Bacillus and biopolymer: Prospects and challenges

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A B S T R A C T
The microbially derived polyhydroxyalkanoates biopolymers could impact the global climate scenario by replacing the conventional non-degradable, petrochemical-based polymer. The biogenesis, characterization and properties of PHAs by Bacillus species using renewable substrates have been elaborated by many for their wide applications. On the other hand Bacillus species are advantageous over other bacteria due to their abundance even in extreme ecological conditions, higher growth rates even on cheap substrates, higher PHAs production ability, and the ease of extracting the PHAs. Bacillus species possess hydrolytic enzymes that can be exploited for economical PHAs production. This review summarizes the recent trends in both non-growth and growth associated PHAs production by Bacillus species which may provide direction leading to future research towards this growing quest for biodegradable plastics, one more critical step ahead towards sustainable development.

1. Introduction

In developing countries several activities are transforming local problems into international issues in this global village. Plastics with favourable mechanical integrity and excellent durability have been one of the fall-outs of the rapid progress in material science technology. Having its utility in diverse sectors, plastics have become an essential part of the modern life. In the global commodity petrochemical based plastic production has grown two hundred fold from 1.5 million tons in 1950 to 299 million tons with an annual growth rate of 9% in 2013 [1,2]. These are typical petroleum-based, non-biodegradable polymers gather or aggregate around our ecosystem which is a far cry from few years back ecosystem [2]. Degradation of such solid wastes is a global concern. Even though it is difficult to completely ban the use of plastics due to their versatile utilities, it is possible to replace or reduce their use with alternative biodegradable polymers with similar properties.

Among the entire bio-based and bio-degradable polymer, polyhydroxyalkanoates (PHAs) are well-known. These are bio-based and biodegradable without waste and also recycled to CO2 and water. The endocellular PHAs are biosynthesized hydroxy-fatty-acids stored as lipid inclusions when carbon source is in abundance and nutrients like nitrogen, phosphorus, oxygen or sulphur are limited. These are secondary metabolites produced by various microbes in response to environmental stress. Such microorganisms can be located in diverse ecological niches like coastal water body sediments, marine region, rhizospheric soil, water sediments and sludge [3]. These environments are often brimming over with organic nutrients and poor in other nutrients to support active growth and meet the metabolic requirements of the starving PHAs accumulating microbial population [4]. Extensive research provides a clear vision on several PHAs producers, that these microbes synthesize PHAs inclusions in the late log phase of growth cycle. Then, in later stage of their life cycle they use it as a carbonosomes [5,6]. Through metabolic activities, PHA is normally depolymerized to D-hydroxy-butyrate on demand, and then metabolized to acetoacetate and acetocacetyl-CoA [7] to provide energy to the cell.

Though these carbonosomes accumulation has been investigated in various bacterial isolates, Bacillus species are extensively studied in PHAs world since the exploration of poly-β-hydroxybutyrate (PHB) in the cytosol of Bacillus megaterium by the French Lemoigne, in 1926 [8]. Some Bacillus species have been reported to produce as much as 90% (w/w) PHAs of dry cells during nutrients imbalance [9]. Bacillus species becoming model organisms in industry and academic world attributed primarily to its genetic stability [10]. Apart from higher growth rate compared to other bacteria, the use of Bacillus species to produce PHAs is advantageous over others due to the absence of lipopolysaccharides external layer in them which makes PHAs extraction much simple [11]. Bacillus species are also capable of producing PHAs copolymers utilizing the relatively simple, inexpensive and structurally unrelated carbon

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scales. Moreover, the isolates possess the ability to secrete number of hydrolytic enzymes that can be exploited for cost affordable PHAs production by utilizing, for instance, agro-industrial and other waste materials [12].

The major drawback of Bacillus species in PHAs production is their sporulating nature. Practically the fact of sporulation and deposition of PHAs granules provoked due to stress factors [13]. To overcome the predicament research on pilot scale PHB productions by B. cereus in the media that depresses sporulation, under acidic pH [14] and potassium deficiency [15] conditions. These pores over strategies not only inhibit spore formation in Bacillus but also can enhance the PHAs productivity. Several studies of PHAs are dealing with mostly on upstream and downstream process, its applications [16,17] and with genetic modifications or mutations to increase the yield [9,18]. Now these experts become an impediment, being economically nonfeasible to market. This review summarizes these recent trends in PHAs production by Bacillus species as an effort to provide direction and leads to future research and development towards the growing quest for biodegradable plastics, one more critical step ahead towards an eco-sustainable development.

2. Biogenesis and chemistry

2.1. Diversity and synthesis of biopolymers by Bacillus

The genus Bacillus is capable of producing organic and inorganic intracellular spherical inclusion bodies enclosed by phospholipid-protein membrane in the cytosol. The inorganic inclusion bodies are magnetosomes surrounded by iron oxide core and the organic hydrophobic inclusion is PHAs surrounded by polyester core [19]. Evidently, magnetosomes surrounded by iron oxide core and the organic hydrophobic inclusion is PHAs surrounded by polyester core [19].

The presence of PHAs granules in the microbial cytosol have also been served as a chemotaxonomic signature for detection of various isolates [20]. A wide array of PHAs producer Bacillus species (Table 1a) are recorded in the last few years with diverse biosynthetic mechanism, structural, thermal and functional properties.

2.2. Forms and taxonomy of biopolymers from Bacillus

The accumulated biopolymer PHAs comprises of 3-hydroxy fatty ester representing not only divergence but also complexity in their monomer classes. It is fascinating and the largest group of biopolymesters with more than 150 monomer compositions exhibiting diverse physical and chemical properties, and functionalities [43,44]. Till now PHAs are grouped into three different categories based on the size, arrangements and number of carbon atom in the polymer, such as short chain length (scl-PHAs with C5 monomer), medium chain length (mcl-PHAs; with C6–C14 monomers) and long chain length (lcl-PHA; with ≥ C14 monomers) respectively [45].

Moreover, the homo and heteropolymers of PHAs corresponds to the presence of more than one type of hydroxalkanoate monomers. The molecular weight of the polymer ranges from 2 × 10^5 to 3 × 10^6 Da, which is based on the type of microbial strain, upstream and downstream processing employed in the production method [46]. Bacillus species are also reported to accumulate heteropolymers of scl- to mcl-PHAs including P(3HB-co-3HV), P(3HB-co-3HHx) and P(3HB-co-4HB) with c-butyrolactone or e-caprolactone as C-source in the production media [47]. Though various PHA monomers are produced by Bacillus species in vitro, very few such as PHB, PHBV and PHBH have en routed for pilot-scale production [48].

2.3. Biochemical pathway of PHAs synthesis

Bacteria have the ability to synthesize PHAs in the stationary as well as exponential growth phases. Non-growth associated PHAs accumulation occurs in the stationary phase of bacterial growth with limitation of N, P, Mg and oxygen and excess carbon sources; however growth associated PHAs production takes place under balanced condition. Notably, most of the Bacillus species accumulate PHAs by adopting growth associated and non-growth associated mechanism [6,49] as compared to other genera. Biosynthetic pathway of PHAs production varies among microbial groups. So far eight different pathways of microbial PHAs synthesis have been reported [13]. PHB, the most common homopolymer of PHAs synthesis starts from metabolism of glucose to generate acetyl-CoA and NADPH through the glycolytic and pentose phosphate pathways. Then, the two acetyl-CoA molecules condensed by β-ketothiolase (PhaA) into acetoacetyl-CoA and subsequently reduced to 3-hydroxybutyryl-CoA by acetoacetyl-CoA dehydrogenase (PhaB) using NADPH as a cofactor and finally polymerized into PHB by P(3HB) polymerase (PhaC) [6,45,50]. Thus, the NADPH is involved in reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA due to over expression of the zwf and gnd genes that encode glucose 6-phosphate and 6-phosphogluconate dehydrogenase respectively [51]. As a matter of fact, the PHB production has been increased by raising the ratio of NADPH to NADP+.

Carbon sources in bacteria are metabolized differentially. So far three pathways for the synthesis of monomers of PHAs in bacteria have been well-studied (Fig. 1). Pathway I utilize sugars like glucose and fructose to yield PHB homopolymer. Copolymers are produced through pathway II and III [53,54]. A contemporary hypothesis for the reaction mechanism of PHA synthases was proposed based on a model by

| Bacillus sp.          | Substrate               | PHAs yield (% of DCW) | Fermentation | PHAs type | Reference |
|-----------------------|-------------------------|-----------------------|--------------|-----------|-----------|
| Bacillus aryabhattai  | Sucrose, glucose & fructose | 57.62                  | Batch        | PHAs      | [40]      |
| Bacillus cereus SPV   | Glucose                 | 38.00                  | Batch        | 3HB & 3HV | [14]      |
| Bacillus cereus       | Glucose                 | 13.77                  | –            | PHB-3HHX  | [34]      |
| Bacillus licheniformis| Glucose                 | 53.01                  | Batch        | PHB       | [42]      |
| Bacillus megaterium   | Glucose                 | 70.00                  | Feed Batch   | PHB       | [37]      |
| Bacillus mycoides DFCI| Glucose                 | 57.20                  | Batch        | PHB       | [28]      |
| Bacillus mycoides DFCI| Glucose                 | 76.32                  | –            | PHB       | [35]      |
| Bacillus sp.          | Glucose                 | 68.85                  | –            | PHB       | [26]      |
| Bacillus sp.          | Raffinose               | 60.57                  | Batch        | P(3HB)    | [27]      |
| Bacillus sp.          | Glucose                 | 80                     | –            | PHA       | [57]      |
| Bacillus sp.          | Sucrose                 | 51.49                  | Batch        | PHAs      | [41]      |
| Bacillus sp. SW1-2    | Glucose                 | 36.00                  | Feed Batch   | PHB       | [36]      |
| Bacillus sp. Ti3      | Starch                  | 58.73                  | Batch        | PHB       | [12]      |
| Bacillus subtilis     | Glucose                 | 69.01                  | Batch        | PHB       | [3]       |
| Bacillus durigmoides  | Glucose                 | 11.30                  | Batch        | PHB       | [32]      |
| Bacillus durigmoides  | Glucose                 | 64.16                  | –            | PHB       | [38]      |
| Lyssimbacillusp.      | 3HHX                    | 80.94                  | Batch        | PHB       | [1]       |
| Paenibacillus duratus | Fructose                | 0.93 g/l               | –            | PHB       | [39]      |
Griebel et al. [55] with two thiolates taking part in the covalent catalysis of polyester synthesis [56]. PHAs synthesis takes place at the thiolate groups (SIH and S2H) of the PHA synthases (E), where one thiolate participates as loading site and the second thiolate acts as the elongation site. This mechanism suggested that one thiol group (SIB) receives a hydroxyalkanoic acid from Co-A thioester which is covalently bonded to the thiol group (E-S I-COAlkyl-OH) and the Co-A is released. However, the increasing polyester chain remains bonded to the second
thiol group to form \([E-S2\text{-poly(PH)}n-\text{OH}]\). This complex, \([E-S2\text{-poly(PH)}n-\text{OH}]\) then transfers the free hydroxyl group upon a nucleophilic attack of the hydroxyl oxygen atom on the carbonyl carbon atom giving rise to \((E-S,\text{-poly(HA)n}+1-\text{OH}) + (E-S1B)\), and the latter can now accept the next hydroxyalkanoic acid from a Co-A thioester (Fig. 2). Moreover, Bacillus species [31,57–60] not only synthesize PHB by non-growth associated mechanism which is operated in nitrogen imitating condition but also by growth associated mechanism [61–64], where high amount of nitrogen doesn’t affect the PHB production negatively [65,66], reasonably it diverts the TCA cycle intermediates towards PHB biosynthesis [67].

2.4. Genes and operons of Bacillus involved in PHAs synthesis

The genes and enzymes regulating biogenesis of polyhydroxyalkanoates have distinct characteristics and depend on the bacterial strain. The ability of a bacterial isolate to synthesize a particular PHA is due to substrate specificity of the key enzyme PHA synthase. Extensive research to study PHA synthases present in the bacterial domain has been fervent, where these are categorized into four different classes. Moreover, the classification is not only based on substrate specificity of enzyme but also subunit composition [45]. These are PHA synthases class I that utilizes CoA thio-esters of 3-HAs, 4-HAs and 5-HAs, and class II polymerases that have specificity towards CoA thio-esters of 3-HAs, 4-HAs and 5-HAs. Notably, these classes of enzymes are expressed by \(\text{phaC}\) gene. The Class III synthase enzyme is composed of two subunits such as \(\text{PhaE}\) and \(\text{PhaC}\), with molecular weight 40 kDa and that have parallel substrate specificities to class I and have the potentiality to polymerise 3-HAs. However, Class IV synthases enzyme bear a resemblance to the class III PHA synthases, but the \(\text{PhaE}\) subunit is replaced by \(\text{PhaR}\) coded by \(\text{phaC}\) and \(\text{phaR}\) gene to synthesize polyhydroxyalkanoates [45].

Research findings [45] also revealed the presence of more than 59 genes associated with PHA synthesis from 45 distinguished bacterial species with varying nucleotide sequence. Though PHA synthase genes vary in number, they mainly occur in two or more different copies in different bacterial strains. These distinct types of the \(\text{phaC}\) gene present in some bacteria also regulate PHAs biosynthesis [68]. Certain PHAs producing bacteria possess a type I-PHA synthase gene clusters composed of the \(\beta\)-ketothiolase \(\text{(phaA)}\), acetacetyl-CoA reductase \(\text{(phaB)}\), PHA synthase \(\text{(phaC)}\) and structural genes occurring in varying arrangements, as observed in Pseudomonas spp. 61-3, R. eutropha, 

\(\text{Acinetobacter}\) sp. RA3849, A. latus and B. cepacia [69–73] (Fig. 3). These three genes constitute an operon in certain bacterial strains, however other bacterial strains have some additional genes involved in PHAs metabolism are also present in the particular clusters [69,74,75]. Majority of PHAs synthesizing bacteria possess type I PHA synthase not positioned close to each other [71,76]. On the other hand, few \(\text{Pseudomonas}\) species have two dissimilar PHA synthase genes clustered in the genome with the same point of reference and separated by a gene that encodes PHA depolymerize \(\text{(phaZ)}\) as revealed in \(\text{P. putida}\) strain U, \(\text{P. oleovorans}\), \(\text{P. aeruginosa}\) and \(\text{P. mendocina}\) [77].

3. Production and characterization of Bacillus generated biopolymer

3.1. Use of suitable raw materials for biopolymer production

PHAs production has been observed by culturing Bacillus species in synthetic culture media [14,24,32,35] since last more than three decades, but the media composition, biopolymer yield and the production cost vary greatly between reports. The raw material cost is predominantly an important factor affecting the overall economics of large-scale PHAs production [78]. Thus, the cost-effective mass production of PHAs production is inherently tied with the development of efficient submerged fermentation technology from low-cost carbon sources. Utilization of cheap raw materials as carbon sources concurrently reduces the cost of manufacture of value-added products [79]. Thus, several inexpensive carbon sources such as sugarcane molasses, beet molasses, date syrup, whey and activated sludge are most commonly used for PHAs production.

Moreover, a number of Bacillus species have been reported to produce PHAs from different low cost substrates or crude raw materials [11,29–31]. The comparative PHB yields by Bacillus species in activated sludge to synthetic medium were 74% and 76.32% (DCW) [29,35] as depicted in Table 1b. This validates the ability of \(\text{Bacillus}\) species to utilize diverse complex starch substrates and its dependence on type of the substrate and enzyme involved in fermentation process. It is pertinent to mention that, \(\text{Bacillus}\) species are well recognized for their capability to hydrolyze starch into simple sugars such as maltose & glucose by amylase and pullulanases enzyme, favoring growth as well as for PHAs production [80,81]. PHAs productions employing starch raw materials also require less amount of energy for liquefaction and saccharification of starch. Narayanan and Ramana (2012) [35] have reported an enhanced PHB production using \(\text{Bacillus mycoides}\) DFC1.
Though, predominant Gram negative bacteria like *Cupriavidus necator* and *Alcaligenes latus* have the potential to produce significant amount of PHB such as 80% and 88% DCW respectively, however, the level of endotoxin in the commercial PHAs can reach up to 120 U/g [82,83]. Thus, endotoxin free PHAs production is highly indispensable using *Bacillus* species.

### 3.2. Techniques involved in characterization of biopolymers

Although several methods used in extraction of PHAs content in bacteria have been described, many are time-consuming, procedurally tough, dependent on organic solvents, involve multiple purification steps and arduous dispersal approach of sodium hypochlorite, chloroform & digesting enzymes [84,85]. These technologies are primarily cost and time intensive thus decreasing the efficacy of downstream processing as well as causing eco-pollution. Strazzullo et al. [86] proposed an efficient, downstream processing for PHAs extraction using sodium dodecyl sulphate with shaking to disperse microbial biomass in distilled water, heat treatment and washing. PHAs are structurally and chemically composition, temperature, humidity and the active microbial consortia. PHAs degradation is enhanced by a decrease in the molecular weight of polymer and an increase in the degree of crystallinity. The number of potential PHAs degrader evolving at the surface of the polymer is lower than the number of associated bacteria. Some dominant soil PHA-degraders are the bacterial genera *Bacillus*, *Xanthomonas*, *Stenotrophomonas*, *Pseudomonas*, *Acinetobacter & Variovorax*, *Schlegelella*, *Azospirillum* and moulds like *Acremonium*, *Penicillium*, *Verticillium*, *Paecilomyces*, and *Zygosporium* [88–90].

### 4.2. Blending: an alternative approach for strengthening biopolymers

Various blends and composites to make the biopolymers suitable to market by enhancing their mechanical strength and reducing the cost have been tried since long. Their hydrophilic nature has helped in developing eco-friendly composites. Blends and multilayers of natural biopolymers with other polymers from sustainable resources can be targeted for their property improvisation. This process also helps to develop cost affordable biopolymer with significant performance. Most widely used natural polymer blends include starch, cellulose and rubber. Starch is the most accepted blending material because of its intrinsic biodegradability and renewability. Aliphatic polyesters are also recognized for their biodegradability and susceptibility for hydrolytic degradation. Such blending significantly increases the thermomechanical stability of PHB. Other advantage of blending is the cytocompatibility to use these materials as biomaterials. Mechanical properties such as elevated Young's modulus and elongation to break the biopolymer matrix are the added advantages of the blended materials.

### 4.3. Commercial applications of biopolymer

Due to its biocompatibility and biodegradability PHAs have a wide range of potential applications such as in packaging, coating material, polymer films, non-woven materials, sutures and pharmaceutical products [91–97] to its negligible cytotoxicity, it is also being used in surgery, pharmacology, trans-plantology and tissue engineering [92,98]. P(3HB-co-4HB) have been validated as scaffold in tissue engineering [83], P(3HB) as the pulmonary artery for the regeneration of arterial tissue [99], P(4HB) for preparing autologous cardiovascular tissue [100] and P(3HB-co-3Hx) in tissue engineering as well as for controlled drug-release [101–103] respectively. PHAs are also being used as cosmetic oil-blotting film [104], skincare products, potential source of organic acids supplement in animal feed and acts as an antimicrobial agent in animal production.

### 5. Prospects and challenge of using *Bacillus* for biopolymer production

As most *Bacillus* species are recognized as safe by the Food and Drug Administration (USFDA), it is an additional benefit for its biotechnological applications. Use of *Bacillus* species has been widely appreciated owing to their many other properties like production of extracellular metabolites, bioremediation and bioenergy production. *Bacillus* species reportedly produce 11–69% higher amount of PHAs compared to other bacterial strains [28], the most potent ones being *B. amylovorans*, *B. latarsoporus*, *B. mycoides*, *B. licheniformis*, *B. circulans*, *B. macerans*, *B. cereus*, *B. firmus*, *B. subtilis*, *B. coagulans*, *B. sphaericus*, *B. brevis*, *B. megaterium*, and *B. thuringiensis*. Another advantage of *Bacillus* species as PHAs producer is its heterogeneous representation. As *B. subtilis* is the...
first Gram-positive to be sequenced completely, it has opened a plethora of functional analysis of Gram-positive bacteria. *Bacillus* species reportedly produces PHAs homo-polymers and co-polymers that increase the diverse nature of the synthesized PHAs [14,68]. They are easily grown utilizing simple sugars to complex industrial wastes. Predominance in the nature and lack of the lipopolysaccharide layer are the added advantages for the use of *Bacillus* species in industrial scale preparation of biopolymers (Fig. 4).

6. Future perspectives

Bacterial biopolymer production and its numerous alternate applications have pushed the bio-industrial sector for its possible commercial-scale production. Wide use of bioplastics can potentially address many potential environmental hazards overcoming the dependence on petroleum to produce plastics, and reduction in CO₂ emission thereby many potential environmental hazards overcoming the dependence on petroleum to produce plastics, and reduction in CO₂ emission thereby. A major problem regarding the use of bioplastics, however, is its high cost. Though many works have been carried out to decrease their production cost, still miles to go to achieve a gold standard in this regard. High value-added applications could be of an immediate interest explored them in surgical and therapeutic applications. Another potential application could be the use of their surface-binding proteins like PhaP, PhaZ and PhaC as drug delivery tools, a possible application in nano-medicine. Genetic modification of bacterial strains to maximize production of biopolymers can be a future research target.

7. Conclusion

In view of the recent advances in biopolymer research, primarily the PHAs have significant impact as a potential substitute of petro-chemical based plastics. The major challenge for the economical production of biopolymers (PHAs) depends on the selection of potential microbes by polyphasic approach and a cost-effective production approach. This suggests selection of suitable *Bacillus* species capable of efficient consumption and bioconversion of inexpensive substrates into a broad range of PHAs with diverse properties and applications. Among the various explored waste material, activated sludge seems to be the most promising for the *Bacillus* species. Combining the batch and fed-batch fermentations for enhanced productivity compared to the other methods available in the public domain can be another process intervention. Considering the controllable nature of chemostat, fed-batch fermentation seems to great potential to enhance productivities. All such efforts at the laboratory scale will need to be validated at pilot-scale for future industrial production and wide application of this biopolymer to tap the application potential of such bacterial species in general, and the genus *Bacillus* in particular.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.10.001.

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