petrochemical polymer, accounting for more than 29% of world petrochemical production and that only 10% of plastic waste is currently recycled (Miskolczi *et al*., 2004, Guzik *et al*., 2014). Polyethylene (PE) is a chemosynthetic polymer derived mainly from fossil fuels and recently also from renewable sources (Ziem *et al*., 2016 , Radecka *et al*., 2016). Rapid depletion of fossil fuels has sparked renewed interest in developing alternative processes to produce biologically derived polymers that meet human needs (Aeschelmann and Carus, 2015).

Hundreds of microorganisms possess the ability to produce different types of Polyhydroxyalkanoates (PHAs). They can be recognized as gram-positive and gram-negative bacteria and in archea. Most of them cannot be considered as hosts in industrial production because their ability to synthesize Polyhydroxyalkanoates (PHAs) is insufficient. Only bacterial species that can accumulate Polyhydroxyalkanoates (PHAs) in satisfactory amounts is Cupriavidus necator. It commonly synthesizes Poly-(3-hydroxybutyrate) (PHB) in the process regulated by three enzymes: β-ketatiolaza (PhaA), acetoacetyl-CoA reductase NADPH-dependent (PhaB) and PHA polymerases (PhaC) (Chen, 2010).

Polyhydroxyalkanoates (PHAs) are biopolymers that is present intracellular in numerous microorganisms in limiting conditions of Sulfur (S), Nitrogen (N), Magnesium (Mg), Phosphorus (P), Oxygen (O), Potassium (K) and excess carbon source (García *et al.*, 2013). The most studied form of Polyhydroxyalkanoate (PHA) is Poly-(3-hydroxybutyrate) (PHB). Poly-(3-hydroxybutyrate) (PHB) is a biocompatible thermoplastic with excellent mechanical strength similar to polypropylene and polyethylene. Poly-(3-hydroxybutyrate) (PHB) has high melting temperature, superior resistance to organic solvents, and excellent modulus (Barud *et al.*, 2011, Laycock *et al.*, 2014). The high crystallinity makes them relatively rigid and fragile. The melting point (T m) ranges from 173 ° to 180 ° C where as the glass transition temperature (T g) is between 5 ° and 9 ° C (Mo˙zejko-Ciesielska *et al., 2016*). Polyhydroxyalkanoates (PHAs) belongs to the family of polyhydroxyesters synthesized by numerous bacteria as an intracellular carbon and energy during excess carbon and nutrient-limiting conditions (Tian *et al.*, 2009).

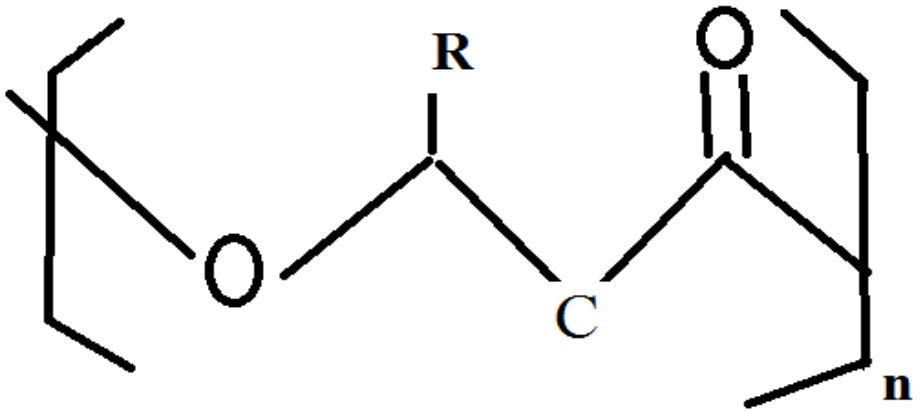

Fig. 1. CHEMICAL STRUCTURE OF POLYHYDROXYALKANOATES (PHAs)

A. MICRO ORGANISMS PRODUCING POLYHYDROXYALKANOATES (PHAs)

Ralstonia eutropha H16 is referred to as chemolithoautotrophs belonging to the β-subclass of the Proteobacteria. *Ralstonia eutropha* or say *Cupriavidus necator* or *Wautersia eutropha* is a Gram-negative, lithoautotrophic organism (Pohlmann *et al.*, 2006). C. necator can use volatile fatty acids (acetic, propionic and butyric acids) as its sole carbon and energy source for growth and Polyhydroxyalkanoate (PHA) synthesis (Du et al., 2002, Chakraborty et al., 2009). On the other hand, acetate and other short chain fatty acids inhibit cell growth and deteriorate the production of metabolites as they uncouple the trans-membrane protein potential and thus interfere with the efficient energy metabolism (Shimizu et al., 2009). An ideal organism to study the biosynthesis of polyhydroxybutyrate (PHB). This bacteria stockpiles organic carbon in the form of poly[R-(–)-3-hydroxybutyrate] (PHB) in special storage granules or say vacuole (Steinbüchel & Füchtenbusch, 1998). During nutrient stress condition that is, in the presence of excess carbon source wild-type *R. eutropha* can accumulate approximately 80 % of its cell dry weight (CDW) as polyhydroxybutyrate (PHB) is an intracellular carbon storage material (Yang *et al.*, 2010; Budde *et al.*, 2011). *R. eutropha H16* can make use of the transiently available supplies of H_2 which arise by the activity of N_2-fixing microbes, because it is equipped with two energy-conserving hydrogenases (Schwartz & Friedrich, 2006). The best known application of *R. eutropha* strains is the production of the biodegradable thermoplastic Biopol at commercial level (Steinbüchel & Doi, 2002).

Several researchers have been interested in the production of PHAs in cultures supplemented with oily substrates. Chee et al. (2010) demonstrated that *Burkholderia* sp. USM (JCM15050) was identified as a producer of effective P (3HB) and was able to synthesize 70% P (3HB) using crude palm kernel oil. Promising results have been published by Chen et al. (2014) in which the newly isolated strain belongs to *Pseudomonas mosselli* grown on the palm kernel and soybean oil has been able to accumulate mcl-PHA at a concentration of 50% CDW. In addition, it was revealed that glycerol could be used successfully for the production of PHA. Hermann-Kraus et al. (2013) by applying pure glycerol to the culture of *Haloferax mediterranei* obtained 75.4% copolyester P (3HB-co-3HV). In addition, by providing butyrolactone as a 4-hydroxybutyrate (4HB) precursor, this bacterium was able to biosynthesize the synthesis of P (3HB-co-3HV-co-4HB) copolyester in the same concentration. Ibrahim and Steinbüchel (2009) used *Zobellella denitrificans* strain MW1 for the production of polyhydroxyalkanoates using glycerol, resulting in a concentration of P (3HB) at 80.4% CDW. Furthermore, it was evaluated that this bacterium is a good producer of P (3HB-co-3HV) copolymer during sodium gluconate co-feeding together with glycerol as sole substrate.

It has also been shown that the application of other waste oils could improve PHA production. It has been found that waste rapeseed oil improves the production of P (3HB-co-3HV) copolymer by adding precursors such as propanol, propionate and valerate during the cultivation of *Cupriavidus necator* H16. Obruca et al. (2010) demonstrated that propanol improved the synthesis of the biopolymer up to 80% CDW. Feeding bacteria with valeric acid positively influenced the mole fraction of 3HV, resulting in a concentration of 18 mol%. In addition, the *Cupriavidus necator* is capable of incorporating the 4-hydroxybutyrate monomer to form the P (3HB-co-4HB) copolymer when grown on vegetable oils. This bacterium was found to synthesize up to 83% P (3HB-co-4HB) with a mole fraction of 4HB in the range of 6-10 mole% growth on soybean oil (Park and Kim, 2011). Moreover, the use of waste vegetable oils seems to be profitable during the fermentations of *Pseudomonas* species. Możejko and Ciesielski (2013) reported that the newly isolated Gl01 strain belonging to the *Pseudomonas* species was capable of producing up to 48% mcl-PHA at 17 hrs from the culture using saponified palm oil as source of carbon. A high concentration of PHAs was reached by Możejko et al. (2011, 2012) during culture of *Cupriavidus necator* using waste rapeseed oil which was supplied as a sole substrate in the bioreactor.

In addition, the use of the digestion liquor as a nutrient medium is as determined to be an ideal substrate for the production of PHA by *Cupriavidus necator*. Passanha et al. (2013) showed that a mixture of microfiltered liquors from food waste and wheat digesters stimulated PHA accumulation up to 90% CDW. It is interesting to note that ammonia, potassium, magnesium, sulphate and phosphate present in media is essential for the initial growth of C. *necator*, while copper, iron and nickel plays role in the accumulation of PHA. In addition, grass biomass was tested for the production of biopolymers. Davis et al. (2013) reported that *Pseudomonas fluorescens* 555 growing on hydrolysates (3.6 g / L total sugar) as the sole source of carbon could produce 32.7% PHA.

B. MAIN CARBON SOURCES

Carbon source is important but is not involved in induction of Polyhydroxyalkanoate (PHA) biosynthesis, although is required for polymer production maximization (Saxena & Tiwari, 2011; Park *et al.*, 2012). The synthesis of polymer is governed by the bacterial strain and the carbon source used for the proper bacteria growth (Santhanam & Sasidharan, 2010).

Agriculture and food industries have a large amount of carbon and other nutrient-rich waste that can be used for the economical production of Polyhydroxyalkanoate (PHA). As this waste can be used as a renewable source by many microorganisms thus giving it more ecological and attractive alternative use like elimination of these waste in the environment (Song et al. 2008, Chee et al. 2010, Prasad & Sethi 2013, Abid et al. 2016). Vegetable oils (such as palm oil, soybean oil, sunflower oil, etc.) are preferred to sugars as the only carbon source for Polyhydroxyalkanoate (PHA) production because they are cheaper and produce more Polyhydroxyalkanoate (PHA) per gram of carbon source. For example 0.3-0.4 g of Polyhydroxyalkanoates (PHAs) were reported per 1 g of glucose. On the other hand, vegetable oils give 0.6 to 0.8 g of Polyhydroxyalkanoate (PHA) per gram of oil. This higher production could be attributed to the higher carbon content per unit mass of the plant Oils versus sugars (Chee et al. 2010, Abid et al. 2016).

The main fermentation strategies used are two-stage fermentation, fed-batch culture, continuous culture and batch culture. Two-stage fermentation is currently the most common method of producing Polyhydroxyalkanoates (PHAs) (Hartmann *et al.*, 2010). In the first stage, biomass is enhanced to the level needed for polyhydroxybutyrate (PHB) production. In the second stage, nutrients are limited to stimulate bacteria so that they synthesis polyhydroxybutyrate (PHB).

For the batch production of Polyhydroxyalkanoate (PHA) in bacterial cells, two modes are normally used. In the first, Polyhydroxyalkanoate (PHA) accumulation begins at the exponential phase and continues to the late stationary phase and is known as a one-step culture. In the second case, the culture of the bacteria is carried out in a growth-promoting medium and when the cells are enriched, they are transferred to the Polyhydroxyalkanoate (PHA) accumulation phase where nutrient depletion is used for this purpose (Kumar & Abe 2010, Abid et al. 2016). For cell survival when bacteria are faced with starvation, fluctuations in environmental conditions, bacteria store important nutrients where PHAs is one of the main compounds of Storage (Haba et al. 2007, Abid et al. 2016).

For the production of Polyhydroxyalkanoate (PHA) in bacterial cells, carbon sources are included and then transmuted to hydroxyalkanoates followed by Polyhydroxyalkanoate (PHA) polymerization and stored in the cell cytoplasm in the form of water-insoluble granules. These granules appear as spherical particles with clear boundaries and are transparent electrons. These granular Polyhydroxyalkanoates (PHAs) are kept in an amorphous state in vivo, otherwise if they are crystallized, these granules cannot serve as a storage compound for the host producing bacterial cell (Loo & Sudesh 2007, Abid et al. 2016).

C. PHB DEGRADATION AND ITS APPLICATION

Polyhydroxyalkanoate (PHA) having bacterial origin so considered natural material and other microorganisms have the ability to degrade these macromolecules. Biodegradable polymer can easily be degraded by anaerobic microorganisms in various environments such as lake water, soil, sewage and sea. Degradation can be categorized as photo degradation and biodegradation. Photo degradation breaks the polymer into smaller fragments which disturbs the structural integrity of the material where as biodegradable means the polymer is completely decomposed (Santhanam & Sasidharan, 2010).

PHAs have useful properties such as: biodegradability, thermoplastic, biocompatibility, non-toxicity, they are considered a replacement for petrochemical polymers. In recent years, companies have been interested in the use of PHAs in packaging, biomedical and agricultural applications. It is well known that PHAs were initially used for the manufacture of cosmetic containers such as shampoo bottles (Hocking and Marchessault 1994), moisture barriers in sanitary products (Lauzier et al., 1993) or Pure chemicals as raw materials for the production of latex paints (Scholz, 2000). In addition, they can be used as carriers for the long-term release of herbicides or insecticides (Galego et al., 2000). The ultra high molecular weight of PHAs may be useful for producing ultrasensitive fibers for the fishing industry (Bugnicourt et al., 2014). However, due to their properties, PHAs are promising materials, particularly in a biomedical field. In particular, the homopolymer P (3HB) and the copolyester of P (3HB-co-3HV) the most studied PHAs for medical applications. In recent years they have been considered as materials in the manufacture of cardiovascular products (cardiac valves, stents, vascular grafts), in the drug delivery system (tablets, microcarriers for cancer therapy), in the treatment of wounds (sutures , Nerve cuffs, orthopedics (bone plates,

vertebral cages) High Immunotolerance, low toxicity and biodegradability are the advantages associated with polyhydroxyalkanoates in tissue engineering.

III. FACTORS DECIDING COST

Various industries try to show interests in Polyhydroxyalkanoate (PHA) fabrication but their cost of production is a major issue. Some researchers are trying to make Polyhydroxyalkanoates (PHAs) production more economical by utilizing inexpensive carbon sources such as plant oils (Shang *et al.*, 2007, Bhubalan *et al.*, 2008, Lee *et al.*, 2008, Kek *et al.*, 2008).

In the recent years, the use of mixed cultures or say consortium being the cheapest method for Polyhydroxyalkanoates (PHAs) production is gaining much attention. Utilizing industrial wastes as carbon source, result in a decrease in the cost of production of Polyhydroxyalkanoates (PHAs) (Lü, 2007).Several factors that might influence mixed culture processes are carbon resources, substrate concentration, temperature, pH and retention time (Kasemsap & Wantawin, 2007, Bengtsson *et al.*, 2008, Serafim *et al.*, 2008).

The final costs of PHAs depend mainly on the price of added substrates as a source of carbon for microbial growth. Moreover, PHAs is produced in the presence of carbon source, PHA productivity and downstream costs determine their introduction into the global market (Choi et al., 1999). Costs of carbon sources accounted for about 50% of final production costs. Analysis and economic evaluation confirmed that large-scale production of PHA from octane would cost about US$ 5-10 per kilogram (Hazenberg and Witholt, 1997). Obruca et al. (2010) calculated that the theoretical price of PHAs produced in waste mode could reach 3.51 Eur/kg PHA, while synthetic alternatives such as polypropylene and polyethylene cost 1.47 and 1.15 Eur/Kg. The conversion of raw materials into biopolymers appears to be an important point in the development of a sustainable biotechnology process and a solution to cost constraints. However, the costs of bioprocesses depend on the price of raw materials such as glycerol waste, vegetable oils, starch, molasses. In addition, downstream processes affect the price of the entire process. Separation processes are particularly difficult. As indicated above, the final calculations were based on the assumption that the PHA recovery price after fermentation represents 30% of total production costs (Sun et al., 2007). Several factors should be considered while selecting the PHAs recovery method, e.g. Producers of PHA, type and composition of biopolymers, product purity requirements, impact on PHA properties (Koller et al., 2013). Several strategies for the isolation and purification of PHAs have been studied. Downstream processing involves microbial mass separation and the supernatant usually by filtration followed by chemical or enzymatic digestion, precipitation or chromatography. The widely used strategy is the extraction of PHA with a solvent, such as chloroform or acetone. These separation methods are costly and involve costs disposal of used solvents. In addition, PHAs are considered environmentally friendly polymers, solvent extraction of PHA from biomass creates additional harmful waste for the environment. Other methods involving enzymatic digestion are too expensive. Therefore, it is necessary to optimize extraction processes based on recyclable solutions which do not create a health risk, for example lactic acid esters based on biological basis can be considered as a promising method. It has been reported that large amounts of PHA in bacterial cells render them brittle and that the biopolymer can be effectively purified by treatment with, for example, a light alkaline solution (Koller et al., 2013). Extraction processes are difficult, especially when PHAs are considered to be used in the biomedical field where high purity of the product is a crucial factor.

Mixed culture or consortium is estimated to have high potential to produce large amounts of Polyhydroxyalkanoates (PHAs) at relatively low cost and minimum requirements. Its ability to use a wide range of cheap substrates including industrial and agricultural wastes makes it environment friendly (Tian et al., 2009).

The cost of production of bioplastic will be determined based on the cost of actual raw material used, electricity consumed, processing charges and chemicals used for its recovery and drying. Processing charges will constitute 20% of the total cost, while the depreciation charges will constitute 10% of the total cost (Girdhar *et al.*, 2013).

TABLE 1. PARAMETERS TO DECIDE COST OF PRODUCTION

Parameters	**Biodegradable Plastics**	**Synthetic Plastics**
Synthesis	**Micro Organisms**	**Crude Oil**
	Waste Carbon Source	**Flash Distillation**
	Culturing	**Column Distillation**
	Biomass Separation	**Cracking (use of catalyst)**
	Polymer Recovery	**Polymerization**
	Heat Required for Film Preparation and Drying	**Heat required for every step**
Degradation	**Biological Compounds are Produced**	**Ethylene, CO_2, CH_4 production**
	Environment friendly Technique	**Toxic to Environment**
	Time consuming process	**Non Degradable**

IV. CONCLUSION

The use of waste polyethylene as carbon source for Polyhydroxyalkanoate (PHA) production could be a double advantage of reducing the environmental impact of polyethylene plastic and producing an environment friendly and biodegradable substitute. According to literature maximum yield of Polyhydroxyalkanoate (PHA) was estimated when the substrate concentration used is 20% v/v. *Ralstonia eutropha* can accumulate 80% to 85% Polyhydroxyalkanoate (PHA) by dry cell weight with about 8-12 granules of polyhydroxybutyrate (PHB) per cell. The inexpensive substrates have been explored for polyhydroxybutyrate (PHB) production to reduce the feedstock cost. On the other hand, the industry requires optimization of fermentation processes for well-known microbes in order to improve the concentration of the biomass and the PHA content with the desired properties. In order to reduce the costs of this bioproduct, additional work should be carried out, in particular, on a high cell density culture method in conjunction with bioproduction of PHAs using cheap carbon sources and easier and non-harmful isolation and purification of Polymers of PHA. It is expected that there is an increasing demand for bacterial polymers with special properties that are suitable for applications in many fields. Using current knowledge and advances in genetic engineering and synthetic biology, it seems possible to build an ideal PHA producer who can biosynthesize new biopolymers and could be used profitably for industrial production.

V. FUTURE OF PHA

For the first time PHA was observed in 1888 by Beijerincka. However, he was unable to define their role and composition. In 1926, the French researcher Lemoigne obtained the poly-3-hydroxybutyric acid (PHB) from Bacillus megaterium (Lemoigne, 1926). Macrae and Wilkinson in 1958 proved that PHAs in bacterial cells act as carbon and energy source and they are collected only in an increased carbon-to-nitrogen ratio. Beginning in 1959, many companies were established to commercialize PHAs as environment friendly bioplastic, completely independent of petroleum sources. W.R. Grace & Company was the first company that tried to produce poly-3-hydroxybutyric acid (PHB). However, the low efficiency of the synthesis and the purification problems of the PHA forced the closure of the company. Beginning in the 1980s, PHAs was produced under the trade names of BiopolTM, NodaxTM, BiocycleTM, BiomerTM, BioGreenTM. Nowadays, the PHA market is very small. Recently, the Telles joint venture, set up by Metabolix and ADM in 2006, targeted a large capacity but sold practically no PHA and then collapsed in 2012. PHA producers are optimistic and still looking for potential at Polyhydroxyalkanoate as biomaterial of new generation biopolymers. Its market needs time to grow it is estimated that demand for PHA will increase tenfold by 2020 (Aeschelmann and Carus, 2015).

In recent years, polyhydroxyalkanoates have attracted much interest in research on the development of Nanoparticles laden with drugs for the pharmaceutical industry. Recently, polyhydroxyalkanoates have been claimed to provide a multifunctional platform for prolonged release of drugs based on microencapsulation technology. They have been demonstrated for the preparation of Nanoparticles (NP) which can be used in the controlled release of various drugs (Mo˙zejko-Ciesielska *et al., 2016*).

The cost of production of Polyhydroxyalkanoates (PHAs) is very high still there are several companies which produce Polyhydroxyalkanoates (PHAs) to meet the market demand. polyhydroxybutyrate (PHB) and PHBV are major components of Polyhydroxyalkanoates (PHAs) that are produced on a commercial scale. Some of the Polyhydroxyalkanoates (PHAs) products in the markets are Mirel, Biopol and Nodax manufactured in USA, Biocycle in Brazil, Biomer in Germany, Tianan PHBV, DegraPol in Italy and polyhydroxybutyrate (PHB) in China. Company name Tianjin Green Biosciences Limited has built a new factory to produce Polyhydroxyalkanoates (PHAs) having capacity of 10 thousand tons per year(Tian et al., 2009). Soon new era of Polyhydroxyalkanoates (PHAs) producing industry is going to come.

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