Renewable Feedstocks on PHA Production by Extremophiles

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Polyhydroxyalkanoates (PHAs) are biodegradable polymers with immense potential in addressing the global plastic pollution crisis and advancing sustainable bioplastics production. Extremophiles are capable of utilizing a broad range of carbonaceous substrates for their growth and metabolism. Production of PHA using refined or pure sugar substrates leads to an increase in overall production cost (approximately 30–50%). Thus, the use of renewable feedstocks may reduce the overall cost, provided that the processing of such biomass to generate simple sugars should not be complex and/or expensive. Few studies have shown PHA production by extremophiles fed on renewable feedstocks such as those from agricultural wastes and industrial wastes. Among them, spent cooking oils, crude glycerol, and cheese whey are some of the important and low-cost substrates that come from various industries. In addition, it has been argued that the use of methane by thermophilic methanotrophs results in a reduction of up to 22% in PHA production cost. Other C1 carbon sources such as CO_2 can also be used for PHA production.

Keywords: biopolymers ; extremophiles ; polyhydroxyalkanoates ; copolymers

1. Waste Oils

Several extremophiles, mostly halophiles, were found to use waste oils and lipid-rich substrate for PHA production. Among the most notable bacterial species, *Halomonas hydrothermalis* and *Halomonas neptunia* were capable of synthesizing PHA copolymer with an HV content of up to 50.15 mol % [1]. Supplementation of mild surfactant such as Tween-80 helps in the oil dispersion and facilitates its consumption by microbes. *Paracoccus* sp. LL1 effectively consumes waste frying oil in the presence of Tween-80 to produce PHA copolymer of up to 30.89% of its total biomass, along with valuable pigments [2]. However, the PHA yield of *Paracoccus* sp. was relatively lower than other halophilic species (**Table 1**). Another halophile belonging to genus *Salinivibrio* spp. grows very well using waste fish oil supplemented with glycerol as carbon sources. Under fed-batch culture, a very high biomass of 69.1 g/L (51.5% PHA copolymer) was obtained within 78 h of cultivation [3].

Table 1. Production of PHAs by extremophilic bacteria using various renewable substrates.

Carbon Source	Microorganism	PHA Types	PHA Yield (% CDM)	Fermentation Condition	Remarks	References
Waste oils						
Palm oil mill effluent	*Salinivibrio* sp.	P(3HB)	nd	15% (*v*/*v*) POME, 5% (*w*/*v*) NaCl, 0.1% (*w*/*v*) yeast, and 0.1% (*w*/*v*) ammonium sulfate.	P(3HB) with high thermal stability	[4]
Palm oil mill effluent	*Bacillus licheniformis* M2–12	P(3HB)	88.7	pH 7, 45 °C	PHA production using cheap raw materials	[5]
Waste fish oil and glycerol	*Salinivibrio* sp. M318	P(3HB-*co*-3HV)	51.5	pH 6.5, 30 °C, 0.5 g/L KH_2PO_4, 600 rpm	Higher biomass of 69.1 g/L was obtained after 78 h in fed -batch culture.	[3]

Carbon Source	Microorganism	PHA Types	PHA Yield (% CDM)	Fermentation Condition	Remarks	References
Waste frying oil	*Halomonas hydrothermalis*	P(3HB)	61.98	pH 7.5, 30 °C, 40 g/L NaCl, 300 rpm	Supplementation of valerate led to an HV content of 50.15 mol%	[1]
	Halomonas neptunia	P(3HB)	55.71	pH 7.5, 30 °C, 60 g/L NaCl, 300 rpm	Supplementation of n-propanol led to an HV content of 29.5 mol%	
Waste cooking oil	*Paracoccus* sp. LL1	P(3HB-*co*-3HV)	30.89	pH 7.5, 30 °C, 1.0 g/L $(NH_4)_2SO_4$, 0.1% Tween-80, 300 rpm, 2.5 vvm	Batch culture with 3.24 g/L biomass and 0.89 mg/L carotenoids	[2]
Oil Palm Empty Fruit Bunch	*Halomonas boliviensis*	P(3HB)	35.7	pH 7, 31 °C, 2.5 g/L KH_2PO_4, 200 rpm	Non-conventional nutrients for cost-effective PHA production	[6]
Crude glycerol						
Biodiesel fuel by-product	*Thermotolerant Pseudomonas* sp. strain SG4502	mcl-PHA	40.6	45 °C, minimal salt media, 160 rpm	PHA production at high temperature	[7]
Jatropha biodiesel byproduct	*Halomonas hydrothermalis* SM-P-3M	P(3HB)	75.0	pH 7, 37 °C, 0.4 g/L KH_2PO_4, 200 rpm	High PHB content by a marine environment isolate	[8]
Crude glycerol	*Paracoccus* sp. LL1	P(3HB-*co*-3HV)	39.3	pH 7.5, 30 °C, 1.0 g/L $(NH_4)_2SO_4$, 300 rpm	Cell-retention culture with 24.2 g/L of biomass and 7.14 mg/L of carotenoids	[9]
Residues from cheese production						
Whey-based media	*Thermus thermophilus* HB8	P(3HB-*co*-3HHp-*co*-3HN-*co*-3HU)	35	initial phosphate concentration of 50 mM	Novel heteropolymer consisting of both *scl*- and *mcl*-PHA	[10]
Cheese whey mother liquor	*Paracoccus homiensis*	P(3HB-*co*-3HV)	29.0	pH 7.6, 28 °C, 3.0 g/L KH_2PO_4, 110 rpm	Utilization and management of dairy wastes	[11]
Acidogenic fermentate of acid whey			17.0	pH 5.5, 30 °C, UASB reactor with autocontrols	Valorization of carboxylic acid rich waste streams	[12]
Other waste materials						
Mixed substrates (Kitchen wastes)	*Halomonas campaniensis* strain LS21	P(3HB)	26	27 g/L NaCl, pH 10, 37 °C for 65 days	Secreted extracellular enzymes for waste hydrolysis	[13]
	Recombinant *H. campaniensis*	P(3HB)	70		Secreted enzymes and also maintained the *phbCAB* plasmid throughout the fermentation process without contamination	
Cassava starch + valerate	*Caldimonas Taiwanensis*	P(3HB-*co*-3HV)	67	Nitrogen limited conditions (C/N = 30)	PHA production by thermophilic bacteria	[14]

CDM: Cell dry mass; P(3HB)—poly(3-hydroxybutyrate); P(3HB-*co*-3HV)—poly(3-hydrobutyrate-*co*-3-hydrovalerate) copolymer; P(3HB-*co*-3HHp-*co*-3HN-*co*-3HU)—poly (3-hydroxyvalerate-*co*-3-hydroxyheptanoate-*co*-3-hydroxynanoate-*co*-3-hydroxyundecanoate) copolymer.

2. Crude Glycerol

Crude glycerol is readily being generated as a byproduct of the biodiesel industry during the transesterification reaction. The effluents from these industries are rich in glycerol content (up to 70% glycerol), with other minor contaminants. After minor pretreatment, or sometimes untreated, effluents can be directly used as a feed for the generation of various

bioproducts, including PHA. Both halophilic and thermotolerant microbes are known to grow on crude glycerol and produce PHA in varying quantities. Among them, *Halomonas* spp. and *Paracoccus* spp. were explored in detail. A marine isolate, *Halomonas hydrothermalis* SM-P-3M is among the highest P(3HB) producer in terms of accumulation per unit biomass (**Table 1**). The isolate could grow on minimal media supplemented with glycerol-rich jatropha biodiesel byproduct and produce P(3HB) up to 75% of its total biomass [8]. Compared to *Halomonas* spp., *Paracoccus* spp. produces PHA up to 40% of cell dry mass (CDM). Yet, both strains have been found to co-produce pigments along with the intracellular polymer accumulation [9]. Co-production of high-value compounds along with PHA, especially the extracellular co-product, is considered to be favorable for economic production of biopolymers [15]. *Pseudomonas* spp. are a well-known producer of mcl-PHA having unique properties. Satoh et al. [7] identified a unique thermotolerant isolate, *Pseudomonas* sp. strain SG4502, that can grow at high temperatures (45 °C was optimal for PHA production) and utilize biodiesel fuel by-products. Biodiesel fuel by-product largely consists of glycerol, which was used as a carbon source by *Pseudomonas* sp. to accumulate up to 40.6% of PHA in CDM (**Table 1**). Composting sites are good source of thermo-tolerant microbes. Recently, a thermotolerant PHA producer identified as *Cupriavidus* sp. CB15 was isolated from corncob compost [16]. This natural isolate showed very high accumulation (PHB content of 75.3 wt % of CDM) using glycerol as a carbon source, which was further optimized to achieve a highest yield of 74.4 wt % of CDM with a 2.12-fold increase compared with unoptimized conditions.

3. Cheese Whey and Cheese Whey Mother Liquor

Cheese whey is another such readily available sugar-/protein-rich source obtained from the dairy industry. However, pretreatment of cheese whey is sometimes required. Compared with chemical hydrolysis, enzymatic hydrolysis of cheese whey was found to be suitable for higher PHA yield. It was found that enzymatic hydrolysate of cheese whey leads to increased PHA yield with higher HV mol % within the copolymer [11][17]. Cheese whey and whey mother liquor were also used as feed to grow *Paracoccus homiensis* during the PHA production process. Mineral salt medium with or without nitrogen limitation was tested at 40 g/L to 70 g/L of cheese whey mother liquor or cheese whey supplementation. The highest PHA content was found with cheese whey mother liquor (**Table 1**). This was relatively suitable substrate (with or without nitrogen limitation) compared to cheese whey. The latter resulted in a maximum of 25% PHA content under non-limiting nitrogen supplementation [11]. Similarly, thermophilic bacteria *Thermus thermophilus* HB8 can grow on whey-based media to produce a novel PHA heteropolymer consisting of scl-mcl monomers [10]. Here, a maximum PHA yield of 35% was obtained (**Table 1**). For large-scale operations, just like halophiles, thermophiles keep contaminants at bay and are favorable for lower operational costs towards cooling.

4. Other Waste Materials as Substrate

Halotolerant microbes such as *Halomonas* spp. are favourable for industrial use as they grow in a saline environment unsuitable for other microbes, eliminating the need of sterilization or aseptic fermentation processes. Another advantage of halophilic microbes is their inherent tendency to lyse in a hypotonic solution, simplifying the PHA recovery and reducing the cost of downstream processes. The most demanding operation is the desalination process for PHA recovery, which needs to be addressed as it also contributes to the overall production cost. Oil palm empty fruit bunch and gluten hydrolysates are also an important waste material available in some regions and rich in carbon as well as other nutrients. In a recent study, media, nutrient, and culture optimization using these substrates as feed for *Halomonas boliviensis* resulted in a maximum of 35.7% PHA accumulation [6]. Another related species, *H. campaniensis* LS21, has the ability to secrete hydrolytic enzymes, and thus is suitable for utilizing mixed kitchen wastes as substrates for P(3HB) production. However, the yield obtained by this strain was uncompetitive with other strains, as it produced 26% P(3HB) on the mixed substrate. Expression of the PHA biosynthesis operon in this strain resulted in more than 2-fold increase in P(3HB) yield under the same cultivation condition [13]. Lignocellulosic hydrolysates are also a common source of cheap organic carbon. The potentials of the halophilic bacterium *Halomonas halophila* and the thermophilic bacterium *Schlegelella thermodepolymerans* for producing PHAs were tested using model media that mimic lignocellulose hydrolysates [18]. When provided with hexose-rich media, *H. halophila* achieved notably higher PHA yields, while *S. thermodepolymerans* exhibited a preference for media abundant in pentoses. Both extremophilic bacteria displayed higher sensitivity to microbial inhibitors compared with the mesophilic strain of *Burkholderia sacchari*. Nevertheless, taking into account their significantly enhanced PHA productivity, even in the presence of microbial inhibitors, as well as other advantageous characteristics associated with extremophiles, such as reduced susceptibility to microbial contamination, both *H. halophila* and *S. thermodepolymerans* emerge as promising candidates for sustainable PHA production. This sustainable approach capitalizes on readily available and cost-effective lignocellulosic resources. Volatile fatty acids (VFAs) are also generated from biomass, especially after anaerobic digestion or similar treatments. These VFAs are direct precursors for PHA monomers and are considered to be economical and environmentally friendly feedstocks for PHA copolymer production.

However, not all the microbial strains can effectively consume large quantities of these substrates, while their higher concentration in the feed medium imposes toxicity for the cell. Very recently, the halophilic bacteria *Salinivibrio* spp. TGB4 and TGB19 were found to grow considerably on acetate or butyrate as feed (even at higher doses of up to 100 g/L). When both acetate and butyrate were fed, P(3HB) production was found to be 6.14 and 6.84 g/L for TGB4 and TGB19, respectively. Under optimum conditions, TGB19 produced a P(3HB) titer of 8.42 g/L (about 88.55% of CDM). This was further improved via fed-batch cultivation, and the P(3HB) titer reached 53.23 g/L by TGB19. Thus, P(3HB) production on VFA as substrate can be a promising way to use effluents from anaerobic digesters for large-scale plants [19]. On a similar theme, a high-concentration propionate-utilizing halophile was identified as *Halomonas* sp. YJ01, producing P(3HB-*co*-3HV) copolymer via a propionate-dependent pathway. Whole-genome analysis revealed multiple genes related to PHA biosynthesis. With 15 g/L of propionate alone, the *Halomonas* sp. was able to produce up to 29 mol% of HV content, while supplementation of glucose decreased the copolymer yield. This suggests that propionyl-CoA conversion to pyruvate must have occurred through 2-methylcitrate cycle that reduced propionate detoxification [20]. On the other hand, another thermophilic bacteria, *Caldimonas taiwanensis*, converted cassava starch to produce the biopolymer under nitrogen-limited conditions. Supplementation of a related carbon source such as valerate led to the copolymer production with a maximum PHA yield of 67% of CDM [14].

Two halophilic bacteria, *Bacillus cereus* LB7 and *Burkholderia gladioli* 2S4R1, were recently found to use mixed sugars obtained from paddy straw hydrolysis. Under optimal C:N ratios of 30:1 and 38:1 and unique growth media, PHB productivities of 0.39 g/L/h and 0.31 g/L/h, respectively [21], were achieved. This work is a unique demonstration showing bioconversion of paddy straw hydrolysate by halophilic bacteria that must be explored further for industrial-scale applications.

With the prevailing knowledge on PHA production using various microbes and feed materials at different operation scales, the success and sustainability of PHA production is now being recommended based on the concept of a circular economy. This model relies on the principle of reduce, reuse, and recycle to achieve a sustainable environment and conserve natural resources for the coming generations. From a bioprocess viewpoint, it emphasizes maximizing substrate utilization, product recovery, and energy efficiency with minimal waste generation. Compared to a linear economy, a circular economy suggests a closed-loop biorefinery design where all bio-based building blocks obtained from biomass are fractionated and converted into multiple products such as bio-fuels, PHAs, organic acids, etc. As these products are of biological origin, their usage in day-to-day life has environmental and socio-economic benefits. Here, the focus is to recycle and reuse the end-life phases for another value chain using technical solutions, modified manufacturing processes, and revised business models to achieve zero waste emission. For instance, during the PHA production process, high quantities of protein-rich effluents are generated as liquid stream. This may amount to up to 50% of the total CDM and may be collected and recovered for other suitable applications such as animal feeds, biocatalysts, or sources of amino acids [22]. There are various co-production strategies available for the simultaneous PHAs and other high-value chemicals that can be explored with integrated biorefineries for successful and sustainable PHA production in practice [23]. Thus, a “cradle-to-cradle” process is important to achieve the targets of a circular economy. The PHA polymers being biodegradable with unique physicochemical properties fits well in the concept of a circular economy. Realization of the circular economy approach on PHA production largely depends on the integration of a lignocellulosic biorefinery with unique green technologies [24][25]. It is expected to mitigate the global challenges of fossil-based fuels and polymers. Thus, factors such as the cost of pretreatment, sugars and inhibitor contents in the hydrolysates, PHA productivity, consistent polymer properties, and life-cycle and techno-economic assessment data are of importance for industrial-scale PHA production using lignocellulosic biomass. For large-scale operations of such integrated process, higher economic investment, technological advancement, and financial subsidies provided by government agencies are required [26].

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