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Biotechnological Production of Polyhydroxyalkonates by Various Isolates: A Review

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ABSTRACT : *Plastics have been the integral part of our life. However, disposal of these non biodegradable (petrochemical derived) plastics poses threat to environment. Since global petroleum reserves are finite, there is a need for the additional new sources of durable materials. Renewable materials from microorganisms can provide the source of sustainable alternative to petroleum derived chemicals including polymers.. Biodegradable polymers release carbon dioxide and water vapour in the air while undergoing biological decomposition and growing biomass during photosynthesis, subsequently absorb the released carbon dioxide. Hence, the problems associated with conventional petroleum-based plastics have brought biodegradable polymers to forefront. PHA (polyhydroxy alkonates) having properties similar to isotactic polypropylene gained importance. But, their wide spread use has been impeded to high production costs. With the isolation of the genes responsible for PHA biosynthesis and in vivo / in vitro metabolic engineering of microorganisms, an enhanced and cost effective production of PHA should be possible is not so a distant future.*

KEYWORDS: *Biodegradablepolymers, Polyhydroxy alkonates, PHA.*

I. INTRODUCTION

Ecological niches are positions designated to microbes where they perform individual role and interact all together to constitute ecosystem. Ecosystem is influenced by biotic and abiotic altercation as result of natural and anthropogenic activities. Microorganisms derive their food via diverse behavioural adaptation in environment to put together thriving existence. PHA accumulation is among such responses towards stress experienced by microorganisms residing at different ecological niches. Ecological niches like estuarine sediments, marine microbial mats, rhizosphere, groundwater sediments, and engineered ecosystems with fluctuating nutrient contents support the population actively involved in PHA accumulation to meet the metabolic energy requirements during starvation period and this concept can be implemented industrially to reduce the cost of biopolymers commercially with sustainable production processes [1]. Synthesis of PHA occurs in a wide variety of organisms, like prokaryotes, eukaryotes, plant and animal tissue [2] [3]. Among prokaryotes, PHA biosynthesis is known to occur in Gram positive and Gram negative bacteria[4] [5], aerobic and anaerobic[6] [7] heterotrophic and also in cyanobacteria [8] [9], archaebacteria [10] [11] [12], marine bacteria [13] [14], bacteroids [15] [16], Azotobacter spp [17] [18], Methylotrophs[19][20] and floc formers like Zoogloea ramigera [21] [22]. More than 80 different forms of PHA have been detected in bacteria [23]. Only two forms of PHA, i.e., PHB homopolymer and 3HB-3HV copolymer are commercially produced by Zeneca. Nature has evolved several different pathways for PHA formation, each optimized for the ecological niche of the PHA producing microorganisms. These include, PHA biosynthesis represented by Ralstonia eutropha, PHA synthesis with an enoyl-CoA hydratase, Methyl malonyl-CoA pathway for P (3HB-3HV) synthesis from sugars, PHA biosynthesis from fatty acids represented by the Pseudomonads and also PHA biosynthesis from carbohydrates represented by the Pseudomonads.

Polyhydroxyalkanoates (PHA) : Polyhydroxyalkanoic acids (PHA) are polyesters synthesized from optically active thiol esters that accumulate as granular inclusions in the cytoplasm of various bacteria [24] [25]. The 3-hydroxyalkanoic acids are all in R- configuration due to the stereo specificity of the polymerizing enzyme PHA synthase. Only in the case of Rhodococcus spp. S-monomers were detected [26].

Physiological role of PHA : PHA are synthesized and accumulated by the cells as a carbon reserve in the presence of excess carbon, but otherwise in growth limiting nutrient environments. Waste disposal creates toxic, growth inhibiting, and unfamiliar environmental conditions [27]. Even though, at such state of physiological stress, PHA producers have been reported to be residing with properties like degrading dyes,

aromatic compounds, and left over organic matter discharge from industries [28] [29] [30] [31]. During starvation, PHA serves as a carbon and energy source and is rapidly oxidized as demonstrated in Staphylococci [32]. Due to its preferable catabolic role as a carbon reserve under stress, PHA retard the degradation of cellular components [33] and thus offer an advantage to the accumulator organism in a mixed flora to survive under adverse condition[34]. PHA plays a significant role in the survival of the microorganism, under conditions of environmental stress, such as, when subjected to osmotic pressure, dessication or UV irradiation[35].In cyanobacteria, glycogen, which is a common storage material, undergoes fermentation in the dark period forming PHA, which is assimilated in the next light period [36]. The advantage of the PHA formation over the other fermentation products is that the stored carbon remains inside the cell. As a carbon and energy reserve, PHA also assist in the sporulation process in Bacillus sp. [37] [38] though the accumulation of PHA is not a prerequisite for the sporulation process.

Growth conditions for accumulation of PHA : PHB has some properties, including tensile strength and flexibility, similar to polyethylene and polystyrene[39]. PHA is mainly composed of poly-β -hydroxybutyric acid (PHB) and poly-β- hydroxyvaleric acid (PHV), although other forms are possible. When nutrients such as, nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low or when the C: N ratio of the feed substrate is high. The nutrient limiting conditions led to PHA accumulation in different microorganisms [40]. Carbon/nitrogen (C/N) ratio is a very important variable to consider during PHA production supported by the fact that optimum C/N ratio of 25 showed maximum PHA productions [41]. In addition to nitrogen, phosphorus, oxygen, and sulfate limitations, limitations of iron, magnesium, manganese, potassium, sodium and also Oleic acid (with C/N ratio of 20) enhanced PHA production in Cupriavidus sp. USMAA2-4 [42].

Apart from PHA, wide variety of organisms can accumulate glycogen and glycogen-like materials. According to the review [43] in addition to nitrogen limiting conditions, glycogen can be accumulated under sulfur limiting or phosphorus limiting conditions or when pH for growth is unfavorable. pH is also a very important factor in relation to PHA productivity and its monomer composition. Study on effect of pH on acidogenic fermentation shows that initial alkaline pH of 9 can generate good amount of VFAs [44]. However, nitrogen limiting condition was reported to be the most stimulating condition for glycogen accumulation in many organisms. Low concentration of phosphorus and nitrogen is favourable to PHB accumulation rather than complete under provided conditions. PHA production under low nitrogen and phosphorus concentration was reported to be 45.1% and 54.2% of CDW, respectively. Optimizing the same cultural conditions enhanced copolymer P 3(HB-co-HV) content by 14%, constituting 88% of HB and 8% of HV [45].

Classification of Polyhydroxyalkanoates : Bacteria synthesize a wide range of PHA and approximately 150 different constituents of PHA have been identified [46]. PHA produced by bacteria consist of three main types : (i) polymers composed of short-chain- length (scl) monomers(ii) polymers composed of medium-chain-length (mcl) monomers and (iii) polymers composed of scl-mcl monomers. Scl-PHA consists of monomeric subunits 3 to 5 carbons in length, while mcl PHA consists of monomers 6 to14 carbons in length and scl-mcl PHA copolymer consists of monomeric subunits 4 to12 carbons in length[47]. Polyhydroxybutyrate (PHB) is the most common type of scl- PHA and this homopolymer of 3-hydroxybutyric acid has been studied most extensively. Copolymers of PHA can be formed containing 3-hydroxybutyrate (HB) 3- hydroxyvalerate (HV), or 4-hydroxybutyrate (4HB) monomers. Most of the microbes synthesize either scl-PHA containing primarily 3HB units or mcl- PHA containing 3- hydroxyhexanoate (HH) 3-hydro-xyoctanoate (HO) or 3-hydroxydecanoate (HD) as the major monomers [48] [49].

Properties of Polyhydroxyalkanoates :PHA extracted from bacterial cells has properties similar to conventional plastics, such as polypropylene [50]. PHA can be degraded at a high rate (3-9 months) by many microorganisms into carbon dioxide and water using their own secreted PHA depolymerises [51]. Most of the studies on physical and thermal properties of bacterial PHA have been carried out with PHB and PHV. Since the presence of functional groups, epoxy-, hydroxy-, aromatic- side chains, opens up a wide range of possibilities for further chemical modification of the polymer structure, their incorporation to polymers influences the physical [52] and material properties of PHA [53]. PHB has a melt temperature of 1750C and a glass transition temperature (Tg) of 40C [54]. PHB, a short chain length polymer, is optically pure i.e. it is 100% stereo specific with D (-) configuration and isotactic in nature. It is relatively stiff and highly crystalline with crystallanity ranging between 55 to 88% [55]. The co- polymer PHB-co-HV has much improved mechanical properties than the homo polymer P (3HB).

Biosynthesis of PHA : The PHA biosynthesis of bacteria can apparently be divided into two major types based on the biosynthesis of bacteria can be the monomer composition of PHA produced by various wild-type bacteria. R. eutropha represents one group while the Pseudomonads represent the other major type of PHA biosynthesis. The chemical diversity of PHA is large of which the most well-known and widely produced form is PHB [56]. The synthesis of PHB is considered the simplest biosynthetic pathway. The process involves three enzymes and their encoding genes [57] [58] [59]. PhaA gene encodes-ketothiolase, the first enzyme for the condensation of two acetyl-CoA molecules to form acetoacetyl-CoA .The next step is the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA catalyzed by the acetoacetyl-CoA reductase [60]. The last reaction is the polymerization of (R)-3-hydroxybutyryl-CoA monomers catalyzed by PHA synthase, which is encoded by the phaC gene [61].

II. BIOSYNTHESIS IN GRAM NEGATIVE (-VE) BACTERIA

a. Ralstonia eutropha (Cupriavidus necator) : The enzymes involved in this biosynthesis pathway have been extensively studied [62]. R. rubrum, which shows similar PHA biosynthesis pathway as R. eutropha, the reductase gives rise to (S)-isomer of 3-HB-CoA. This is converted to (R)-type by two enoyl-CoA hydratases [63]. The (R)-3HB-CoA is then converted to (R)-P (3HB) by PHA synthase. In R. eutropha accumulates copolymer of 3HB and 3HH when grown on between C 3 -5 substrates with odd number of carbon atoms [64].In C. necator, the PHA formed generally contains monomers having only 3-5 carbon atoms. This has led to the conclusion that in C. necator, the PHA synthase enzyme is only active towards scl-HA [65].

b. Pseudomonas : This pathway is exhibited in Pseudomonads (P. oleovorans) belonging to various alkanes, alkanols or rRNA homology group I. In this alkanoates PHA SCL -C are obtained from intermediates of group 3-hydroxyacyl CoA substrates of C 6-14 fatty acid -oxidation pathway [66]. P. aeruginosa, P. aureofaciens, P. citronellis, P. mendocina and P. putida are shown to possess this pathway. Even over expression of transacylating enzyme such as malonyl-CoA-ACP transacylase (FabD) also seems to generate monomers for PHA biosynthesis [67].

Pseudomonas putida KT2440 growing on nonionic acid under nitrogen limitation produced 27% mcl PHA at specific ratio of 0.48/h [68]. Palm oil utilization for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by Pseudomonas aeruginosa IFO3924 was reported [69]. The bacterium P. guezennei produces a novel PHA mcl with elastomeric properties [70]. Adrian et al. (2010) reported as Jatropha curcas seed oil can be used as a substrate to produce the copolymer poly (3- hydroxybutyrate-co-3-hydroxyvalerate) [71]. P.putida, the site-specific mutagenesis for PHA synthase PhaC2Ps1317 from Pseudomonas stutzeri 1317. Recombinant R. eutropha PHB-4 containing phaC2PsQKST could be used as a strain for production of copolymers consisting of dominated HB and medium-chain-length 3-hydroxyalkanoates (HA) with better application properties [72].

PHA synthesis with an enoyl-CoA hydratase : In Rhodospirillum rubrum, Moskowitz and Merrick proposed a the pathway that included two hydratases, one specific for the R enantiomer and the other specific for the S enantiomer [73] . In Aeromonas caviae, the PHA biosynthetic pathway proceeds from enoyl-CoA derivatives of the fatty acid oxidation pathway . PhaJ converts crotonyl-CoA, pentenoyl-CoA and hexenoyl-CoA to PHA precursors, but it does not convert octenoyl-CoA [74][75]. Methylobacterium rhodesenium again uses two hydratases for PHB synthesis. Along with two hydratases, this bacterium has two constitutive acetoacetyl-CoA reductases, one NADH dependent and the other NADPH dependent [76]. These four enzymes work in combination to synthesize 3-hydroxybutyryl-CoA needed for PHB formation.

Methylmalonyl-CoA pathway for P (3HB-3HV) synthesis from sugars : In Rhodococcus ruber and Nocardia corallina, during P (3HB-3HV) synthesis monomers are derived from both the fatty acid degradation pathway and the traditional C.necator PHB biosynthetic pathway [77]. Recently, Aid used Cupriavidus necator as production strain, polymer content (77%, w/w) and polymer properties (Mw=665 kg/mol, PI=2.6) [78].

Biosynthesis of PHA in Gram Positive Bacteria : Among Gram-positive bacteria, Bacillus spp.[79][80][81], Clostridium spp [82], Corynebacterium spp.[83], Nocardia spp.[84], Rhodococcus spp. [85], Streptomyces spp. [86]and Staphylococcus spp. [87] accumulate PHA. Further, interestingly, the Gram-positive genera Corynebacterium, Nocardia and Rhodococcus are the only wild-type bacteria, which naturally synthesize the commercially important co-polymer P (3HB-co-3HV) from simple carbon sources such as glucose [88][89]. This could allow a considerable decrease in the production cost of the co-polymer. Demonstration of Bacillus sphaericus was done using statistical experimental design and RSM in submerged fermentation with jackfruit seed hydrolysate as the sole source of carbon [90].

a. Bacillus spp. : During the initial stages of PHA research, the polymer was particularly studied in the genus Bacillus. P (3HB), the best characterized PHA, was identified and isolated from a Bacillus sp [91]. P (3HB) was formed and degraded by Bacillus cereus and Bacillus megaterium in washed suspensions [92]. The production of PHV along with PHB from Bacillus megaterium NCIM 2475 and the results show that this was made up of PHB-co-PHV when grown in presence of sucrose [93]. Biodiversity of Bacillus spp. using phenotype microarray panels which allowed the testing of the effect of more than 90 different carbon, nitrogen, sulfur and phosphorus sources as well as pH on the growth characteristics of these strains[94]. Bacillus spp. MC1 from the gut of termites, was able to accumulate PHA utilizing different carbon sources like glucose, fructose, sodium acetate, sodium valerate and 1,4-butanediol. Gas chromatography analysis of the polymer has shown it to be basically composed of poly (3-hydroxybutyrate) [95].

b. Clostridium spp. : Clostridium botulinum accumulates P (3HB) as an energy source for sporulation [96]. Clostridium acetobutylicum ATCC824 has a thiolase homologous to b-ketothiolase from other PHA accumulating bacteria.

c. Corynebacterium spp. : Corynebacterium spp. accumulate PHA co-polymers containing 3HV monomer units from a range of unrelated carbon sources such as glucose (PHA content of 8% w/w dcw) and fructose (PHA content of 1% w/w dcw)[97].

d. Nocardia spp. : Accumulation of PHA co-polymers containing 3HV monomer units from a range of unrelated carbon sources such as acetate (PHA content of 20% w/w dcw) and fructose (PHA content of 0.3% w/w dcw) by Nocardia lucida NCIB10980 [98].

e. Rhodococcus spp. : Species of Rhodococcus have attracted great interest in recent years due to their unusual and diverse abilities to catalyze biotransformation and degradation of various hydrophobic substances such as hydrocarbons, chlorinated phenols, steroids, lignin, coal and crude oil [99]. This interesting finding led to further metabolic analysis which proposed that succinate may be a precursor of the 3HV monomer unit [100] [101].

f. Staphylococcus spp. : P (3HB) has been extracted from various species of Staphylococci such as Staphylococcus aureus, Staphylococcus xylosus, Staphylococcus simulans, Staphylococcu swarneri, Staphylococcus sepidermidis and Staphylococcus haemolyticus [102]. P (3HB) is present in cells in trace quantities (such as 0.15% w/w dcw for S.aureus grown in fructose) and most importantly it does not form granules as in other bacteria indicating possible existence as cPHB. However, the metabolic role of PHA in this genus is poorly understood [103].

PHA synthesis by other bacterial spp. :PHA productions from Azotobacter and Methylotrophs were also investigated earlier. However, PHA with low yield and molecular weight was produced from Methylotrophs. Renner studied the production of copolymer by 13 bacteria from the rRNA super family III [104]. Sinorhizobium fredii strain grown on glucose and sodium dodecanoate in a two stage fed batch fermentation produced poly (3-hydroxybutyrate-co-3hydroxyoctonoate) [105]. Comamonas acidovorans JCM10181 with additional copies of its PHA synthase gene and β-Ketothiolase gene produced poly (4- hydroxybutyrate) [106]. A Hydrogenophaga pseudoflava strain was able to synthesize P (3HB-co-4HB) having a high level of 4-hydroxybutyric acid monomer unit (4HB) from γ- butyrolactone during 3 to 4 days of incubation [107].

Azotobacter chroococcum H23 when grown in culture media amended with alpechin (waste water from olive oil mills) as the sole carbon source. A strain of Azotobacter chroococcum H23 (CECT 4435), Azotobacter vinelandii UWD, and Azotobacter vinelandii (ATCC 12837), members of the family Pseudomonadaceae, were used to evaluate their capacity to grow and accumulate polyhydroxyalkanoates (PHA) using two-phase olive mill wastewater diluted at different concentrations as the sole carbon source [108]. Fernanda analyzed the PHB production in Bradyrhizobium spp [109]. A strain of Serratia sp. showed intracellular electron-transparent inclusion bodies when incubated in the presence of citrate and glycerol 2-phosphate without nitrogen source following pre growth under carbon-limitation in continuous culture [110].

Twenty strains from the collection of the luminescent bacteria CCIBSO (WDCM839) of the Institute of Biophysics, Siberian Branch, Russian Academy of Sciences, assigned to different taxa (Photobacterium leiognathi, Ph. phosphoreum, Vibrio harveyi, and V. fischeri) were analyzed for polyhydroxyalkanoates production. The most productive strains were identified, and the conditions ensuring high polymer yields in batch culture (40–70% of the cell dry mass weight) were determined [111]. In different systems like,

cyanobacteria and actinomycetes PHB has been reported to be produced indigenously. This includes report about PHB in Spirulina sp [112].

PHA synthesis by recombinant bacteria : In recent years, a combination of genetic engineering and molecular microbiology techniques has been applied to enhance PHA production in microorganisms. Growth of the recombinant cells was impaired in many of these studies, especially in nutrient-rich medium [113]. Recombinant E.coli cultured under optimal conditions has been shown to accumulate PHB up to 85% of the cell dry weight. PHB formed in these E. coli, however, were of higher molecular weight than PHB produced by natural producers [114].

Even after extensive attempts at maximizing PHB production in non-PHB producing microorganisms, the PHB accumulation level was not as high as what could be obtained with the natural producers of the biopolymer. One of the major obstacles in producing PHB in recombinant organisms is associated with the instability of the introduced pha genes and loss of the plasmid due to metabolic load of ten limits high yields of the biopolymer [115]. PHA polymerase phaC1/C2 genes were successfully amplified from genomic DNA of three bacterial isolates showing 540 bp DNA fragment which confirmed the presence of phaC1/C2 gene presence [116].

Biosynthesis of PHA in eukaryotic cells : The production of bioplastics in bacteria is limited by its high cost compared to the costs associated with petroleum-derived plastics production. Studies of PHA formation in yeast and insect cells can provide valuable information about how these pathways can be incorporated into plants. Synthesis of PHB has been demonstrated in Saccharomyces cerevisiae by expressing the PHB synthase gene from R.eutropha [117]. Poirier introduced a modified phaC1 gene from P.aeruginosa into S.cerevisiae. [118]. The above PTS1-modified P.aeruginosa phaC1 gene into P.pastoris and achieved mcl-PHA synthesis in this yeast system with fattyacids in the growth medium [119].

III. FACTORS AFFECTING PHA SYNTHESIS AND COMPOSITION

It is considered to be well known that copolymer composition, i.e., %PHB and %PHV is primarily influenced by the substrate used [120]. The author reported the concentration of a substrate supplied also affects the amount of polymer produced. When recombinant E.coli expressing PHA biosynthesis genes from Alcaligenes eutrophus was grown by adding 1 mM valine to 1% glucose medium, growth ceased and upto 2.5% of the incorporated monomers were HV; upto 4% were HV when 1 mM threonine was added as well. The novel growth conditions were useful for the production of heteropolymeric PHA in the recombinant E. coli strain from a single carbon source [121].Wheat bran was hydrolysed by a crude enzyme preparation from Aspergillus oryzae NM1 to provide a mixture of reducing sugars composed mainly of glucose, mannose, xylose and arabinose. Growth of Halomonas boliviensis using a mixture of glucose (0·75% w/v) and xylose (0·25% w/v) in the medium led to a PHB content and concentration of 45 wt% and 1 g l−1, respectively, after 30 h. Butyric acid and sodium acetate for PHB production could also be provided by anaerobic digestion of solid potato waste [122].

Effect of nitrogen limitation during acclimatization period of biomass on production of polyhydroxyalkanoate was investigated. Polymer yields for both biomasses decreased with substrate shift however, biomass enriched with nitrogen deficiency adapted well to acetate–propionate mixture. The results presented in this study showed that polymer storage ability of biomass was improved more under dynamic conditions with nitrogen deficiency when compared to that without nitrogen deficiency. Limiting ammonia availability during batch experiments also caused higher polymer production by suppressing growth, as well as during enrichment of biomass [123].

IV. PHA RECOVERY PROCESS

In addition to the costs of maintaining pure cultures and the high costs of organic substrates, polymer recovery process is another factor that contributes to the overall high cost of PHA production. In the past two decades, several recovery processes have been investigated and studied in order to find an economic way to isolate and purify PHA. Several methods have been used as a recovery process for PHA. These methods include solvent extraction, sodium hypochlorite digestion, and enzymatic digestion [124] [125] [126] [127].

V. CONCLUSION

Currently, European consumption of biodegradable polymers is roughly estimated at 50 000 tonne year. Both government and industries have intensified their efforts in the development of biodegradable polymers. However, the production of biodegradable polymers still accounts for less than 1% of the world annual production of polymers (>100 million tonnes), with maximum usage in low-grade applications such as

food packaging. This slow growth in production is attributed to the high production costs and inferior thermo-mechanical, physical and processing properties of biodegradable polymers compared with petrochemical types. Therefore, intensive work is needed to both reduce costs and improve properties of PHA.Today, a broad range of PHA covering a spectrum of physical properties can be synthesized by a large number of microbial species. Many different products and applications ranging from everyday used products to medical applications are now being explored. The challenge for the future application of the PHA materials depends mostly on the increase in the level of production of these PHA with desired properties in an economical fashion.

Although, the cost of production of PHA is still quite high (4–6 USD kg−1), current advances in fermentation, extraction and purification technology as well as the development of superior bacterial strains are likely to lower the price of PHA, close to that of other biodegradable polymers such as polylactide and aliphatic polyesters. The isolation and development of bacterial strains that can utilize cheap carbon substrates need to be pursued intensively. The isolation of bacterial strains that can synthesize P (3HB-co-3HV) from simple sugar substrates and waste materials is a good example of reducing the substrate cost by eliminating the necessity for supplementing with the more expensive propionic acid. Several of the bacteria described earlier can be employed to enhance PHA production over the next few years. However, metabolically engineered recombinant bacterial strains are more likely to surpass the wild type strains currently in use. In this case, the stability of recombinant bacteria is an important factor for successful PHA production. A combination of more efficient metabolically engineered strains capable of utilizing cheap carbon sources and efficient fermentation strategies is certainly the way forward for increased efficiency of PHA production. It is possible that PHA will become one of the leading biodegradable polymers in the next decade, once the advances mentioned above are in place.

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