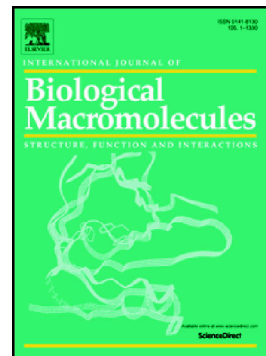# Journal Pre-proof

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Polyhydroxyalkanoates synthesis using acidogenic fermentative effluents

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Abstract

Polyhydroxyalkanoates (PHA) are natural polyesters synthesized by microbes which consume excess amount of carbon and less amount of nutrients. It is biodegradable in nature, and it synthesized from renewable resources. It is considered as a future polymer, which act as an attractive replacement to petrochemical based polymers. The main hindrance to the commercial application of PHA is the high manufacturing cost. This article provides an overview of different cost-effective substrates, their characteristics and composition, major strains involved in economical production of PHA and biosynthetic pathways leading to accumulation of PHA. This review also covers the operational parameters, various fermentative modes including batch, fed-batch, repeated fed-batch and continuous fed-batch systems, along with advanced feeding strategies such as single pulse carbon feeding, feed forward control, intermittent carbon feeding, feast famine conditions to observe their effects for improving PHA synthesis and associated challenges. In addition, it also presents the economic analysis and future perspectives for the commercialization of PHA production process thereby making the process sustainable and lucrative with the possibility of commercial biomanufacturing.

Keywords: PHA; biosynthetic pathways; fermentative modes

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1. Introduction

The use of plastics, which are necessary in all current systems, is highly acquired utilizing fossil feedstocks. Although conventional synthetic plastics are broadly used, yet they have many drawbacks; for instance, the dependence on exhausting fossil fuels for their generation, their non-biodegradable nature and the integrated formation of toxic materials during their production. The production of synthetic plastic is not only non-sustainable but their continuous accumulation in nature is posing a big threat to the environment. Polyhydroxyalkanoates (PHA) being sustainable and environmentally friendly are emerging potential alternative to fossil-based plastics. [1]. PHA are a group of biologically produced polymers having an extensive variety of desirable physical properties based on the monomeric components present in the polymer [3]. They are produced from sustainable sources and therefore, considered as environmental friendly unlike conventional synthetic polymers [4]. Moreover, these PHA are biodegradable which make them eminent option for replacing fossil-based synthetic plastics in chemical and material industry [18,19]. The biopolymers such as polylactic acid and polybutylene succinate can replace traditional polymer with identical characteristics such as polypropylene and low-density polyethylene [20]. White biotechnology, particularly regarded for the PHA synthesis depends on the exploitation of whole cells or enzymes as catalysts [21]. The applications of PHA particularly in recently developing fields, include tissue engineering, targeted drug delivery, and agricultural fields [22].

PHA is generally produced under certain situations of limited growth caused by absence of a key nutrient and carbon flux under nutrient limitation is diverted towards PHA accumulation [5]. The production of PHA consists of two stages where in first stage cells are initially cultivated to a high

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cell density culture with a well-proportioned nourishment approach, followed by second stage with the limitation of at least one of the vital nutrients and surplus carbon. This imbalanced nutritional condition provokes intracellular production and accumulation of PHA [6]. To acquire the commercial feasibility of PHA, there is a need to reduce the production costs and one of the key elements responsible for high costs is the utilization of extravagant fermentable carbon sources, which accounts for roughly 40% of the total PHA manufacturing expenditure. In last few decades, alternative manufacturing processes of polyhydroxyalkanoates (PHA) is being developed depending on the utilization of wastes from industries and their derivatives as a substrate [2]. Numerous sustainable carbon sources, mostly agro-industrial wastes like starch residue, soy molasses, whey, glycerol, butchery residue and waste oils have been examined for PHA production [8]. Also, mixed microbial cultures (MMC) are used rather than pure cultures for PHA production to cut down the manufacturing costs as the maintenance requirements with MMC is low [9]. The soluble acid rich effluents, known as volatile fatty acids (VFA) obtained from the dark fermentative effluents (DFE) have the potential to serve as a carbon source for microbial growth and intracellular PHA accumulation [7]. The study by Mohammadi et al. [10] describe that organic acid obtained from acidogenic fermentation of palm oil mill effluent is an advantageous and affordable substrate for PHA synthesis. Whey is the main derivative of the dairy production possessing lactose as main sugar. The utilization of whey lactose for the manufacturing of PHA may possibly reduce the costs of production, without conflicting with the provisions for human food chain and concomitantly resolving an environmental hassle [1,11]. Rowaihi et al. [12] illustrated a two-phase submerged fermentation process where carbon dioxide is converted into PHB adopting hydrogen as source of electrons and energy. They utilized the strain acetogen *Acetobaterium woodii* in the first phase to transform CO_2 into acetic acid whereas in the second phase, another strain *Ralstonia eutropha* H16 was employed to transform acetate into PHB. There are numerous halophilic bacteria capable of using inexpensive substrates for PHA production proficiently [10]. It has been reported that

Halomonas halophila was able to harness hydrolysates of spent coffee residues for the production of PHB and achieved a PHB content of 61.95% of cell dry weight [13]. Huang et al. [14] utilized *Haloferax mediterranei* for PHA production. This organism can persist in a high-saline condition and this immensely decreases the sterility demands of a manufacturing amenity thereby reducing the capital cost.

The PHA producing bacteria use volatile fatty acids as substrates existing in the waste and in accordance with the variety of VFA provided to the culture, the PHA constituents/monomers vary which acts as a significant part in determining the characteristics of PHA [8]. The extensive application of PHB is largely restricted as PHB exhibits the characteristics of rigidity, fragility, and thermal instability [15]. Besides propionic acid, valeric acid has been selected as a precursor for 3HV units to attain a larger fraction of HV. Acetic acid generally contributes between 30 to 80% of VFA formed from waste effluents while the valeric acid and other kinds of VFA are generally produced or incorporated in fewer amounts. This variation in VFA composition may due to the presence of variety of the organic compounds present in the waste [16]. Reddy et al. [17] determined the composition of PHA restored from the mixed culture cultivated with the effluent produced from the hydrogen generating reactor at 6 g/L VFA concentration with varying time frame of 12–48 h. It was reported that the synthesized polymer acquired more hydroxybutyrate content (95%) and less hydroxyvalerate content (3%) for all the period of time because of large acetic acid and less butyric and propionic acid concentrations in the composition of VFAs.

2. Biochemistry and metabolism for PHA production

2.1. Major strains involved in PHA production (VFA utilizers)

PHA are synthesised by an extensive variety of microbes and are generally stored as intracellular energy under nutrient limiting conditions. Different types of bacterial strains are suitable for generating PHA. *Ralstonia eutropha* previously known as *Alcaligenes eutrophus* and currently

designated as *Cupriavidus necator*, is capable of producing PHA commercially on account of its maximum polymer components proportionate to the carbonaceous substrates. Furthermore, *R. eutropha* has the ability to grow on a variety of carbon sources [23]. In another literature, a newly separated Comamonas sp. EB172 was noticed to be a pertinent microbe for commercial production of PHA as it has the ability to concentrate the copolymers poly(3-hydroxybuyrate-co-3-hydroxyvalerate) from mixed organic acids [10]. Nowadays, the separation or identification of new halophilic microbial strains is an emerging trend which generally exhibit efficient PHA production from low-cost substrates. A large number of wastewater plants generate significant salty wastewater and halophilic microorganisms are broadly explored for their capability to withstand high salinity [24]. Archaea are regarded as extremophiles as they are revealed to exist at hot springs, swamps, oceans, salt lake, etc., to maintain their growth and used for producing PHA. They can be cultivated ideally at 5% and withstand less than 10% of sodium chloride [25]. The PHA can be generated utilizing the bacteria photosynthetically as they can agglomerate PHA enriched with carbon [26]. In a literature, it was shown that *Saccharophagus degradans* which is a gram-negative motile-proteobacterium effectively degraded and used cellulose as the main carbon source for cultivation and PHB generation [20]. Ciesielski and Przybylek, [18] found that *Thauera* sp. was dominant in a mixed culture supplied with high amount of acetic acid whereas *Paracoccus denitrificans* was identified as main biocatalyst when amount of acetic acid was very less compared to propionic acid. The red alga *Gelidium amansii* has gained concern in recent years as feedstock for PHA production. It can develop rapidly than land plants and can be grown in wide areas of the ocean in the absence of nitrogen fertilizers. Moreover, lignin is absent in *G. amansii* and there is no need to eliminate lignin, resulting in an inexpensive production of mono-sugar [27].

2.1.1. Acetic acid metabolic pathway

Mostly, the acetic acid is formed through chemical catalysis beginning from acetaldehyde, methanol, butane or ethylene, and presently, production of acetic acid by fermentation is procuring

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much interest. The free sugars are metabolized via glycolysis pathway into pyruvate followed by oxidative decarboxylation into acetyl-CoA mediated through either pyruvate dehydrogenase complex or pyrivate-formate lyase. The acetyl-CoA is further transformed into acetate by phosphoacetyl transferase and acetate kinase and emits out. There is another route via pyruvate oxidase for direct oxidation of pyruvate into acetate [28]. The synthesis of PHA involves coenzyme-A, and redox cofactors NAD(P)H/NAD(P)$^{+}$. The concentrations of acetyl-CoA and free coenzyme-A are crucial in regulating metabolic routes, especially PHA storage. NADH is involved in catabolic reactions, whereas NADPH has a significant role in reductive biosynthesis including PHA [29,30]. The mechanism consists of a series of three catalytic reactions. In the first step, catalytic condensation of two acetyl-coenzyme A molecules with β-ketothiolase encoded by phaA results in the generation of acetoacetyl-CoA with the subsequent release of a co-enzyme (R)-3-hydroxybutyryl-CoA by NADPH dependent acetoacetyl-CoA reductase encoded by phaB and finally, the co-enzyme (R)-3-hydroxybutyryl-CoA is polymerized to PHB by PHA synthase encoded by phaC [31]. Under conditions of nutrient limitation, acetyl-CoA is formed by the depolymerisation of PHB into 3-HB and this acetyl-CoA is acts as a carbon and energy source via TCA cycle. The amino acid metabolism also favours the agglomeration of PHB. A major fraction of acetyl-CoA formed from acetic acid is transformed to pyruvate and thereafter it is converted to serine and lipids necessary for the production of cell membranes. The PHB synthesis is thus enhanced by the energy produced by the remaining fraction of acetyl-CoA [32].

2.1.2. Butyric acid metabolic pathway

Generally, glucose or fatty acids are usually used as substrates for the PHA synthesis. For instance, PHA comprising of non 3-hydroxubutyrate monomers requires fatty acids as precursors for the generation of monomers of short chain (scl) or medium chain length (mcl). Cells will likely undergo β-oxidization to convert fatty acids into acetyl-CoA for cell growth. Through the β-oxidation route, there is an enhancement in the conversion of substrate into PHA. In this pathway,

the two significant enzymes involved are 3-ketoacyl-CoA thiolase (FadA) and 3-hydroxyacyl-CoA

dehydrogenase (FadB). The mcl PHA is formed by the removal of enzymes FadA and FadB where

the fatty acids are transformed into 3-hydroxyacyl-CoA [33]. Tobin and O'Connor, [34] studied the

accumulation of PHA by Pseudomonas strains namely *P. putida, P. oleovorans, P. Fluorescens, P.

jessenii and P. Citronellolis* from various aromatic hydrocarbons. It was reported that the PHA

accumulated from styrene and phenylacetic acid consisted of aliphatic monomers alone. There

emerges a wide variety among Pseudomonas strains regarding the relation between the amount of

accumulated PHA and the number of carbons on the side chain of phenylalkanoic acids. In another

literature report, Yoon et al. [35] investigated the generation of pure aromatic polysters, where

butyric acid was utilised as a co-substrate for a maximum production of cell biomass. Two phenyl

substituted alkanoic acids 5- phenylvalerate 5 PV and 6-phenylhexanoic acid were utilised as the

precursors of aromatic monomers. A putative metabolic pathway of 6-phenylhexanoate is

recommended based on the metabolic analysis. Subsequent to the passage into the cell, acy-CoA

synthetase transformed 6 PC into 6-phenylhexnoyl-CoA that moves into the ß-oxidation pathway

which in turn is transformed to (R)-3HPC-CoA through (R)-specific hydration catalyzed by (R)-

specific enoyl-CoA hydratase, eventually polymerised into PHA.

2.1.3. Propionic acid metabolic pathway

A. eutrophus can accumulate PHB up to 80% (wt/wt) on glucose as carbon source. It was observed

that a variable copolymer containing 3HB and 3HV monomer fractions is formed by the addition of

propionic acid with glucose [36]. When propionate is used, it is transformed to propionyl-

coenzymeA and undergoes condensation with acetyl-coenzymeA to produce PHV [37]. Catalán et

al. [38] generated 3HV utilising propionic acid as the source of carbon. A variant obtained from

Herbaspirillum seropedicae Z69 can produce P (3HB-*co*-3HV) from propionic acid, was

constituted to enhance the effectiveness of 3HV synthesis. The results indicated that the propionic

acid yielded 0.80 g g^{-1} of 3HV that was below the highest hypothetical value (1.35 g g^{-1}). In another

report, Andreeßen et al. [39] synthesized poly(3HP) biotechnologically using propionyl-CoA synthase, like (*pduP*) from *S. enterica*, and a PHA synthase, like (*phaC1*) from *R. eutropha*, by using 3-hydroxypropionate (3HP) as a precursor molecule.

Lactic acid metabolic pathway

Lactic acid is one of the major byproducts obtained during microbial fermentation, especially Enterobacter, and can be utilised for the production of biopolymeric materials. The most significant microorganisms pertaining to the species are *Lactobacillus*, *Bacillus* and *Rhizopus*. During the fermentation of lactic acid (LA), low pH has an hampering impact on the metabolic actions of the LA generating microbial cells. For the biological production of PHA, two fundamental routes should be developed for the production of lactate-containing polyesters. Initially, lactyl-CoA is formed by host strains via heterologous metabolic routes. Subsequently, the lactyl-CoA acts as the substrate and it is used by the PHA synthase. Lactate-containing polyesters possessing a variety of co-monomers have been formed utilising recombinant *E. coli* furnished with the biosynthesis of PHA comprising lactate. An extensive variety of PHA comprised of different hydroxycarboxylic acid monomers such as 3-HP, 3-HV, 4-HB has been produced by *E. coli* [40]. Tran and Charles, [41] recommended that the engineered PhaC enzyme has a wider substrate range as compared to PhaC from native *P*. *putida*. The engineered PHA synthase was primarily Type II PhaC1 synthase that acquires and polymerizes medium chain length 3HA monomers.

2.1.4. Valeric acid metabolic pathway

For the production of larger HV units, valeric acid was selected instead of propionic acid as an indicator for 3HV fractions. The catabolism of the acid to a shorter alkyl-CoA is not included for the production of 3HV from valeric acid. The PHB is produced by the formation of the intermediate 3-hydroxyvaleryl-CoA which is then degraded to propionyl-CoA and acetyl-CoA [42]. Glycolysis transformed glucose to pyruvate and then changed to acetyl-CoA resulting in PHB

synthesis, whereas the PHV is synthesized by the production of propionyl-CoA from pyruvate and valeryl-CoA formed from valerate under anaerobic condition [43].

2.1.5. Bioengineering of PHA synthesis pathways

Due to the high production cost and uncertainty on thermo-mechanical characteristics results in the limitation of large-scale commercialization of PHA. In recent times, advanced engineering for the manipulation of genetics on halophilic bacteria has been developed. Molecular engineering equipments have been developed to produce recombinant Halomonas spp. for the generation of copolymers consisting of 3HB and 3HV with the effective transformation of high substrate to PHA [44]. *Pseudomonas* species is regarded as a great example for the manipulation of PHA bioproduction from various substrates. For example, *P. putida*, a natural PHA manufacturer, is regarded as a model bacterium for biodegradation exploration and a construction path, mostly due to its high tolerance to solvents and oxidative stress conditions. Synthetic biology provided new tools for proposed genome engineering, for the formation of novel biopolymers [45]. **Table 1** shows the various biosynthetic pathways and engineering approach employed for PHA production.

2.1.6. Key enzymes involved

Research has been conducted extensively for analysing the abilities of enzymes for manufacturing different types of polymers including the ones which are not generally generated by the traditional inorganic catalysts. The enzyme catalysed polymerization contributes many benefits relative to the chemical processes like occurrence of reactions under moderate conditions of temperature, pressure and pH [40]. The enzyme PHA synthase (PhaC) is rather critical for the production of PHA in every microbial species, particularly for the species namely *Rhizobia, R, eutropha* etc. [7]. It has been reported that a very few synthases are capable of polymerizing both scl and mcl PHA. These enzymes are called low-specificity PHA synthases [54]. Furthermore, purple and non-sulphur bacteria produce and agglomerate PHB as an intracellular storage of carbon and energy under

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limited nutrient environment [55]. Another intracellular enzyme, dehydrogenase is necessary for the oxidation and reduction of various substrates that encompasses the transformation of acetate to acetyl-CoA, which is again transformed to PHA [17]. The synthesis of 3HB in microbes takes place with the presence of acetyl-CoA using PhaA (ß-ketothiolase) and PhaB (acetoacetyl Co-A reductase) [56]. The β-ketothiolase enzymes function "bio-Claisen" condensations on two CoA thioester molecules, thereby inducing a carbon-carbon bond [57]. Generally, the -ketothiolases/acetyl-CoA acetyltransferases pertain to the group of transferases or acyltransferases that convert groups excepting aminoacyl groups [58].

3. Effects of mixture and individual VFA rich streams on PHA production and its copolymers

3.1. Mixture of organic acids

Dark fermentation is the conversion of organic feedstocks into hydrogen through fermentative route with series of biochemical reactions. The process is manifested by numerous species of bacteria utilising the carbohydrates present in wastewaters and generating biohydrogen and an effluent enriched with VFA consisting of acetic, propionic, and butyric acids. In a literature, Cardeña et al. [55] studied the production of hydrogen and PHB by *Rhodopseudomonas palustris* using mixture of acetic, propionic and butyric acids. It was observed that a maximum specific production of PHB of 16.4 mg-PHB/g-TSS/day was obtained with mixture containing1370 mg/L acetic acid, 618 mg/L propionic acid, and 133 mg/L butyric acid. Acetic acid was used for the formation of PHB as it was the major constituent in the mixture acids (1235 mg/L) [55]. Ali Hassan et al. [26] ascertained the influence of organic acids on PHA formation. They obtained the organic acids, particularly acetic, propionic acid and formic acid by the anaerobic treatment of POME by palm oil sludge. They noticed that the formation of formic acid decreased the PHA synthesis and content whereas 0.50 g-g^{-1} of PHA yield and 67% of content were attained in the absence of formic acid. Lian-Ngit et al. [59] determined the potential of recombinant *E. coli* JM109 capable of utilizing both glucose and acid-based carbon sources and it was observed that the

PHA accumulation was noticed to be same but the cell dry weight was observed to be enhanced by the provision of nitrogen in the medium however; the 3HV synthesis was negatively affected in production of copolymer. Luangthongkam et al. [60] ascertained PHB and PHBV production using methanotroph-dominated mixed culture rich in methane and without methane with a blend of propionic and valeric acid. They reported that the formation of 3HV was influenced by propionic and valeric acid, however, a larger fraction of 3HV and PHA content was attained from the valeric acid. Boonsawang and Thongchai, [23] examined the impacts of propionic and butyric acid on the production of PHA by xxx. It was noticed that there was a reduction in biomass and PHA concentration, however, there was an increase in PHA content. Moreover, the influence of propionic acid was low on PHA content compared to butyric acid. **Table 2** shows the effect of individual and mixture VFA streams on PHA production.

3.2. Acetic acid

Acetic acid is considered as the most preferred VFA for the synthesis of PHA. Acetogenic bacteria, a former collection of specifically anaerobic microbes, are able to reduce carbon dioxide using hydrogen as the only source of energy and electron to produce acetate. Padovani et al. [67] employed either blue-green (cyanobacteria) or purple non-sulphur photosynthetic microorganisms (PNSB) for the production of bioplastics through photofermentation. It was observed that there was no generation of PHB while growing cyanobacteria in autotrophic mode. In contrast, it was feasible to attain a PHB concentration of 317 mg/L with the addition of acetate in mixotrophic mode. There was also a viable cogeneration of PHB and H_2 while cultivating PNSB, namely, *R. Palustris*. Acetic acid can be utilised thereafter by *C. necator* for accumulating biomass and PHA [42]. In a literature, Rowaihi et al. [12] investigated the transformation of acetic acid (3 g L^{-1} acetate) into PHB (0.5 g L^{-1} HB) using *R. eutropha* H16. Ciesielski and Przybylek, [18] exploited acetic and propionic acid as substrates. It was reported that in MMC supplied with higher amount of acetic acid, the prominent strain was related to the *Thauera* sp. whereas *Paracoccus denitrificans* was identified other than *Thauera* sp. with large fraction of propionic acid and little amount of acetic acid. Thus it was determined that acetic acid can be better substrate for bacteria related to *Thauera* species, while *P. denitrificans* is efficient for PHA production from

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propionate. Wang et al. [32] examined the enhancement in production of PHA using halotolerant bacteria, *B. cereus* strain HY-3. Acetic acid is a chief substrate acquired and perhaps utilised as the major carbon source for the 3HB formations. It was observed that for 0.5-5.0 g/L of acetic acid concentration, the highest 3HB units for the medium were found to be $41.0 \pm 0.415\%$ and $49.2 \pm 1.21\%$, respectively. Desouky et al. [62] enhanced the production of PHA by determining the optimum concentration of acetic acid using *Bacillus flexus* strain AZU-A2 as microbial cell factory. It was revealed that when acetic acid was added, there was a maximum production of PHA of 3.97 g/L was obtained in batch fermentation whereas in fed batch fermentation, 6.13 g/L of PHA production was achieved.

3.3. Butyric acid

Butyric acid is a possible building block for commercially important chemicals. Several microbial strains related to various species have been separated from different environments for the production of butyric acid, for instance, Butyrivibrio, Butyribacterium, Clostridium, Eubacterium, Fusobacterium, Megasphera, and Sarcina [28]. Although, butyric acid can be generally formed through chemical oxidation of butyraldehyde, there has been a growing attention in the bioproduction of butyric acid through fermentative route. During fermentation of sugars, butyric acid is obtained as the main end product produced by numerous anaerobic bacteria. Amongst them, *Clostridium* species have been utilised ideally for the formation of butyric acid since it requires simple medium for cell growth and reasonably high production [68]. Chakraborty et al. [6] examined the influence of individual VFA on PHA synthesis using the condensed corn solubles (CCS) as a means for the cultivation of *R. eutropha*. According to the results, the maximum cell population was attained at 5 g/L of butyric acid.

3.4. Propionic acid

Propionic or pentanoic acids are needed as substrates for the formation of copolymer P (HB-co-HV). The influence of propionic acid on cultivation and agglomeration of copolymer differs with every microbe. Ramsay et al. [69] studied the copolymer concentration by *A. latus, A. eutrophus, B.*

cereus, P. pseudoflava, P. cepacia, and M. halodenitrificans cultured on glucose and propionic acid. It was noted that the synthesis of P (HB-co-HV) by *A. latus* contained up to 3.4 mol% HV monomers, whereas *B. Cereus* stored up to 50 mol% HV monomers. The copolymer production by *A. Latus* was decreased with reduction in levels of propionic acid (0.01% (wt/vol)). When the concentration of propionic acid was above 0.05% (w/v), the production of PHA was completely inhibited. In contrast, with *B. cereus*, PHA production was inhibited at 0.1% (wt/vol) propionic acid and was completely stopped above 3% (wt/vol). Chung et al. [70] determined the production of the biopolymer, poly(3-HB-co-3-HV) acid, by *A. eutrophus* using propionic acid as a co-substrate. It was revealed that the cultivation of *A. eutrophus* was found to be inhibited by propionic acid at concentrations above 0.5 g/L. The growth inhibition was overcome by the adjustment of the pH. For the pH of 7.5, there was a continuous growth of *A. eutrophus NCIMB* 11599 up to 5 g/L of propionic acid concentration, while no growth took place at pH 6.5. Munir and Jamil, [71] utilised VFA and glucose as carbon source to check the potential of *Pseudomonas* sp. and *Bacillus* sp. as co-culture. They reported that the production of PHA was found to be less with pure culture but when the selected cultures were mixed, there was an enhancement in the production of PHA.

3.5. Lactic acid

Polylactic acids are a distinct bioplastic that can be utilised as a replacement to fossil-based polymers. Aslım et al. [72] recognized lactic acid bacteria and reported that the formation of poly-L-hydroxybutyrate based on percentage of cell dry weight of Lactobacillus strain was 6.6-35.8%. Jiang et al. [73] examined the formation of PHB using lactate and a blend of lactate/acetate. The mixed culture nourished on lactate was predominated by a new gamma proteobacterium and with this nourishment, the accumulation of over 90 wt% PHB was obtained within 6 h. Poly(lactate-*co*-ethylene glycol) copolymer is biocompatible and thus can be used for medicinal applications with improved properties [40]. Chakraborty et al. [6] determined the effects of VFA on the production of PHA using condensed corn soluble, a by-product of bioethanol production process for the cultivation of *R. eutropha*. It was reported

that the metabolism of the VFA support the cell growth and the lactic acid was exhausted at the fast rate superseded by acetic, succinic and propionic acids.

3.6. Valeric acid

Kemavongse et al. [74] determined the influence of propionate and valerate on the generation of copolymer. They found that the combination of acetate with propionate and valerate instigated the agglomeration of poly-β-hydroxybutyrate-co-β-hydroxyvalerate within the cell and achieved PHA content of 82.3% within cultivation period of 60 h. The results pointed out that when valeric acid was utilised as co-substrate, there was an increase in HV fraction from 0 to 84.77 mole %. Khanna and Srivastava, [75] examined the influence of adding valeric acid in the production of copolymer resulting in a maximum percentage of HV unit. It was reported that there was a reduction in the quantity of biomass production with increase in acid concentration because of hampering effect of toxic organic acids. At 30 h of valeric acid addition, synthesis of polymer was retarded due to the accumulation of huge quantity of PHB in the cells which resulted in the less uptake of valeric acid. Luangthongkam et al. [60] obtained a higher PHA content and 3 HV fraction with the addition of valeric acid. Chen et al. [76] developed a biosynthetic route for PHBV using carbon through the biosynthesis of threonine in *E. coli*. They over expressed the main genes occupied in threonine metabolism and created the determined routes of propionyl-CoA to 3-hydroxyvaleryl-CoA. It was found that 17.5 mol% 3HV fractions was produced by engineered *E. coli* from xylose.

4. Factors affecting PHA production using effluents from dark fermentation

4.1. pH

Among the feasible parameters, pH is considered as important parameter influencing the concentration of VFA. Chen et al. [77] assessed the formation of VFA by the combined fermentation of food waste and sludge subjected to varying pH conditions. The results revealed that there was an increase in total VFA production at pH 9, 7 and 11 but reduction was observed at pH 5 and also in the blank test. At pH 9, the maximum generation of total VFA achieved was 25,934 ± 1458 mg COD/L. Choonut et al. [61]

determined the effect of pH for the synthesis of PHB by *Alcaligenes eutrophus*. *A. eutrophus* was grown under the optimum conditions comprising of 50% stillage and 5 g/L yeast extract at a temperature of 30°C. The influence of pH on the production of PHB was studied by carrying out cultivation at different pH at (4, 7 and 9). It was reported that the highest PHB production was attained at pH 7 followed by pH 9 and 4. Pittmann and Steinmetz, [78] utilised primary sludge as substrate to examine various tests for the production of PHA and analysed the effect of pH (6, 7, 8, 9). It was observed that there is no much variability in the peak VFA concentration at pH 6and 8. The maximum VFA concentration of 18,286 mg/L was attained at a pH 7. Desouky et al. [62] examined the effects of pH on cultivation and accumulation of PHA by *Bacillus flexus*strain AZU-A2 with varying pH between 5.0 and 9.0 during fermentation. It was observed that the DCW was found to increase at pH 5. On the contrary, at pH 7.5, there was a maximum production of PHA (2.04 g/L), recovery yield (46.4 (%, w/w)), and productivity (0.057 g/L/h). Chakraborty et al. [6] optimized the CCS as a medium for the culture of *C. necator* and to assess the influences of individual VFA on PHA production. It was noted that the optimum pH for *R. eutropha* is 7. The value of pH for 240 & 80 g/L CCS media and nutrient broth, was 8.2, 8.7 and 8.5, respectively. The reason for the increment in pH of the CCS medium was probably due to utilization of organic acids. Villano et al. [47] determined the effect of pH on PHA synthesis by mixed cultures on the enriched biomass in a sequential batch rector (SBR) and also accumulation of PHA in batch tests. The rates and yields of copolymer P (HB/HV) were found to be decreased as the pH increased from 7.5 to 9.5. It was reported that there exists steadiness in the composition of microbial population at pH 7.5, with *hyalina* as the predominant bacterial strain.

4.2. Feeding regimes

The generation and constituents of PHA differed with feeding regime applied. Feeding regime alters the monomers compositions of the generated PHA. Chen et al. [77] correlated the impact of various feeding regimes viz. one time (A1), three times (A2, A3) and continuous pulsed feeding regimes (A4) on PHA production. It was found that feeding one time increase the VFA concentration rapidly resulting in the synthesis of high PHA and subsequently reduced because of consumption of VFA. For three times and

continuous pulsed feeding, the rate of PHA production was usually enhanced through feeding in batches related to the feeding one-time. During the continuous pulsed feeding regime, there was an enhancement in the PHA and VSS concentrations showing the maximum PHA production with the reduction in dissolved oxygen (DO). Albuquerque et al. [4] assessed the impact of continuous feeding regimen on the composition of polymer. It was reported that the HV content increased from 31 to 39% compared to pulse wise feeding. This is probably due to the fact that fractions of propionate and valerate in pulse feeding assays were considerably less than acetate and butyrate and consumed initially and the presence of these organic acids restrict the formation of HV. Employing certain techniques during feeding may enhance the PHA productivity and constituents with the selected substrate [8]. The primary concern related with fed-batch fermentation is to control the concentration of substrate in the optimum value in connection with restricting and hampering levels. Hence, the feeding of substrate approach performs a vital role in acquiring high cell density cultures with PHB effectively. The batch and fed-batch cultures were executed for the PHA accumulation. It was reported that among three feeding regimes namely pulse, stepwise and continuous feeding regime, the maximum PHA accumulation was attained by continuous feeding (we have already said it above). The maximum PHB production is accelerated by the continuous fermentations especially for the cultures of maximum distinct growth rates, yet the implementation of this growing strategy commercially for the synthesis of PHB has been restricted as the continuous feeding is greatly susceptible to contamination resulting in big losses financially [79]. **Table 3** shows the various fermentation process, operational conditions and enhancement strategies for PHA production.

4.3. HRT

Hydraulic retention time (HRT) can be stated as the average duration where the substrate and biomass persists in a reactor. Hence, it is considered as a significant factor, because it determines the daily flow rate clarified into the reactor. HRT must be sufficient for the solubilisation of complex organic substances, hence encouraging the successive acidogenic fermentation of hydrolysates [85]. In a literature report, it was reported that the retention time is based on the nature of substrate as a

consequence of hydrolysis. On the other hand, HRT can vary with the same substrate because retention

time not only relies on the nature of substrate but on other operating parameters [86]. In a literature ,

Mohammadi et al. [10] recovered PHA by means of NaOH at HRT greater than 1 h to obtain maximum

purity and yield of recovery from a novel strain *Comamonas* sp. EB172. It was identified that PHA

yield of recovery from *Comamonas* sp. EB172 had no prominent variation in purity when incubated at

varying digestion times. This is because of rapid rate of hydrolysis of PHA fragments using sodium

hydroxide. In addition, the increase in digestion time from 1 to 5 h makes the quantity of protein

discharged from the cells to the supernatant constant at 65–66%. Consequently, the recovery of PHA

from Comamonas sp. EB172 by NaOH having the purity of 62.4% and recovery yield of 79.3% was

obtained with optimum digestion time of 1 h. Pittmann and Steinmetz, [78] conducted   the experiments

with the cycle time of 24, 48 and 72 h. It was observed that the quantity of PHA produced depends on

the span of the feast/famine-stage illustrating a longer cycle time resulting in a shorter feast-stage. This

may be due to long starvation period. The result revealed that the highest PHA concentration of 28.4%

cell dry weight was achieved.

4.4.   OLR

Organic loading rate (OLR) represents the quantity of food wastes subjected into the reactor per day and

per unit of working volume. The selection of an optimum OLR is essential for attaining a balanced

production of VFA. For this, OLR must be substantial to contribute an sufficient quantity of carbon

source to metabolism of fermentation, based on working temperature [85]. In literatures, it was

recommended that there is a direct relationship between the increases in organic load to PHB

accumulation. In feast phase when there is an availability of high substrate, it takes much time to

accumulate high PHB whereas less organic loads lead to rapid production of PHB. Amulya et al. [87]

exploited the spent wash wastes as substrate for the generation of PHA using *Bacillus tequilensis* as

biocatalyst along with five different OLRs as 0.66; 1.32; 1.98; 2.64 kg COD/m^3-day. For all the OLRs,

it was noticed that the maximum PHA production occurred within 36^{th}h of operation besides OLR4. The

maximum production of PHA (40%) was attained for OLR2. The high concentration of substrate creates

an osmotic shock which constrains bacterial growth. Mohan [88] examined the utilisation of novel isolated bacterial strain *Serratia ureilytica* for PHA production with VFA as substrate at varying OLRs. The maximum PHA production (51% dry cell weight) was achieved with OLR2 at 24 h, while the OLR1exhibited the maximum removal of substrate (84%). Reddy and Mohan, [43] synthesized PHA utilising wastewater as substrate and mixed culture as biocatalyst and studied the effect of substrate load and concentration nutrient (N and P). At high substrate load, the accumulation of PHA was observed to be maximum for OLR3 with 40.3% of dry cell weight, under N and P limited conditions.

4.5.   Molecular mass distribution and polydispersity index

PHB has been applied in the field of medical and pharmaceuticals depending on the biocompatibility and biodegradability where the molecular mass is considered as a significant aspect as it demonstrates the mechanical properties of the polymer. The determination of the molecular mass of PHB using novel strains substantially enhance the properties of PHB, extending the possible utilization of this polymer particularly in the medical field [89]. The thermoplastic and crystallization properties of Poly-(3-hydroxybutyrate) are largely based on its molecular mass. The low molecular masses ($<1 \times 10^3$ kDa) of P(3HB) are described by their fragility and thermal degradation when the melting temperature is above 180°C. In general, PHA synthases can be classified into four types having molecular masses within 61 to 73 kDa. Higher molecular weight polymers are synthesized by Class I enzymes relative to class II enzymes, within 500 kDa to many millions and from 50 kDa to 500 kDa, respectively. The intermediate PHA are produced by class III and class IV synthases [90]. The bacterial production of P(3HB) and other PHA possess high molecular mass with the polymer characteristics identical to traditional plastics namely polypropylene [91]. Fernández et al. [65] generated a calibration curve using polystyrene standards with low polydispersity to determine the product molecular weights of PHA (polyhydroxyalkanoate) using TOA (technical oleic acid) as the source of carbon. The average molecular weights were found to be 39.148 Da Mn and 54.783 Da Mw with a low polydispersity index of 1.4 whereas the values for Mw and Mn are less for TOA by *P. putida* with 2.53 as polydispersity index. Prieto et al. [66] examined that in a recombinant PHB producing *E. coli* strain, the molecular

weight of the PHB is managed by the activity of PHB synthase. The polymer chain length is controlled by the enzymes and it was found that bigger the synthase molecules, the chain length of the polymer is shorter. The production of medium chain length PHA using *P. oleovorans* POMC1 had molecular weights varying between 180,000 and 230,000 relying on the concentration of the inducer. Matsumoto and Kageyama, [92] noticed the enhancement of molecular weight of P(2HB)at a threshold temperature of 32–34^{0}C whose value was near to the glass transition temperature (*T*g) (30∘C).

4.6.   Physical properties

PHA vary in composition and properties as homo or copolymers based on the variations in structure of monomers [93]. The side chain length and the molecular weight affect the properties of the polymers. The short chain length PHB is a extremely crystalline polymer which is brittle, whereas medium chain length PHA vary from rubber to sticky polymers [94]. PHB shows identical properties as polypropylene which includes melting point, tensile strength and degree of crystallinity, nevertheless the polymer on crystallization becomes fragile and shows less resistance to stress. Therefore copolymerization with 3-HV enhance the properties of polymer by increasing ductility and exert influence on resistance and reduce processing temperatures [95].PHA can dissolve in chloroform and other chlorinated solvents. The elastic modulus specifies the stiffness of PHA and it varies from flexible medium chain length to brittle short chain length PHA. The capability of any substance that can be elongated without any breakage is known as elongation at break [8]. The copolymer P (3HB-*co*-LA) showed appropriate thermal behaviour and also possesses enhanced mechanical properties, including less viscosity and dynamic moduli. The properties, such as rigidity, malleability and optical lucidity is enhanced by mixing medium chain length with polylactic acid by means of melt-mixing method to obtain a different copolymer [41]. The physical and thermal properties of copolymer poly (3-HB/3-HV) is improved, based on the HV unit. As the portion of hydroxyvalerate increases, there is a considerable reduction in melting temperature without having any impact on degradation temperature, thereby providing the structural properties and process ability of polymer with identical to those of polypropylene [47]. The production of PHA by fermentation using the substrates containing aromatic groups, allows reasonable

changes in its physical properties due to the possible interactions among aromatic rings. The temperature of decomposition and crystallinity of polymers is modified as a consequence of the length of the acyl side chain of substrate utilised in the fermentations [96].

4.7. Biocompatibility and biodegradability of PHA

Biodegradability and biocompatibility are essential aspects of PHA. An attractive aspect of PHA that make a difference from conventional petroleum based plastics is their biodegradability. The ability of the polymers to be deteriorated by microorganisms is known as biodegradation. Biocompatibility is described by the form and surface porosity of the component and also the devoid of any harmful compound produced during the degradation of polymer. These biodegradable polyesters are steady in air, idle and are insoluble in water. The extracellular PHA depolymerases produced by the degrading microorganisms transform the polyesters into water-soluble oligomers and monomers which are utilized by the microorganisms as a carbon source [97]. The renewable resources used for the production of bioplastics do not contribute to the reduction of limited resources. Numerous bioplastic materials such as polyhydroxyalkanoates, polylactides, aliphatic polyesters, polysaccharides, and mixtures of these, have been produced effectively and are of immense significant with several possible commercial applications [95]. PHA are degraded biologically by a huge group of microorganisms using the enzymes PHA hydrolases and PHA depolymerases. Depending on the composition of the polymer, its physical nature, and significantly, the environmental conditions, the activities of these enzymes differ [91].

5. Various fermentative mode and kinetics of PHA production

5.1. Batch system

In batch fermentation, the essential nutrients and carbon sources are supplied at the commencement of fermentation which is inadequate to support the growth demands of the bacterial cells exponentially. This turn out to be a constraint for the effective progression of the growth of bacterial population and production of copolymer, although the cell density is supported by the medium [49]. Desouky et al. [62] specified or identified *Bacillus flexus* strain AZU-A2 by 16S rRNA gene sequence and the factors such as physiology and nutrition that affect the generation of PHA were optimized in batch

fermentations. At 24 h of batch fermentation, a maximum of 3.97 g/L of PHA production and 88.0 (%, w/w) of the recovery yield was attained in optimized medium. Alkotaini et al. [27] produced PHA utilizing the acid-pretreated red alga (*Gelidium amansii*) as an inexpensive source of carbon existing abundantly through batch and fed-batch cultivation. It was noticed that the dry cell weight was increased to 5.5 g/L which is less in comparison to fed-batch fermentation (10.1 g/L). In another study, He et al. [68] produced butyric acid in controlled pH batch fermentation and determined the optimum pH for cell growth as 6.5 and the formation of butyric acid as 12.25 g/L with a cell yield of 3.65 g/L.

5.2. Fed batch system

Fed-batch culture is widely applied practice in industry and can avoid the inhibition caused by high substrate levels through intermittent feeding to maintain the substrate concentration below the toxic levels. The substrate inhibition caused due to high organic acids concentration results in the destruction of the transmembrane pH gradient of the bacterial cells whereas the cell growth and PHA accumulation occurs at lesser concentration [98]. Through the fed-batch culture, consumption of dissolved oxygen occurs rapidly and there was a quick decline in redox potential accompanied by surfacing off the redox potential signal which perhaps associated with the fast accumulation of PHA [82]. Zhao et al. [15] determined the concentration of PHBV as 14 g/L and CDW of 29 g/L in fed batch fermentation whose value was four and eight-fold more than that attained in the batch process respectively. Desouky et al. [62] obtained a highest production of PHA as 6.13 g/L in fed-batch fermentation. Wisuthiphaet and Napathorn [21] obtained the PHB production of 16.9±0.2 g/L with a productivity of 0.234 g PHB/(L h) through fed-batch cultivation and observed 70% reduction in production related to batch cultivation. To increase the PHB concentration and to evade inhibitions of substrate simultaneously, fed-batch fermentation approach has been studied by many researchers. Nevertheless, the performance of fed-batch strategy requires advancement of an on-line monitoring and control system for assessment of remaining substrates and addition of fresh nutrients [22].

5.3. Repeated fed batch

An easy and widely known cultivation approach, which may be advantageous in improving generation of PHB, appreciably high cell growth is repeated-batch cultivation [99]. Write few lines about what is repeated fed-batch cultivation. Huang et al. [14] studied the repeated fed-batch culture for the production of PHA by an archaea, *Haloferax mediterranei* and acquired 140 g/L of cell concentration, 77.8 g/L of PHA concentration, and PHA yield of 55.6 wt.%. For the bulk production of PHA under highly alkaline condition and without nitrogen limitation, this process can be withstood over a long period. Gahlawat, [22] employed a drain-and-fill approach of repeated-batch cultivation for the improved generation of PHB utilising *Azohydromonas australica*, about 20 % (v/v) of the cultivation stock was extracted from the reactor and provided with an equal volume of fresh medium. This approach indicated a 3.3 and 1.8 times enhancement in concentration and productivity of PHB respectively as related to batch cultivation.

5.4. Continuous Fed batch

Continuous fermentation strategy has attained attraction of sustaining steady nutrient condition and is beneficial to explore the impact of nutrient constraint on the cultivation of bacteria and productivity in a rapid and contemporaneous way [98]. The continuous fermentation strategy is a widely known technology for attaining maximum productivities, particularly for strains with high rate of specific growth, lesser manufacturing costs and a stable product quality in biological engineering processes [3].

5.5. Feeding strategies

5.5.1. Single pulse carbon (glucose) feeding

Hafuka et al. [100] examined the production of PHB and studied the influences of various feeding regimes namely single pulse, stepwise, and continuous pulse of fermented food-waste effluent. The extracted fermentation broth was strained with a membrane filter to eliminate anaerobic microbes and solids and utilised as a carbon source for *C. necator*. It was indicated that the single pulse feeding produced the maximum cell concentration of *C.necator*. But the PHB concentration was more in the stepwise and continuous feeding regimes. The single pulse feeding regime led to higher growth rate of *C. necator* possibly due to more concentration of VFA.

5.5.2. Feed-forward control

During the generation of PHA, it is not possible to measure the PHA concentration online; hence, the indirect method generally controls the storage process. The dissolved oxygen indicates to handle the reserve of PHA, and the excessive DO represents the substrate consumption and high PHA composition [101]. Zeng et al. [83] determined the PHB production in SBR and feed on demand control (FD) with the length of feast stage of the SBR and FD experiments were 5.6 h. It was noticed that the substrate fed by FD control was about 1.5 times more suggesting maximum uptake rate of substrate. Thus, the FD control was therefore beneficial to substrate metabolic rate and storage process, subsequently checking the impact of substrate hindrance on storing PHB.

5.5.3. Intermittent carbon feeding

Chakraborty et al. [84] determined the cell dry weight, concentration and content of PHA both by intermittent and continuous feeding strategies utilising the substrate condensed corn soluble for the culture of *R. Eutropha*. The results revealed that there was no much difference in both the feeding strategies except the value of PHA productivity was found to be higher in the intermittent feeding strategy. Beccari et al. [102] characterised the storage response of mixed culture cultivated on an acetate-limited medium using intermittent feeding and they noticed maximum storage capacity for the selected bulking sludge. Omar et al. [64] generated PHB by *Cupriavidus necator* utilizing organic acids arising out of fermented kitchen waste employing intermittent feeding strategy. It was reported that 0.242 g/L/h of PHB production was obtained in fed-batch culture by applying intermittent feeding which was found to be 4 times larger than that of PHB productivity in batch culture.

5.5.4. Feast -Famine conditions

In feast-famine condition, intermittent feeding was adopted. In the feast phase, excess carbon source was provided where the growth of microorganisms occurs and the carbon is stored in the form of polymers and in the famine phase, there is no addition of carbon with the exhaustion of reserved carbon for maintaining growth. The metabolic RNA and enzymatic activity of cells are low due to the famine

phase and when exposed to feast phase leads to carbon storage thereby making a selection pressure for microorganisms suitable for storing PHA [81,103]. The feast and famine regime is widely identified as an prominent method for PHA accumulation by mixed microbial culture within aerobic systems [104]. Fradinho et al. [81] suggested a novel approach for selecting photosynthetic PHA accumulating bacteria through a permanent feast method. The results revealed that the implementation of permanent feast strategy with the availability of higher light is regarded as the best choice that results in an enhanced production of PHB with photosynthetic mixed cultures. Whereas, there was a reduction in PHB productivity per light input when the system is exposed to feast and famine strategy under dark/light conditions. Morgan-Sagastume et al. [105] examined the viability of producing PHA under feast–famine and it was found to increase up to 34% (g PHA g VSS^{-1}) with acetate during 20 h in batch accumulations. Amulya et al. [80] examined the PHA production using food waste as feedstock via feast famine strategies. The reaction (R1) was first performed for a cycle span of 24 h. They reported that there was an enhancement in PHA production until 8 h in 24 h operation and decreased gradually afterwards till the end of the run. The highest production of PHA was observed to be 12.6 ± 0.25% DCW during the feast phase and at the end of the cycle, the production of PHA was decreased to 2.7 ± 0.05% DCW. It was noticed that by reducing the cycle span from 24 to 12 h resulted in an improved PHA production. Korkakaki et al. [19] determined the possibility of utilising a mixed substrate. The activated sludge, fed with a blend of acetate–methanol is introduced into a bioreactor and handled aerobically causing a feast/famine regime in a period of two weeks of operation. The DO concentration was found to decrease distinctly subsequent to the feeding phase denoting the reduction of the exhausted carbon, acetate. The concentration of PHB was observed to reach its maximum value at 108 min at the end of the feast phase when acetate was depleted.

6. Commercial potential of microbial PHA production

The economic analysis is assessed by computing the cost of raw material which contributes to the PHA's production cost. The high production costs of PHA are determined to be 20–80% more than petrochemical based polymer, act as a main predicament for their commoditization Fernández-Dacosta

et al. [106] investigated the economic importance by including total capital, component and exploitation of energy costs that consider the major costs of total PHB production. They determined the total investment cost using distinct aspects for delivered equipment costs. The result revealed that the total synthesis costs, inclusive of monetary credits, were: alkali surfactant - 1.40 €/kg PHB, surfactant hypochlorite - 1.56 €/kg PHB and dichloromethane solvent - 1.95 €/kg PHB. It was observed that the costs of downstream is more than upstream. In another literature, Dacosta et al. [107] assessed the economic analysis which indicated the total synthesis costs of 1.56 €/kg PHB including fermentation, extraction of PHB and the credits attained without wastewater treatment. They also reported that the downstream processing costs exceeded the fermentation cost, accounting for 73% of the whole manufacturing costs. Akiyama et al. [108] forecasted the cost of PHA copolyester synthesis through fermentation utilizing soybean oil as the source of carbon. They calculated the yearly cost of PHA production copolyester as 3.5-4.5 US$/kg, relying on the assumed biosynthetic process efficiencies. Likewise, the cost of large-scale synthesis of P (3HB) from glucose was determined as 3.8-4.2 US$/kg. Leong et al. [109] reported that the cost of crude glycerol as 0.362 US$/kg PHA considering it as the cost-effective substrate, subsequent to refined glycerol (0.484 US$/kg PHA) because they provide a good yield of biodegradable polymer. Leong et al. [110] assessed the purification and recovery strategies of PHA production process economically and environmentally. The economic analysis is based on the annual production of PHA of 9000 tons and it was estimated at 5.77 US$/kg with a remuneration period of less than 4 years and return on investment of 25.2%. They demonstrated that the utilization of ATPE results in the reduction of consumption of chemicals and also the cost of downstream material and wastewater treatment. **Table 4** shows the economics of PHA production using individual VFA and VFA rich streams.

7.   Challenges associated with PHA production

7.1.   Downstream processing cost

The recovery process of intracellular product becomes more challenging compared to metabolite secreted in extracellular medium as PHA are the reservoirs of energy and carbon sources that are

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stored within the host cells. Generally, the PHA recovery is a complex operation which needs more than one unit operation. The factor that will be much affected by the extraction process is properties of PHA. The recovery system of PHA from the cell biomass is a significant aspect that affects the characteristics of the constituents of biopolymer PHA and the processing cost [117]. Moreover, the recovery process affects the type of microorganism and culture conditions. Mixed microbial cultures (MMC) are said to be more immune to cell hydrolysis compared to pure cultures. Chloroform, propylene carbonate, methylene chloride and dichloroethane are the solvents used for the recovery of PHA in an immensely pure form [118]. The main restraining factor for the commercialisation of PHA production is their high cost [117]. It has been assessed that the recovery and processing of the polymer accounts for half of the production cost. The recovery process is found to have a huge impact on generation costs, therefore there is a need for the effective methods for the recovery and purification of PHA to produce biodegradable plastics from sustainable resources in an economical and ecofriendly system [119]. It is therefore obvious, that the downstream processing chain of PHA starts from cell lysis of the biomass isolated from the matrix by centrifugation, in order to reveal the entrapped cell product [120]. López-Abelairas et al. [113] compared the acid and alkaline treatments for the PHB recovery from *C. necator* H16 cells and they reported that the cost of downstream processing with the use of sodium hydroxide (1.02 €/kg) and sulphuric acid (1.11 €/kg) is found to be low when compared with other alkaline solutions such as sodium chlorite and sodium hypochlorite and selected the acid treatment as the best option for the recovery of PHA.The PHA composition affects the effectiveness of the downstream process and the yield and they are interrelated based on the composition of PHA. It was observed that a comparatively less P(3HB) content of 50 wt% of the cell dry weight resulted in an expensive recovery of $4.8/kg P(3HB) relative to the cost of recovery process of 88 wt% P(3HB) content as $ 0.92/kg P(3HB) [121].

7.2.   Using mixed microbial culture

The choice of culture with a prominent storage capacity of PHA is of major concern in the process of production by mixed culture. The culture exploited for PHA synthesis greatly effects the performance system, along with the capital and operation cost. For the commercial production of PHA, mixed cultures have been recommended because they are cost-effective and they do not need aseptic environment and facilities for an axenic biological process [103,122]. The major bottleneck that are met using MMC are the cell concentrations and volume productivities [20]. The culture selection is another main challenge for PHA accumulation and in order to enhance the volumetric productions, selecting conditions should be focussed towards the enrichment of cultures with maximum storage and cultivation rate. Two types of enrichments such as anaerobic/aerobic and aerobic dynamic feeding (ADF) are adopted for the production of PHA using mixed culture. The main restraining factor under ADF is the availability of carbon substrate for enhancing the accumulation of PHA. Johnson et al. [123] utilised enriched MMC for the production of PHA with 89 wt % of cellular content in fed-batch mode within 7.6 h under growth limiting conditions. Verlinden et al. [124] evaluated that the utilisation of mixed cultures namely activated sludge can enhance the effectiveness of fermentation in the production of PHA that impart a reduction in the cost of PHA and hence improve their market potential along with the reduction in environmental impact of PHA production.

8. Conclusions

The utilization of synthetic plastics extensively is a universal concern to our ecosytem and biopolymer production by microorganisms is a prominent key to prevent this predicament. PHA is a promising biopolymer which is not only biodegradable but also sustainable as it can be manufactured using a variety of pure as well as crude renewable sources. The two major factors which are holding back the commercial production of PHA are high cost of feedstocks and expensive downstream processing. Therefore, a lot of research and development work is needed to curb the elevated manufacturing cost of PHA and make it cost-competitive opposed to fossil-based

plastics. The use of inexpensive substrates, designing of promising microbial systems using metabolic engineering and synthetic biology tools to generate PHA rich biomass in short time, bioprocess optimization and an economical product recovery might pave the way for industrial production of PHA.

Acknowledgements

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Fig. 1 The concept diagram of biopolymer production from dark fermentative effluent.
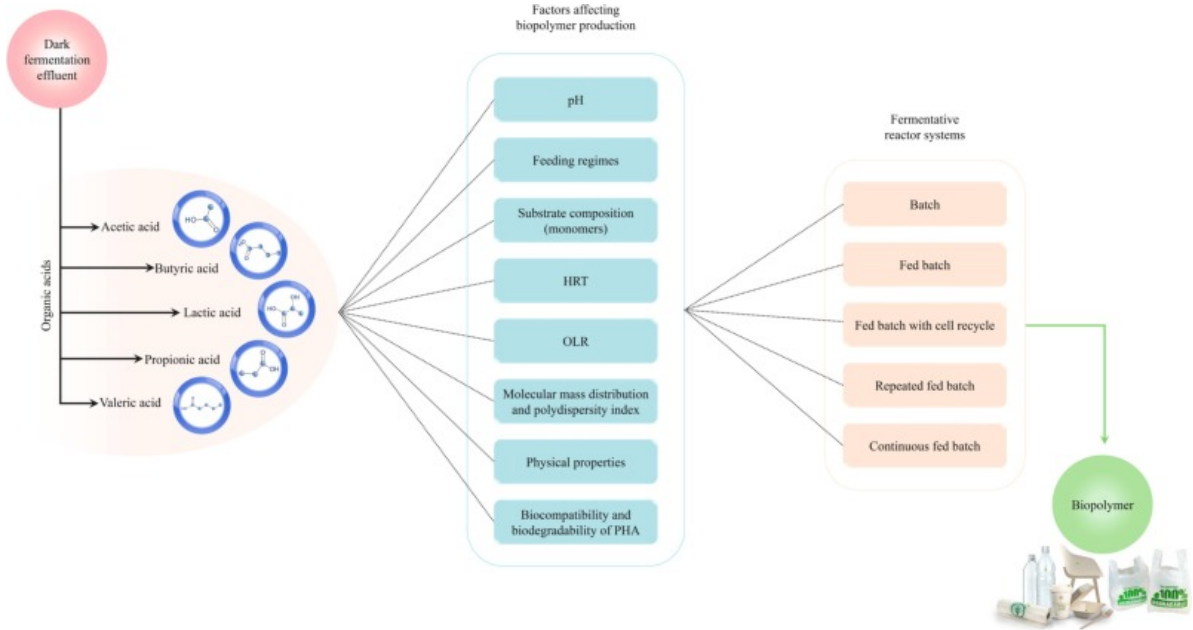

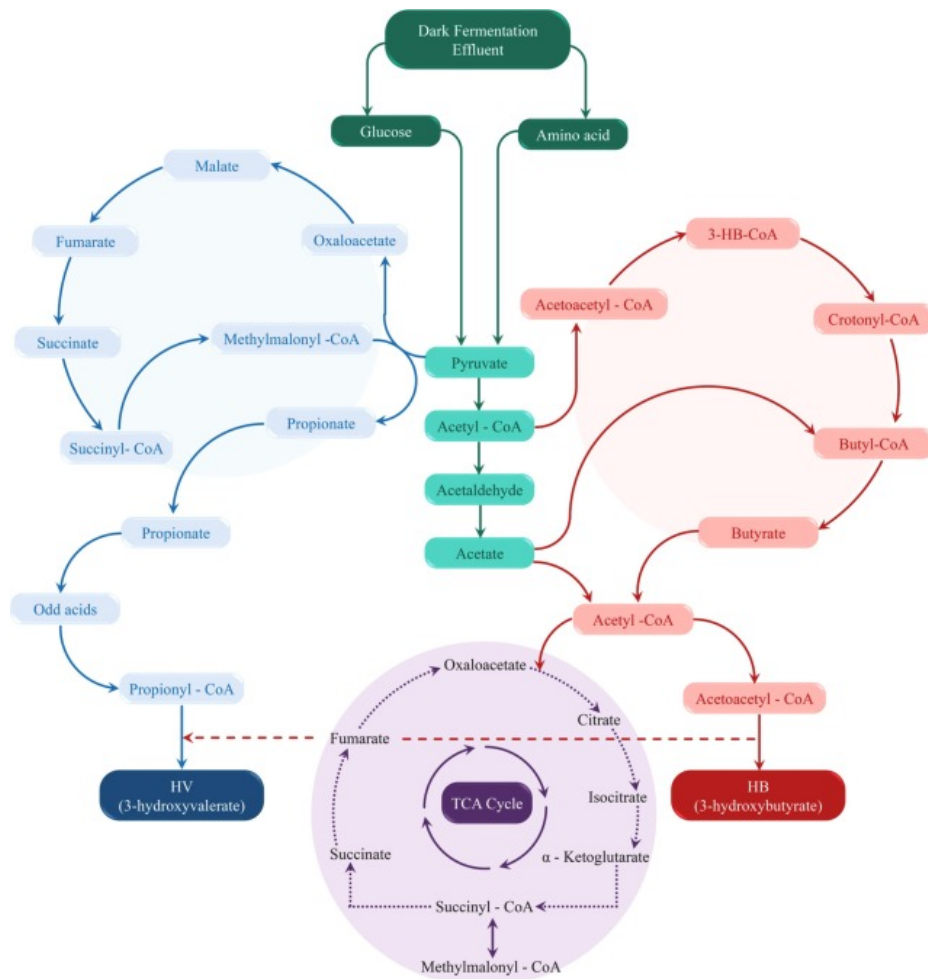**Fig. 2** Biosynthetic pathways for PHA production.

Table 1 various biosynthetic pathways and its bioengineering/modifications for PHA production.

S No	Type of VFA	Strain involved	Biosynthetic pathway	Bioengineering/modification	Type of PHA	Key findings	Reference
1	Acetic acid	*Bacillus cereus* strain HY-3	PHB biosynthetic	threonine, serine, malate, and pyruvate,	PHB	PHB content - 49.2 ± 1.21%	[32]
2	Propionic acid	*Herbaspirillum seropedicae* Z69	propionyl-CoA	2-methylcitrate synthase (PrpC) gene	3 HV	3HV yield - 0.80 g/g	[38]
3	Fatty acid	*E. coli*	ß-oxidation	yeast extract improved cell growth	P(3HD)	5.4 g/L P(3HD) containing 99.6 mol% 3HD units	[46]
4	Acetic and propionic acid	*Lampropedia hyalina*	Acetate and propionate	-	Hydroxyl valerate (HV)	HB/HV - 0.53 COD/COD HV - 48% (mol/mol)	[47]
5	Glycerol	Recombinant *E.* XL1-Blue	PHB biosynthetic	filamentation-suppressed FtsZ	PHB	PHB concentration - 149 g/L	[48]
6	1,4-butanediol 1,6-hexanediol	*C. malaysiensis* USMAA1020	TCA pathway	additional *phaC* gene into *C. malaysiensis*	PHA	PHA concentration – 46 g/L yield (Yp/x) - 11.5 g/g	[49]
7	Soy bean oil	*Cupriavidus necator* DSM 545	ß-oxidation	Carbon source	PHB	PHB content - 81% (w/w) PHB productivity - 2.5 g l^{-1} h^{-1} Yp/s value was 0.85 g g^{-1}	[50]
8	Acetate	*Escherichia coli*	Acetic acid metabolic	overexpression of *pta-ackA* and *acs* genes	P3HB	1.27 g/L P3HB	[51]
9	Glycerol with propionic acid	*S. enterica*	propionyl-CoA	*prpC*, as a host	PHBV	34.2 ± 15 (%DCW)	[52]

| 10 | Propionic acid | *Burkholderia* sp. IPT 101 | a-oxidation of propionate | *prp mutants* | 3 HV | Y3HV:Prop - 1.20 g g^{-1} | [53] |

Table 2 Effect of individual and mixture VFA streams on PHA production.

S No	VFA derivatives/type	Reactor employed	Microbial strain used	Operational conditions	PHA yield (g/L)	Accumulation efficiency (%)	Characterization employed	Reference
1	Acetic acid	Single use stirred tank reactors	*A. woodii* (DSM 1030), *R. eutropha* H16 (DSM 428)	Temp - 30℃ Speed – 2800 rpm	PHB – 0.5 g/L	PHB -33.3%	-	[12]
2	Acetic, propionic,butyric	SBR	*Thauera* sp *P. denitrificans*	Temp - 39℃ pH -7	3 HB – 227.8 mg/L, 3 HB and 3 HV – 673 mg/L	PHA – 34.2%	-	[18]
3	Propionic and butyric acid	-	*R. eutropha*	Temp - 30℃ HRT – 7 days Speed – 200 rpm	PHA -0.7 g/L	PHA – 46.5%	-	[23]
4	Acetic acid	flask	*Bacillus cereus* strain HY-3	pH – 7 Temp – 30℃ Speed – 160 rpm	-	PHB – 49.2%	-	[32]
5	Acetic, propionic, butyric	-	*Alcaligenes eutrophus*	Temp –30℃ pH – 4,7 9	PHB – 5.75 g/L	PHB – 86.50%	-	[61]

6	Acetic acid	Flask – 250 mL	*Bacillus flexus* strain AZU-A2	Temp - 37℃ Speed – 150 rpm	PHA – 3.97 g/L	-	-	[62]
7	Lactic acid and acetic acid	Glass jar	*R. eutropha*	Temp– 30℃ Speed – 200 rpm	PHA – 1.30 g/Lh	PHA – 73.1%	Mw - 0.1–2.0 × 10^6 PI - 1.6	[63]
8	Lactic and acetic acids	Bioreactor	*C. necator* CGUG 52238	Temp – 30℃ Speed – 160 rpm	0.79 g/g	PHB - 84.54% w/w	-	[64]
9	Oleic acid	flask	*Pseudomonas* 42A2 NCIMB 40045	pH 6.80, Temp - 30℃ Speed – 100 rpm	-	54.6%	Mw - 54.7 Da	[65]
10	Bacteria	bioreactor	*Pseudomonas oleovorans*	Temp - 37℃ flow rate - 1.4 L min^{-1}	-	20.6% of CDW	Mw - 180,000, PI – 2.7	[66]

Table 3 Various fermentation process, operational conditions and enhancement strategies for PHA production

S No	Fermentation process	Enhancement strategy regime	Microbial strain used	Operational conditions	PHA yield	Highlights	Reference
1	Repeated fed batch	pH-stat control strategy	*Haloferax mediterranei*	pH – 6.9 – 7.1 Temperature – 37℃ Speed – 800 rpm	PHA – 77.8 g/L	Under a hyper-saline condition and with no nitrogen limitation restriction, results in the mass production of PHA.	[14]
2	Continuous batch	Feast-famine	Mixed culture	Temperature – 30℃ pH – 7 stirring speed – 750rpm	0.62 C-mol C-mol^{-1}	kinetic properties of the PHA-producers determine the efficiency of the process	[19]
3	Fed batch	-	Azohydromonas lataDSM 1123	Temperature – 30℃ pH – 7 agitation – 200 rpm	PHB - 0.40 g PHB/g sucrose	77% cost reduction with 60-Brix syrup when compared with using refined cane sugar	[21]
4	Fed batch	Single pulse	*Halomonas venusta* KT832796	pH – 7 Temperature – 30℃ speed - 500 -900 rpm	0.194	PHA productivity increased to 8.65-fold in the fed-batch than batch mode	[24]
5	Fed batch	intermittent	*Bacillus megaterium* KCTC 2194	Temperature – 30℃ Speed – 175 rpm	PHA - 10.1 g/L	By intermittent fedding, PHA content was enhanced	[27]

6	Fed batch	Pulse, constant, mixed	*Cupriavidus malaysiensis* USMAA1020	Temperature – 30°C pH – 7 agitation – 300 rpm	PHA – 11.5 g/g	1. The pulse-feed of carbon and nitrogen resulted in higher PHA Concentration 2. Constant feeding was found to be a more feasible strategy than mixed feeding	[49]
7	Batch	Feast-famine		OLR - 17.64 kg COD/m^3-day cycle period - 48 h.	0.168 g PHACOD/g WWCOD	Reducing the cycle length enhanced PHA production	[80]
8	Continuous batch	Feast-famine	Photosynthetic consortium of bacteria and algae	light intensity of 227W/m^2 Temperature – 30°C pH – 6.5	0.67 Cmol PHB/Cmol Acet	1.PMC also presented high phosphate removal rates 2.no aeration inputs are required	[81]
9	Fed- batch	pH stat fed batch	*H. mediterranei* ATCC 33500	Agitation – 800 rpm Air flow rate – 10 L/min Temperature – 37°C	50.8 % (w/w) dry cell	The melting temperatures of the PHBV < PHB	[82]
10	Feast-famine	Feed on demand	Mixed cuture	Feast phase – 360 min Famine phase- 695 min	0.81 ± 0.02 Cmol/Cmol	Pulsed feeding strongly improved the PHA storage efficiency in cell	[83]
11	Dual fermentation	24 h, 3 h and continuous	*R. eutropha*	Temperature – 30°C pH – 6.5 HRT – 24 h	0.0697 g L−1 h−1	Utilization of inexpensive carbon sources may lead to economically viable PHA production	[84]

Table 4 Economics of PHA production using individual VFA and VFA rich streams.

S No	Type of fermentation	VFA profile/ VFA rich stream	PHA production cost	Separation cost	Utilities cost	PHA yield	Reference
1	Fed batch	Wastewater	2 €/kg	-	1.0 €/kg	0.4 gPHA/gCOD	[107]
2	Two stage fed batch	Soy bean oil	3.5 - 4.5 US$/kg	3.82 US$/kg	6.9 thousand US$/ year	0.70 g/g	[108]
3	Two phase extraction process	Glycerol	5.77 US$/kg	61,000 $/year	1,052,081 $/year	-	[110]
4	Batch	Activated sludge	US$ 11.8/kg	-	0.9 US$	44%	[111]
5	Fed batch	Sucrose	$ 5.58/kg	-	$ 5.58/kg PHB	0.29 g PHB/g substrate	[112]
6	Fed batch	Gluconate	-	NaOH - 1.02 €/kg H2SO4 - 1.11 €/kg	-	-	[113]
7	Fed batch	Crude glycerol	1.937 US$/kg	-	0.658 US$/kg	-	[114]
8	Batch	Crude glycerol	2.6US$/kgPHB	-	0.8 US$/kg PHB	0.3 g/g	[115]
9	Fed batch	Sucrose	2.6 USD/kg PHB	-	-	0.36 g PHB/g sucrose	[116]

Highlights

- Utilizing dark fermentation effluent for biopolymer production is cost effective.

- Metabolic pathway of different organic acid to biopolymer has been reviewed.

- Economy and commercialization aspect of biopolymer production is discussed.

Polyhydroxyalkanoates synthesis using acidogenic fermentative effluents

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