# Strategies for Large-scale Production of Polyhydroxyalkanoates

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Review
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Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible intracellular polyesters that are accumulated as energy and carbon reserves by bacterial species, under nutrient limiting conditions. Successful large-scale production of PHAs is dependent on three crucial factors, which include the cost of substrate, downstream processing cost, and process development. In this respect, design and implementation of bioprocess strategies for efficient PHA bioconversions enable high PHA concentrations, yields and productivities. Additionally, development of PHA fermentation processes using inexpensive substrates, such as agro-industrial wastes, facilitates further cost reduction, thus benefitting large-scale PHA production. Thus, the aim of this review is to highlight various bioprocess strategies for high production of PHAs and their novel copolymers in relatively large quantities. This review also discusses the application of kinetic analysis and mathematical modelling as important tools for process optimization and thus improvement of the overall process economics for large-scale production of PHAs.

*Key words:*

polyhydroxyalkanoates, PHA copolymers, large-scale production, PHA optimization, bioprocess strategies

## Introduction

Polyhroxyalkanoates (PHAs) are macromolecular storage polyesters produced by metabolic transformation of carbon sources by microorganisms, under nutrient limiting conditions. The latter involves excess of carbon source and limiting concentrations of at least one other essential nutrient, such as nitrogen, phosphorous, and/or sulphur.[1] PHA accumulation as intracellular carbon and energy reserves can be rationalised due to their oxidized state and consequent high calorific value of >20 KJ $g^{-1}$.[2] The ever increasing popularity of PHAs is based on their biodegradable and biocompatible nature along with the possibility of tailored molecular structure and/or composition, which makes them highly versatile candidate materials for a range of different applications, including bulk and medical.[3,4] This has led to the development of more than 150 different types of biotechnologically produced PHAs to date.[5] PHAs offer several distinct advantages over other popular biopolymers such as poly-L-lactic acid (PLLA) or starch-based polymers e.g., starch-polyethylene. PHA degradation mechanism involves surface erosion rather than bulk degradation reported for PLLA. This allows a more predictable degradation profile of PHA-based medical products than PLLA. In addition, PHAs have reduced immune response due to lower acidity of their degradation products as compared to the toxicity resulting from a huge accumulation of lactic acid generated by PLLA degradation. The latter leads to complications in medical applications.[6] The monomeric composition also affects the degradation rate of PHAs. Thus, the possibility of a tailored composition of PHAs provides a means to control their degradation at a desired rate (short or extended period of time) depending upon the application.[7] Both *in-vitro* and *in-vivo* tests have proved PHAs to be biocompatible with bone, cartilage, blood and various other cell lines. Their biocompatibility has been reviewed in detail by Valappil *et al*.[8] Other polymers, such as starch, exhibit unsatisfactory mechanical properties and difficult processing.[9] Thus, the lack of variability in structure, extensive material properties, and controlled degradation for PLLA or starch-based polymers makes them less attractive than PHAs.

Several microorganisms, including both native and recombinant strains, have been employed for PHA production. Industrial production processes for PHAs have generally been developed using Gram negative strains, such as *Cupriavidus necator* and *Alcaligenes latus,* mainly due to the relatively high PHA yields offered by them and the ability of some strains to synthesize PHAs under nutrient non-limiting conditions.[10–11] However, huge efforts have also been directed towards process development based on Gram positive strains such as *Bacillus sp.* and *Corynebacterium glutamicum*. Their primary advantage is the absence of the lipopoly-

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saccharide (LPS) layer in the Gram positive strains, which makes the PHAs derived from them to be ideal for medical applications.[12]

The PHAs can be divided into three main categories based on the number of carbon atoms in their monomeric units i.e., short-chain length (scl-PHAs) containing 3–5 carbon atoms, medium-chain length (mcl-PHAs) containing 6–14 carbon atoms, and long-chain length (lcl-PHAs) containing more than 14 carbon atoms.[13–14] Another type of classification divides PHAs into homopolymers i.e., containing only one type of monomer unit, and heteropolymers (copolymers), which are composed of more than one type of monomer unit.[13] Copolymers could be a combination of only scl-/mcl-PHA monomers or consist of both scl- and mcl-PHA units. Of all the PHAs, the scl-PHA, poly-3-hydroxybutyrate, P(3HB), has been most widely explored.[4] However, one of the major challenges with P(3HB) is its brittle and stiff nature, which limits the applications of this polymer. On the other hand, mcl-PHAs are flexible and elastomeric polymers.[14–15] Their structural diversity allows more flexibility with respect to tailoring their properties to suit specific applications.[16] Additional advantages are offered by PHA scl-mcl copolymers, which interestingly combine specific properties of their constituent monomers and facilitate in catering to more diverse applications.[4,16]

While research efforts towards large-scale PHA production have been continued for more than three decades, the final quantity and quality of the biopolymer are the two major factors that dictate their market applications.[17] The PHA production process involves a series of complex steps beginning from biomass growth, polymer accumulation, cell harvesting to polymer extraction and purification which affect the final polymer quality. The nature of microorganism, inherited metabolic pathway, medium constituents and bioprocess strategy are additional important factors controlling the polymer yield and quality.[18] In fact, the major challenge facing biotechnological polymer production has been the development of economic PHA production competitive with the petrochemical synthesis. Major factors leading to relatively higher cost of bio-based PHA production include high cost of substrate, low polymer concentration, yield, and productivity of the production processes.[19–20] Cost of the substrate is known to account for almost 50 % of the total production cost in any bioprocess. Additionally, low overall product yields contribute to the cost, as a less efficient bioconversion would require more substrate for the production of the same amount of product.[21] The problem is compounded by the fact that mostly PHA production gives low product concentrations and low overall volumetric productivities. The latter leads to higher capital and operating costs, especially for large bioreactors.

Improvement in process economics is possible by design and implementation of efficient bioprocess strategies for improving the overall process kinetics, and thereby resulting in higher PHA concentrations and productivities.[22] Besides natural strains, tremendous efforts in metabolic engineering in the recent years have led to the development of high product-yielding strains, which are now being increasingly used in process development. However, while dealing with recombinant microorganisms, stability of the plasmids is an important aspect that needs to be clearly established and ensured before the engineered strain could be used for large-scale production. Otherwise, there is a risk that the plasmid might lose its function over several runs of fermentation (production cycles).[23–24] Additionally, maintenance of plasmid stability usually requires addition of expensive antibiotics in the medium, which is not very suitable for economic large-scale production. In such a case, other plasmid maintenance strategies, such as creation of an auxotrophic mutant with a deletion and then complementing that mutation with a plasmid containing the deleted gene, could be used.[24]

In addition, the use of thermophilic or thermotolerant strains, especially for industrial production of PHAs, offers several cost advantages. These include operation at medium and elevated temperature, which in turn accelerates the reaction rates, reduces the cost for cooling/heating, and decreases the chances of cross-contamination from other microorganisms.[25] Another method to reduce the fermentation cost is the development of production protocols for the bioconversion of inexpensive and renewable carbon (and/or nitrogen) substrates, including waste and by-products from agriculture and industrial sources, instead of pure and refined substrates.[20] In fact, recent years have witnessed an increasing trend towards exploration of unconventional substrates for cost efficient production of several types of PHAs.

Thus, the present review highlights the importance of process development towards efficient PHA fermentations enabling high PHA concentrations, yields and productivities. Results of various bioprocess strategies including batch, fed-batch with different types of feeding regimes, continuous cultivation, two-(or multi-)stage cultivation in batch, fed-batch and continuous mode for the production of PHAs and their copolymers have been discussed and compared. Application of statistical techniques, kinetic analysis and mathematical modelling for PHA process optimization is also highlighted. This article aims to review the progress in PHA process development in the last 5 years.

## Bioreactor operating strategies for large-scale production of PHAs

Different bioreactor operating strategies have been used by researchers to optimize the production of PHAs. Tables 1, 2, 3 give an overview of the results obtained using various cultivation strategies and employing different native/recombinant PHA and/or copolymer producers. The tables also highlight the recent use of various renewable and inexpensive nutrients for possible economical production of PHAs.

### *Batch fermentation*

Batch fermentation is the simplest and primary investigation method for any bioprocess.[26] It is essentially a closed system involving the addition of the substrate in the bioreactor at the beginning of the cultivation, and subsequent removal of the product at the end. No additional substrate is introduced into the bioreactor during the cultivation nor is the medium with/without cells withdrawn from the reactor at any given point in time. Interestingly, batch fermentation has been the most extensively used strategy, especially to investigate the influence of various process operating conditions, bioconversion of (new) carbon sources, use of different microorganisms for production of different types of PHAs in the last 5 years (see Table 1). As can be seen from Table 1, poly-3-hydroxybutyrate (P(3HB) remains the most widely investigated PHA by batch fermentation, accounting for one-third of the literature reports. However, quite interestingly, there have been only few reports on the utilization of pure or refined substrates (sugars) for P(3HB) production,[27–30] while different types of inexpensive carbon sources such as agro-industrial wastes including cane molasses, sugar beet juice, rice straw hydrolysate, grass biomass hydrolysate, plant oils e.g., coconut oil, have been largely investigated.[31–38] *Alcaligenes and Bacillus sp.* remain the microorganisms of choice for P(3HB) production (Table 1).

In addition to mesophiles, the production of P(3HB) from isolated thermophiles has been investigated. In one of the reports, an isolated thermophilic bacteria *Bacillus shackletoni* K5 was involved in a batch process, which resulted in a satisfactory biopolymer content of 72.6 % dry cell weight (dcw).[28] A similar result with a biomass concentration of 4.8 g $L^{-1}$ and a P(3HB) content of 73 % dcw was obtained using an isolated *Chelatococcus* strain by Ibrahim *et al.*[25] In a recent report, growth-associated and non-nitrogen limited P(3HB) production was reported for *Chelatococcus daeguensis* TAD1. The former helps to reduce the fermentation time thereby improving the productivity of the process. The thermophilic isolate also demonstrated the ability to accumulate inexpensive carbon source i.e., glycerol for P(3HB) production, resulting in a high yield of 80.8 % dry cell weight.[39] Such findings are important for economic bioprocessing due to the operational advantages offered by the thermophiles, as mentioned above in the introduction. The capability of these thermophiles to utilize wastes as substrates is another added advantage. Furthermore, the enzymes from these thermophiles have a significant potential to be technologically advantageous, as thermostable PHA synthases could be a novel feature for *in-vitro* PHA biosynthesis.[25] However, process design to ensure optimum oxygen availability at high operating temperatures requires attention. The authors believe that this issue would be considered appropriately and effective strategies devised when efforts towards *in-depth* research on thermophiles are carried out and more results are available, which at the present, are still limited. Nonetheless, given the above advantages of using thermophiles, this could be an additional alternative method for PHA production.

Although a critical evaluation of the obtained results is rather important, such an assessment is rather difficult in the present review, as one or more essential process parameters such as biomass (g $L^{-1}$), polymer concentration (g $L^{-1}$), polymer content (% dcw), overall productivity (g $L^{-1}$ $h^{-1}$) is usually missing in reports on PHA production (see Table 1, 2, 3). Moreover, the polymer yield in terms of substrate consumption (i.e. gram PHA per gram substrate) and biomass formation (i.e. gram PHA per gram rest biomass) is rarely reported. This kinetic information is crucial because it provides metabolic flow analysis of the substrate to different pathways within the process and serves as a valuable tool to understand the process behaviour for future optimization. Overall, in batch processes, a P(3HB) content of 38–72 % dcw has been achieved by various researchers. The differences in production have mainly been due to differences in substrate and/or microorganism used in the specific batch fermentation. An exception to the above result has been a report involving the use of rice straw hydrolysate by *Bacillus firmus* NI 0830, which yielded a significantly high P(3HB) content of 89 % dcw. [32]

In addition to P(3HB), other homopolymers such as poly(-3-hydroxyoctanoate) P(3HO), poly(-3--hydroxy-4-methylvalerate) P(3H4MV) and several copolymers involving 2–3 monomer units, such as poly(-3-hydroxybutyrate-*co*-3-hydroxyvalerate) P(3HB-*co*-3HV), poly(-3-hydroxybutyare-*co*-4-hydroxybutyrate) P(3HB-*co*-4HB), poly(-3-hydroxybutyrate-*co*-3-hydroxyhexanoate) P(3HB-*co*-3HHx), poly(-3-hydroxypropionate-*co*-4-hydroxybutyrate) P(3HP-*co*-4HB), poly(-3-hydroxybutyrate-co-3-hy-

Table 1 – *Summary of results of batch fermentation for production of PHAs*

| Polymer type | Microorganism used | Substrate | Biomass (g $L^{-1}$) | Polymer (g $L^{-1}$) | Polymer content (% dcw) | Productivity (g $L^{-1}$ $h^{-1}$) | Scale of experiment (L) | Ref. |
|---|---|---|---|---|---|---|---|---|
| scl-mcl copolymer PHA[a] | *Thermus thermophilus* HB8 | Whey | 2.1 | 0.51 | 24.4 | – | 2 | 51 |
| P(3HB) | *Caldimonas taiwanensis* | Gluconate | – | – | – | – | – | 96 |
| P(3HB-*co*-4HB) | *Cupriavidus sp.* USMAA2–4 | 1,4-butanediol, γ-butyrolactone | – | – | 31 | – | – | 40 |
| P(3HB-*co*-4HB) | *Cupriavidus necator* | Spent palm oil, 1,4-butanediol | – | – | 70–81 | – | 1 | 41 |
| P(3H4MV) | *Burkholderia sp.* JCM15050* | Fructose, isocaproic acid | 0.2 | 0.2 | 19 | 0.002 | – | 42 |
| P(3HB-*co*-3HV) | *Halomonas campisalis* | Maltose | – | – | 45–81 | – | 0.25 | 43 |
| P(3HB) | *Chelatococcus sp.* | | 3.0 | – | 59.3 | – | 1 | 25 |
| scl-mcl copolymer PHA[aa] | *Methylobacterium extorquens* | Methanol | – | – | – | – | 14, 75, 150 | 2 |
| P(3HO) | *Pseudomonas mendocina* | Sodium octanoate | 0.64 | 0.2 | 31.38 | 0.003 | 1 | 44 |
| P(3HB-*co*-3HHx) | *Cupriavidus necator* H16 | Jatropha oil | 9.2 | 8.3 | 90 | 0.17 | – | 45 |
| P(3HB) | *Bacillus cereus* SPV | Sugarcane molasses | 6.93 | – | 51.37 | – | 2 | 31 |
| PHA[b] | *Halomonas campisalis* MCM B1027 | Maltose | 1.72<br>1.33 | 0.97<br>0.67 | 56.23<br>49.17 | 0.04<br>0.03 | 14<br>120 | 46 |
| mcl-PHA[b] | *Pseudomonas sp.* TN301 | Naphthalene | 0.48 | – | 23.0 | – | – | 97 |
| mcl-PHA[b] | *Pseudomonas mediterranea* A1 | Crude glycerol | 4.8 | 2.93 | – | 0.04 | 2 | 98 |
| P(3HB) | *Bacillus mycoida* DFC1 | Sucrose | 4.35 | 3.32 | 76.3 | 0.05 | – | 27 |
| P(3HB-*co*-3HV) | *Sinorhizobium meliloti* MTCC100 | Rice bran hydrolysate | 7.45 | 3.6 | – | 0.05 | 0.25 | 52 |
| P(3HP-*co*-4HB) | *Escherichia coli** | 1,3-propanediol, 1,4-butanediol | – | – | 12–82 | – | 0.5 | 47 |
| P(3HB-*co*-3HV) | *Halofenax mediterranei* | Vinasse | – | 19.7 | – | 0.21 | 0.25 | 48 |
| P(3HB-*co*-3HV-*co*-4HB) | *Cupriavidus sp.* USMAA2–4 | Oleic acid, γ-butyrol-actone, 1-pentanol | – | – | 81 | – | – | 49 |
| P(3HB) | *Bacillus firmus* NI 0830 | Rice straw hydrolysate | – | 1.7 | 89 | 0.02 | – | 32 |
| P(3HB-*co*-3HV) | *Escherichia coli** | Glucose, propionic acid | 11 | 5 | 45.4 | 0.1 | – | 50 |
| P(3HB) | *Aeromonas hydroiphila* NIU01 | Coconut oil | 7.3 | 3.6 | 49.6 | – | – | 33 |
| P(3HB-*co*-3HV) | *Cupriavidus necator* DSM545* | Crude glycerol, rapeseed meal hydrolysate | – | 5.5 | 45.2 | – | – | 54 |
| P(3HB-*co*-3HV) | *Bacillus sp.* OU40[T] | Agro/Industrial waste | 3.2–3.5 | – | 57–72 | – | – | 55 |
| P(3HB) | *Bacillus shackletoni* K5 | Glucose | 3.76 | – | 72.6 | – | 5 | 28 |
| P(3HB) | *Alcaligenes sp.* | Cane molasses, urea | 11.8 | 8.8 | 74.6 | 0.17 | 7.5 | 34 |
| mcl-PHA | *Pseudomonas sp.* | Grass biomass hydrolysate | 0.77–0.84 | – | 20–34 | – | 0.25 | 35 |
| P(3HB) | *Bacillus megaterium* MSBN17 | Pulp industry waste | 5.6 | – | 58 | – | – | 62 |
| mcl-PHA[b] | *Pseudomonas putida* CA-3 | VFA | 1.6 | – | 39 | – | – | 53 |
| P(3HB) | *Bacillus megaterium* MSB904 | Agro-industrial waste | – | – | 56.8 | – | 0.25 | 36 |
| P(3HB) | *Bacillus megaterium* R11 | Oil palm empty fruit bunch | – | 12.48 | 51.6 | 0.26 | 0.5 | 37 |
| P(3HP)-*b*-P(4HB) | *Escherichia coli* S17–1* | 1,3-propanediol, 1,4-butanediol[d] | 8.2 | – | 21.1 | – | 0.5 | 71 |
| P(3HB)[e] | *Alcaligenes latus* | Sugarbeet juice[#] | 10.3 | 4.0 | 38.6 | 0.22 | – | 38 |
| P(3HB) | *Alcaligenes latus* | Sucrose | 8.71 | 6.2 | 71.2 | 0.17 | 7 | 29 |
| P(3HB) | *Azotobacter vinelandii* OPNA* | Sucrose | 6.1 | 4.6 | 75.4 | 0.07 | 3 | 30 |
| P(3HB)[e] | *Aeromonas hydrophila* NIU01 | Coconut oil[#] | 16.8 | 10.4 | 62.1 | 0.03 | 3 | 33 |
| P(3H4MV) | *Burkholderia sp.* JCM15050* | Fructose, isocaproic acid[#] | 2.1 | 0.84 | 39 | 0.02 | – | 42 |
| P(3HB) | *Chelatococcus daeguensis* TAD1 | Glycerol | 2.5 | 2.0 | 80.8 | 0.01 | 0.3 | 39 |

References are arranged in chronological order

* recombinant strain used, # 2-stage batch, [a] scl-mcl copolymer containing 3-hydroxyvalerate, 3-hydroxyheptanoate, 3-hydroxynanoate, 3-hydroxyundecanoate,[aa] scl-mcl copolymer with functional group, [b] composition not specified, [c] VFA from anaerobically digested grass, [d]1,4-BD addition followed by 1,3-PD addition at 24 h, [e] results reported only for second stage cultivation, dcw – dry cell weight

Table 2 – *Summary of results of fed-batch fermentation for production of PHAs*

| Polymer type | Microorganism used | Substrate | Biomass (g $L^{-1}$) | Polymer (g $L^{-1}$) | Polymer content (% dcw) | Productivity (g $L^{-1}$ $h^{-1}$) | Scale of experiment (L) | Ref. |
|---|---|---|---|---|---|---|---|---|
| P(3HB) | *Bacillus megaterium* SRKP-3 | Dairy waste | – | 11.32 | – | 0.31 | 3 | 60 |
| P(3HB) | *Cupriavidus necator* | Food waste | – | – | 87 | – | 1.5 | 64 |
| P(3HB-*co*-3HV-*co*-3HO-3HDD) | *Weutersia eutropha* | Canola oil | – | 18.3 | 90 | 0.46 | 5 | 69 |
| mcl-PHA copolymer | *Pseudomonas sp.* SG4502 | Biodiesel waste product | 1.5 | 0.61 | 40.6 | – | 3 | 74 |
| P(3HB) | *Alcaligenes latus* | Sucrose | – | 11.8 | 95 | – | 3 | 87 |
| P(3HB) | *Bacillus cereus* SPV | Sugarcane molasses | ~5.0 | – | ~14 | – | 2 | 31 |
| P(3HB) | *Burkholderia cepacia* | Sugar maple hydrolysate | – | 8.7 | 51.4 | 0.10 | 1 | 61 |
| mcl-PHA[a] | *Pseudomonas putida* KT2440 | Octanoic acid | – | – | 16.2 | – | 3.7 | 68 |
| P(3HB)-*b*-P(3HHx) | *Pseudomonas putida* KTOYO6ΔC* | Sodium butyrate, sodium hexanoate | 5.8 | – | 57.8 | – | 0.5 | 72 |
| P(3HB-*co*-3HV-*co*-4HB) | *Cupriavidus necator* DSM545 | Crude glycerol, γ-caprolactone, propionic acid | – | 16.7 | 37 | 0.25 | 2 | 73 |
| P(3HB) | *Alcaligenes latus* | Sucrose | 29.7 | 22.6 | 76 | 0.59 | 7 | 88 |
| P(3HB) | *Bacillus megaterium* MSBN17 | Pulp industry waste | 26.7 | 19.1 | 71.5 | 0.48 | 2.5 | 62 |
| P(3HB) | *Bacillus megaterium* uyuni S29 | Glucose | 28.6 | 8.5 | 29.5 | 0.25 | – | 63 |
| P(3HB-*co*-3HV) | *Cupraviadus necator* DSM545* | Crude glycerol, rapeseed meal hydrolysate | – | 10.9 | 55.6 | – | – | 54 |
| P(3HB) | *Bacillus shackletoni* K5 | Glucose | 9.46 | – | 44.8 | – | 5 | 28 |
| P(3HB) | *Pseudomonas fluorescens* S48 | Waste frying oil | – | – | 55.3 | – | 10 | 98 |
| P(3HN) | *Pseudomonas putida* KT2440 | Nonanoic acid, glucose, acrylic acid | 71.4<br>34.3 | – | 75.5<br>55.7 | 1.8[c]<br>1.2[c] | 5[#]<br>7 | 67 |
| P(3HB-*co*-3HV) | *Comomonas sp.* | VFA | 5–15 | – | 40–53.0 | – | 2 | 99 |
| P(3HB) | *Azotobacter vinelandii* OPNA * | Sucrose | 37.2 | 27.3 | 73.0 | 0.45 | 3 | 59 |
| P(3HB) | *Cupriavidus necator* DSM 545 | Glycerol | 104.7 | 65.6 | 62.7 | 1.31 | 3 | 65 |
| P(3HB) | *Burkholderia sacchari* DSM 17165 | Wheat straw | – | 105 | 45–68 | 1.3–1.5 | 2 | 66 |
| P(3HB-*co*-3HV) | *Cupriavidus necator* | Propionic acid, butyric acid | 65.9 | 58.0 | 88.0 | 0.65 | 2 | 70 |

References are arranged in chronological order

* recombinant strain used, [a] composition not specified, VFA – Volatile fatty acids, dcw – dry cell weight, [#] different feed concentrations used for two scales, [c] overall productivity reported

Table 3 – *Summary of results of continuous fermentation for production of PHAs*

| Polymer type | Microorganism used | Substrate | Biomass (g $L^{-1}$) | Polymer (g $L^{-1}$) | Polymer content (% dcw) | Productivity (g $L^{-1}$ $h^{-1}$) | Scale of experiment (L) | Ref. |
|---|---|---|---|---|---|---|---|---|
| mcl-PHA[a] | *Pseudomonas putida* KT2440 | Potassium octanoate, Potassium 10-undecenoate | – | – | 42.0 | 0.90 | 16 | 78 |
| P(3HB) | *Halomonas* TD01 | Glucose | 20.0 | 13.0 | 65.0–70.0 | – | 3[b]/1[c] | 79 |
| P(3HB) | *Cupriavidus necator* | Glucose | 19.0 | – | 77.0 | 1.85<br>0.10[d] | 7.5[b]/3.6[c] | 80 |
| P(3HB-*co*-3HV) | *Alcaligenes latus* | Sucrose | 32.4 | 24.6 | 75.9 | 2.18 | 7 | 81 |

References are arranged in chronological order

[a] composition not specified, dcw – dry cell weight, [b] first stage bioreactor, [c] successive stages bioreactor, [d] specific productivity (g $g^{-1}$ $h^{-1}$)

droxyvalerate-*co*-4-hydroxybutyrate) P(3HB-*co*-3HV--*co*-4HB), have been produced by batch fermentation.[40–50] Production of mcl-PHAs and other interesting PHA copolymers including scl-copolymers and scl-mcl copolymers has also been reported using unconventional substrates, such as lignocellulosics e.g., grass mass hydrolysate, rice bran, rapeseed meal hydrolysate, and industrial waste e.g., whey, biodiesel waste, vinnase etc.[35,48,51–56] Utilization of waste whey for PHA production is particularly useful as only 50 % of the whey produced is used for production of food ingredients and animal feed, while the rest remains as a pollutant owing to its high biological oxygen demand. Production of scl-mcl copolymer using whey adds additional merit, as such copolymers are natural blends that combine the interesting chemical, mechanical and thermal properties of both scl and mcl PHAs.[51] In a similar report, various agricultural and industrial wastes such as bagasse, waste starch, whey, and rice bran were used for P(3HB-*co*-3HV) production by an isolated *Bacillus sp.* OU40$^T$ strain.[55] Quite interestingly, batch production resulted in considerably high PHA content of 57–71 % dcw depending on the waste substrate used. It would be interesting to further develop this process using fed-batch and other high cell density cultivation techniques for improvement in biomass and PHA production, considering its potential for high PHA copolymer production using a variety of wastes. Another high-polluting industrial waste is vinasse obtained from the ethanol industry. In a report employing extremely halophilic archaeon *Haloferax mediterranei,* vinasse was successfully used for accumulation of a significantly high concentration of 19.7 g $L^{-1}$ of P(3HB-*co*-3HV). [48] It has been extensively discussed by Koller *et al.* that the usage of inexpensive waste substrates as a means to low-cost large-scale PHA production depends on the availability of potential substrates and the possibility of integration of PHA production into the existing production lines.[57] In this respect, the above examples exhibiting high PHA copolymer production while also demonstrating the potential of integration with an ethanol plant would be worth developing on a large scale.

Another interesting strategy, which has been used to enhance PHA synthesis is two-stage fermentation. This methodology could be used with batch, fed-batch and/or continuous mode of nutrient feeding. It essentially involves physical separation of two phases, i.e., microbial growth in one bioreactor and product formation in a second bioreactor, with an aim to enhance product synthesis over a conventional batch fermentation. This strategy also allows maintenance of different media and/or environmental conditions in two stages, each suited to achieve a particular condition, e.g., high growth rate in first stage followed by high rate of product accumulation in second stage. Adoption of this strategy for improved batch P(3HB) production using *Aeromonas hydrophila* was attempted.[33] Once a high biomass density was achieved in the first stage, the cells were transferred to second stage in which they were grown under nitrogen-limiting conditions to allow maximum accumulation of P(3HB). The authors observed a dcw of 16.8 g $L^{-1}$ with a polymer concentration and content of 10.4 g $L^{-1}$ and 62.1 % dcw, respectively, in the second stage. In a similar report, a two-stage batch fermentation was used to promote P(3H4MV) production by relieving the toxicity of isocaproic acid on cells. A PHA content of 39 % dcw was achieved in this study.[43]

### *Fed-batch fermentation*

In batch fermentation, the product concentration(s) and productivity(ies) are usually limited due to starvation of the culture towards the end of cultivation. Fed-batch fermentations are particularly important as a means to achieve high cell densities, consequently leading to high metabolite production in the bioprocesses.[58] These involve initiation of cultivation as a batch, followed by the addition of substrate into the bioreactor during cultivation. However, the product is removed only at the end of fermentation. Fed-batch fermentations ensure regulation of appropriate nutrient feed into the bioreactor to avoid both substrate limitation and/or inhibition.[26] In general, fed-batch fermentations usually involve the control of nutrient feed through dissolved oxygen (DO), regulation of pH, concentration of specific medium component (for e.g. substrate) in the medium. Various types of fresh nutrient feeding profiles in fed-batch fermentations allow maximum product concentrations and productivities. These include fed-batch with pulse addition of substrate, constant feed rate, linearly increasing feed rate, exponentially increasing/decreasing feed rate, pseudo-steady state of substrate(s), high cell density with highly concentrated feed, and 2-stage fed-batch.[26,59]

As is the case with batch fermentation, P(3HB) has been the most investigated biopolymer by fed-batch fermentation. Pulse feeding of substrate, i.e., addition of small known amounts of substrate when the substrate is at low concentration in the bioreactor, has been a widely used fed-batch strategy for P(3HB) production.[59–63] This could be mainly attributed to the ease of operation of this strategy, which essentially involves measurement of residual substrate in the bioreactor at several periods during cultivation, and subsequent addition of concentrated substrate to the bioreactor when it falls below a limiting value. This addition helps to avoid limitation

of substrate in the fermentation medium, thereby allowing continued microbial growth and product formation. The P(3HB) concentration in all the above cases was measured to be between 8–11 g $L^{-1}$, depending mainly on the microorganism and carbon source used. A relatively high P(3HB) concentration and productivity of 19 g $L^{-1}$and 0.48 g $L^{-1}$ $h^{-1}$, respectively, was reported, primarily as a result of statistical process optimization.[62]

Another fed-batch strategy involving substrate feeding at a constant feed rate for P(3HB) production has been described.[31,64] A novel exponential feeding strategy based on alkali-addition monitoring was developed to maintain waste glycerol (substrate) at a constant level inside the bioreactor.[65] It resulted in a significantly high P(3HB) concentration of 65.6 g $L^{-1}$, yield of 62.7 % dcw, and productivity of 1.3 g $L^{-1}$ $h^{-1}$. Exponential feeding of substrate was combined with pulse addition in a mixed fermentation strategy involving sucrose and yeast extract for increased P(3HB) production.[59] As a result, an improvement in the overall P(3HB) concentration by 7-fold was achieved as compared to batch cultivation (see Table 1). In yet another interesting approach, feeding was controlled by a decrease in the stirring speed, which resulted from exhaustion of the carbon source in the medium.[66] This strategy made it possible to reduce catabolite repression, thus allowing the utilization of sugars other than glucose in the medium by the microorganism. It resulted in achieving a polymer concentration as high as 105 g $L^{-1}$, which translated into high polymer productivity of 1.6 g $L^{-1}$ $h^{-1}$. Another homopolymer that had been produced by fed-batch fermentation involving co-feeding of the substrates was poly(-3-hydroxynonanoate) P(3HN). Overproduction of this homopolymer was attempted by maintenance of specific growth of *Pseudomonas putida* KT2440 at a particular value by co-feeding of three substrates, i.e., nanonoic acid, glucose and acrylic acid in a sequential manner.[67] Under specific conditions of using nonanoic acid, glucose and acrylic acid at a mass ratio of 1.25:1:0.05 in the feed while maintaining a specific growth rate of 0.15 $h^{-1}$, the authors obtained a high mcl-PHA content of 75.5 % dcw with 89 mol % of 3-hydroxynonanoate, and an overall productivity of 1.8 g $L^{-1}$ $h^{-1}$. In a similar strategy, production of an mcl-PHA was attempted using xylose and octanoic acid.[68] It basically involved initiation of the cultivation as a batch fermentation on xylose, with linear feeding of octanoic acid at different point times during the exponential phase of the culture. Xylose was consumed by *Pseudomonas putida* KT2440 (pSML1) only for growth but not for mcl-PHA production, while octanoic acid was used as an mcl-PHA precursor. It was also observed that linear feeding of octanoic acid at the end of exponential phase (10 h of cultivation) resulted in a higher PHA yield of 16.2 % dcw as compared to 12.1 % dcw obtained by feeding in the mid-exponential phase. This was explained by the fact that the accumulation of octanoic acid during mid-exponential phase resulted in it being used for growth rather than mcl production, thus reducing the overall PHA yield.

In addition to homopolymers, particularly P(3HB), there have been a few studies on copolymer production by fed-batch fermentation. A 3-stage fed-batch cultivation has been found to produce copolymer comprising 4 monomeric units, i.e., poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyoctanoate-*co*-3-hydroxydodecanoate P(3HB)-*co*--P(3HV)-*co*-P(3HO)-*co*-P(3HDD), using canola oil as substrate.[69] The strategy successfully yielded a copolymer concentration of 18.3 g $L^{-1}$ with a copolymer productivity and content of 0.46 g $L^{-1}$ $h^{-1}$ and 90 % dcw, respectively. In a recent study, nitrogen deficiency rather than limitation was imposed along with feeding of a mixture of substrates for P(3HB-*co*-3HV) production.[70] This strategy resulted in a high copolymer production of 58 g $L^{-1}$ with a significant polymer accumulation of 88 % dcw.

Production of a di-block copolymer poly-3-hydroxypropionate-*block*-poly-4-hydroxybutyrate (P(3HP)-*b*-P(4HB)) using 1,3-propanediol (1,3-PD) and 1,4-butanediol (1,4-BD) as substrates has also been reported.[71] The di-block copolymer combined soft block P(4HB) and strong block P(3HP) to gain unique and excellent material properties. The time of sequential feeding of two precursors was found to be the most crucial factor for di-block copolymer production. Addition of 1,4-BD as the first precursor followed by feeding of 1,3-PD at different stages of cultivation resulted in different monomer ratios. However, addition of 1,4-BD as second precursor, only at early exponential phase gave the 4HB monomer in the PHA chain. In another report, the sequential yet timely addition of co-substrates was strongly regulated in a fed-batch fermentation to produce a block copolymer poly(-3-hydroxybutyrate--block-poly-3-hydroxyhexanoate) P(3HB)-*b*-P(3HHx) using an engineered strain of *Pseudomonas putida* KTOYO6Δ.[72] Careful adjustment of concentration and addition time of substrates, sodium butyrate and sodium hexanoate yielded a reasonably high block copolymer content of 57.8 % dcw. The block copolymer demonstrated improved structure-related mechanical properties as compared to random copolymer poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate (P(3HB-*co*-3HHx) and a blend sample of P(3HB) and P(3HHx). A graphical representation of the chemical structure of block copolymer comprising P(3HB) and P(3HHx), its random copolymer, and a sample blend is presented in Fig. 1.

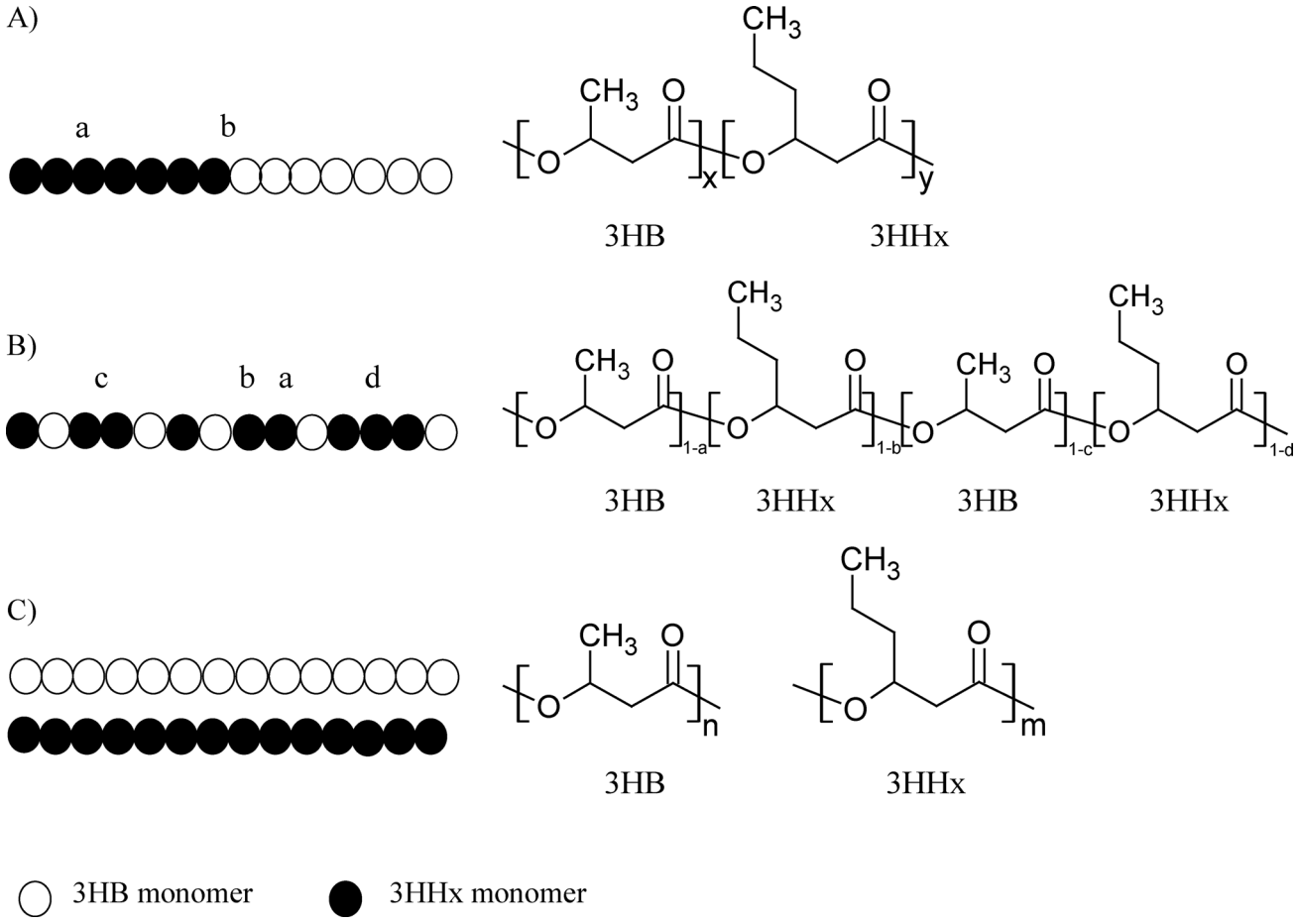


Fig. 1 – *Graphical representation of chemical structure of (A) block copolymer PHB-b-PHHx (B) Random copolymer P(3HB-co-3HHx) (C) Blend of P3HB and P3HHx. Reproduced with permission.[72] Copyright, 2012. BioMed Central.*

The production of PHA copolymers by fed-batch fermentation has also been reported using unconventional substrates such as biodiesel waste product. Production of scl copolymer P(3HB-*co*-4HB) and terpolymers P(3HB-*co*-3HV-*co*-4HB) using untreated raw glycerol along with γ-butyrolactone and propionic acid was attempted by high cell density fed-batch cultivation of *Cupriavidus necator* DSM 545. Dissolved oxygen concentration was found to be the most important parameter to control 4(HB) monomer content and thus tailor the composition of copolymers. 16.7 g $L^{-1}$ terpolymer concentration with a PHA content of 37 % dcw was reported at 20 % dissolved oxygen concentration.[73] Fed-batch cultivation using crude glycerol and rapeseed meal hydrolysate by *C. necator* DSM 545 demonstrated a two-fold increase in production of P(3HB-*co*-3HV) copolymer to 10.9 g $L^{-1}$ as compared to a polymer concentration of 5.5 g $L^{-1}$ obtained in batch cultivation.[54] Production of a PHA copolymer comprising predominantly 4 mcl-PHA monomer units was reported using an isolated thermotolerant *Pseudomonas* SG4502 strain when grown on biodiesel waste. A biomass concentration of 1.5 g $L^{-1}$ and PHA content of 40.6 % dcw was obtained by fed-batch cultivation.[74]

Comparing the above fed-batch fermentation results for various PHAs with those achieved with batch fermentation, it could be stated that though batch fermentation is usually adopted due to its ease of operation, it is equally easy to design and implement simple fed-batch strategies to achieve considerably improved concentration, yield and productivity of PHAs (Table 1, 2). Along with the usage of inexpensive substrates such as agricultural and industrial wastes, these methods would undoubtedly result in a cost reduction of PHAs and therefore benefit their large-scale production.

### *Continuous fermentation*

Continuous fermentations offer the advantage of high productivity, particularly for cultures having high specific growth rates.[26,75] This is primarily due to the fact that the bioreactor could be operated at high dilution rates without any problem of washout, resulting in high product concentrations and thereby high productivities. In petrochemical industries, continuous processes are the rule, while recent

times are also witnessing an increasing trend in the pharmaceutical sector towards continuous production. This is understandable due to the steady states and constant removal rates obtainable by such systems; the former ensuring a constant quality product stream from continuous processes.[76] However, the use of this cultivation strategy for PHA production has been limited with only few examples available from the reports in the last few years (Table 3). A major problem for industrial implementation of this technique has been the fear of high chances of microbial contamination, which would result in huge financial losses.[77] It is expected that such operational difficulties can be overcome by provision of proper cultivation environments and more robust strains, which allows continuous and stable operation of the bioreactor for long periods. This in fact is important for establishing high productivity systems for successful large-scale production.

In a rather unique chemostat strategy involving the use of elevated pressure in bioreactor, a 3-stage continuous cultivation was attempted for production of an mcl-PHA using *Pseudomonas putida* KT2440.[78] The set-up comprised batch cultivation on a C8 carbon source, fed-batch cultivation on C8/C11:1, and finally a continuous cultivation on C8/C11:1 at a dilution rate of 0.15 $h^{-1}$. The mcl-PHA content, yield on substrate and volumetric productivity were all found to increase at elevated pressure. The possibility of implementation of a non-sterile cultivation process in a continuous mode is another useful strategy to reduce the production cost of PHAs. Such a strategy was adopted in a 2-stage continuous cultivation where contents from the first bioreactor were transferred after a week of cultivation to the second stage for subsequent P(3HB) production under nitrogen-deficient conditions. A final P(3HB) concentration of 13 g $L^{-1}$ was obtained in the second stage accounting for 65–70 % dcw yield.[79] Feasibility of a five-stage continuous cultivation with increasing dilution rate in each successive step was investigated in order to improve substrate utilization rate for P(3HB) production by *C. necator* DSM 545.[80] The system featured a biomass concentration, P(3HB) content and volumetric productivity of 19 g $L^{-1}$, 77 % dcw and 1.85 g $L^{-1}$ $h^{-1}$, respectively.

An interesting process involving integration of fed-batch and continuous cultivation was designed and implemented for improved productivity of P(3HB-*co*-3HV).[81] Batch cultivation of *Alcaligenes latus* was followed by operation of the bioreactor under fed-batch mode with nitrogen limitation. Simultaneously, pulses of valeric acid were added to allow 3HV incorporation into the polymer chain. A significantly high biomass and polymer concentration was obtained as a result of fed-batch fermentation prior to the start of continuous cultivation. Such a result is not possible to achieve with batch cultivation. Further improvement in the productivity resulted from conversion of the bioreactor to continuous mode. An overall P(3HB-*co*-3HV) concentration of 24.6 g $L^{-1}$ was obtained at steady state, which translated into a productivity of 2.18 g $L^{-1}$ $h^{-1}$.

Although there are few reports on continuous cultivations, it can be clearly seen and compared that the productivities achieved in continuous set-ups are significantly high than those achieved by batch and even some fed-batch fermentations (Table 3). This demands further investigation, especially the use of non-sterile processes and inexpensive substrates, which would be most suitable for economic production of PHAs, and is thus the way forward for their large-scale production.

## Process optimization using Design-of-Experiments methodology (DoE)

One of the most important aspects of any fermentation process is the right medium composition that would promote biomass growth and metabolite production. In addition, other process operating parameters such as agitation, aeration, medium pH, and temperature also affect the process variables. The classic approach of optimizing 'one-factor-at-a-time' is time-consuming because it requires a large number of experiments to optimize the values of process parameters. In addition, it does not account for interactions between the various factors, and is therefore unable to depict their combined effect on the responses under investigation.[29,82] When dealing with complex, multi-variable systems such as biological processes, such an approach is not useful, as it is unable to screen the significant process factors and optimize their values. This limitation can be overcome by the Design-of-Experiment (DoE) methodology or statistical experimental planning, which involves generation of well-defined output factors or responses, such as biomass/product concentration or productivity, from a set of defined input factors.[83] This approach has been used by many researchers for optimization of PHA production using various carbon sources and microorganisms. Application of DoE is for small-scale experiments mainly due to the cost involved in conducting large-scale experiments. However, it remains an important tool for process optimization to obtain optimal values of culture medium components and operational conditions (pH, temperature, agitation etc.) for a particular process. This is essential for obtaining high 'optimal' PHA concentrations and yields before proceeding to large-scale production based on that process. Some examples of application of DoE for PHA production from recent literature are discussed below.

Johar *et al*. used Central Composite Design (CCD) to optimize the values of biotic and abiotic factors such as media pH, temperature, inoculum size, concentration of carbon and nitrogen source, for maximum PHA production using *Comomonas sp.*[84] Under optimized conditions, a two-fold increase in production of PHA was achieved. Similar results were obtained by Gahlawat and Srivastava, who studied the interactive effects of various medium components on P(3HB) production using *Azohydromonas australica* by DoE methodology. The application of DoE resulted in a >2-fold increase in P(3HB) concentration. DoE was also used by the authors as a tool for estimation of kinetic parameters in a batch process, which facilitated further process optimization in model-based fed-batch cultivation(s).[29]

Statistical optimization of P(3HB) production using two different species of *Bacillus* and different types of agro-industrial wastes as substrates was attempted by Sathiyanarayanan *et al.*[36,62] The studies focussed on the estimation of optimal values of media components for enhanced P(3HB) production. This is the first important step towards development of any bioprocess based on novel isolated or metabolically engineered strains and/or carbon sources. Three-dimensional plots were generated by DoE which highlighted the interactive effects of different variables on P(3HB) production (Fig. 2). A high P(3HB) concentration of ~19 g $L^{-1}$ was obtained by media optimization.[62] In a similar report, an improved batch P(3HB) production of 8.8 g $L^{-1}$ with a P(3HB) content of 80 % dcw was achieved under statistically optimized conditions.[34]

## Kinetics analysis and mathematical modelling for PHA process optimization

One of the most essential fermentation requirements for ensuring high PHA accumulation in most PHA production processes is maintenance of appropriate concentrations of excess carbon, along with limited availability of nitrogen in the fermentation medium during cultivation. On the other hand, the concept of dual nutrient limitation involving both carbon and nitrogen for tailored synthesis of PHAs has also been studied.[85] In some other cases, inhibition of microbial growth might be caused by use of high initial substrate concentrations e.g. volatile fatty acids (VFAs) and waste glycerol, thereby affecting the overall growth and product formation rates. Thus, a thorough understanding of the process kinetics is extremely important for designing fermentation strategies involving appropriate regulation of substrate(s) for maximum PHA concentration and/or productivity.

Mathematical models are invaluable tools in bioprocess engineering. They help to better understand the system, and facilitate an informed optimization of the process conditions in minimum time without trial and error.[86] Mathematical treatment of biological processes is rather tricky and complicated due to their inherent complex, segregated and multivariable nature. Mathematical models are thus the simplification of an actual phenomenon and therefore different models can be developed for the same process depending on the objectives of the model and available measurements. Models adequately describe (*off-line*) the biomass growth, substrate consumption, and product formation kinetics of cultivation. These can be used to simulate the consequences of feeding different concentrations of substrates and their rates on product accumulation. Thus, a number of different feeding strategies can be designed (*in silico*) to optimize the process and the best strategy with maximum PHA production can then be experimentally implemented. The model could be further used to optimize fed-batch and or continuous cultivation(s) e.g. start/stop time of nutrient feeding, substrate concentration in feed, feeding profile for further improvement of PHA concentration and productivity.[26] Fig. 3 presents a schematic of the steps involved in model development and its subsequent application for design of various cultivation strategies in fed-batch and/or continuous fermentation(s).

Mathematical models have been quite widely used for PHA processes. While in some cases, the model has been developed as a simple mathematical representation of the involved production, in other cases it has been successfully applied for process optimization by design of nutrient feeding strategies. Interestingly, the application of mathematical modelling has not been limited to only PHA homopolymers, but also to their copolymers. This is particularly important, as a thorough understanding and consequent development of 'the' right fermentation approach for reliable and reproducible production of copolymers would be highly desirable to exploit the full potential of PHAs for various exciting applications.

A mathematical model predicting biomass growth, substrate consumption, polymer production, as well as average molecular weight of P(3HB) under batch and fed-batch conditions, using *Azohydromonas latus*, was developed by Penloglou *et al*.[87] Process optimization by model-designed fed-batch fermentation involving single feed of sucrose and ammonium sulphate was performed. It resulted in an accumulation of up to 95 % dcw polymer with a concentration of 11.84 g $L^{-1}$ in 25 h. Batch mathematical model for P(3HB) production by *Azohydromonas australica* was developed by Gahlawat and Srivastava.[88] The developed batch model was then extrapolated to fed-batch by incorporation of appro-

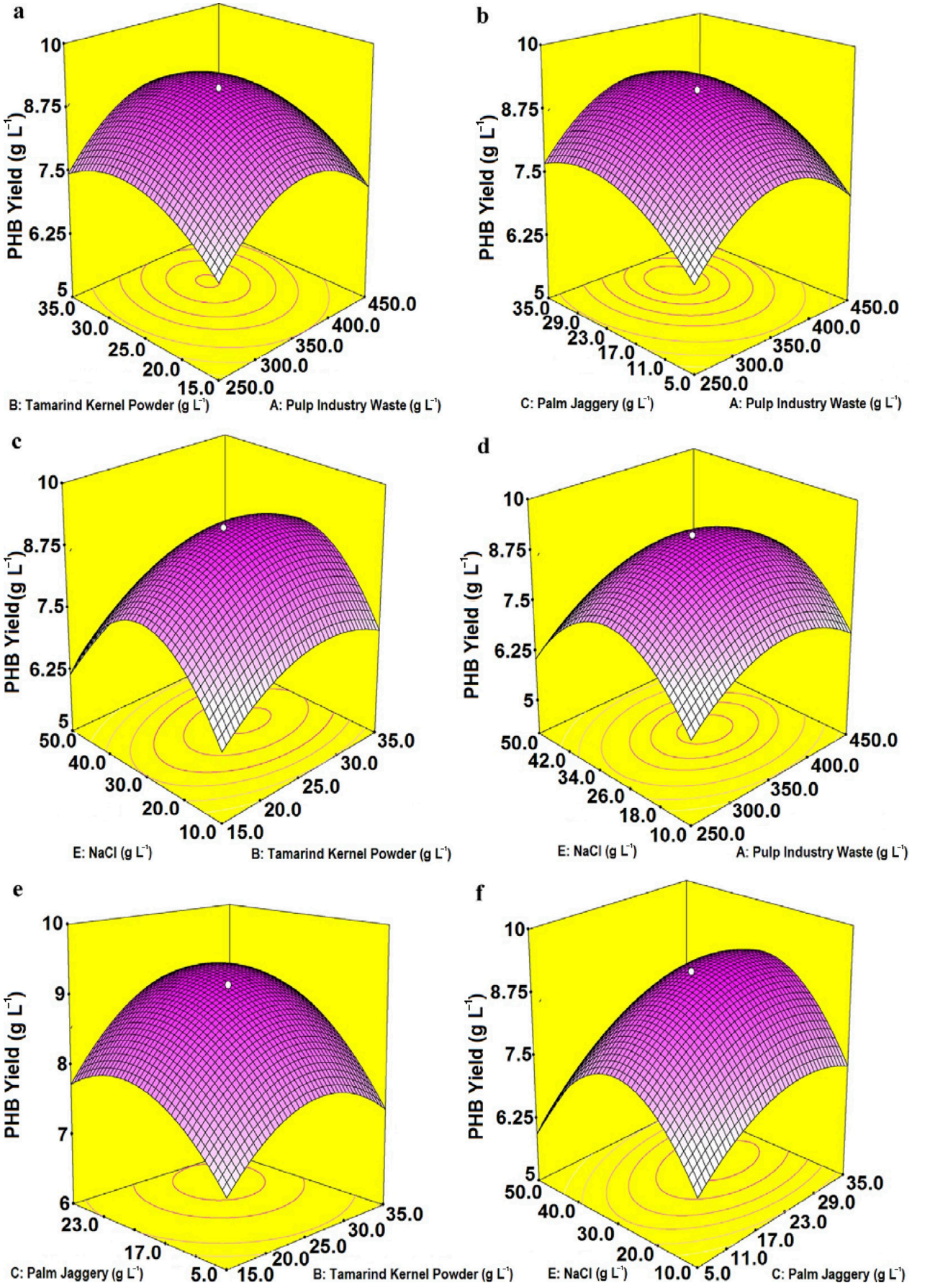


Fig. 2 – *Three-dimensional plots generated by DoE highlighting the interactive effects of different medium components on P(3HB) production. Reproduced with permission.*[62] *Copyright, 2013. Elsevier.*

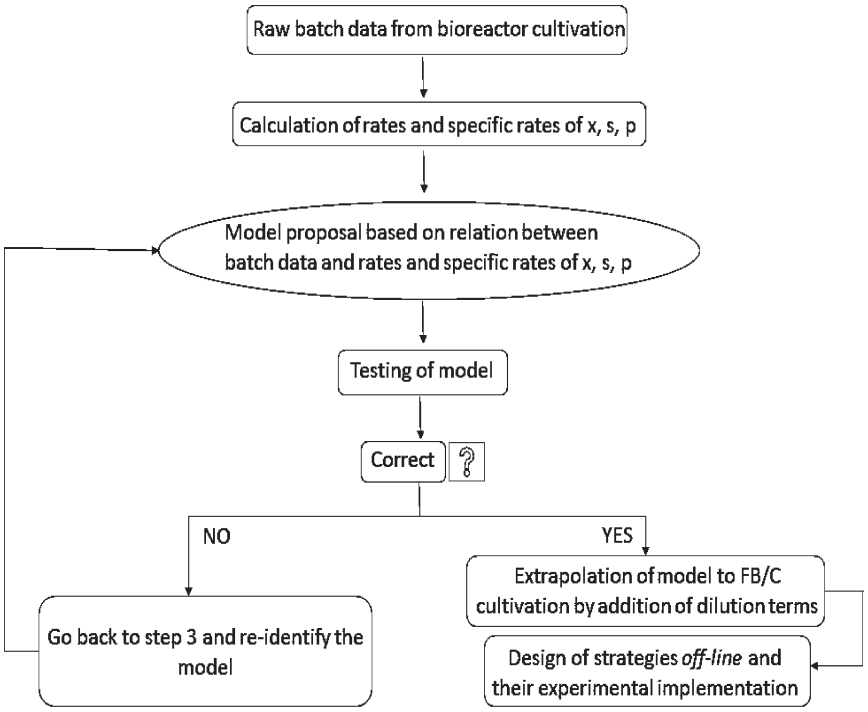


Fig. 3 – *Schematic representation of steps in model development and its application for process optimization. x – biomass, s – substrate, p – product; FB – fed-batch, C – continuous.*

priate dilution terms to the model equations. Various feeding strategies in fed-batch cultivation were designed using the model and implemented for improved P(3HB) production over batch fermentation. These included fed-batch fermentation with constant and decreasing substrate feed rate. A significantly high P(3HB) concentration of 22.65 g $L^{-1}$ with an overall P(3HB) content of 76 % dcw was obtained in constant feed-rate fed-batch cultivation. This represented a 3.5-fold increase in P(3HB) productivity (0.59 g $L^{-1}$ $h^{-1}$ vs 0.17 g $L^{-1}$ $h^{-1}$ in batch) in model-based fed-batch strategy. In yet another work involving *Azohydromonas lata*, a mathematical model was developed and applied for process optimization of P(3HB) production.[89] Though modelling of P(3HB) production had been attempted by several researchers, P(3HB) consumption during cultivation was neglected in all available models. This was incorporated in the model structure by Franz *et al.* who developed a structured, cybernetic model including underlying P(3HB) metabolic processes in continuous bioreactor systems.[90] Non-linear analysis of the continuous P(3HB) production process revealed that its consumption could reduce the final product yields at low dilution rates. In addition to development of mathematical models for *Azohydromonas sp.*, at least in one paper, a simple kinetic model based on Leudeking-Piret expression was used to represent PHA production kinetics by *Bacillus flexus*.[91] Model parameters were found by non-linear regression technique, and a good agreement was found between model predictions and experimental observations. The developed model could be further used to design high cell density fermentations involving *Bacillus* sp.

Wang *et al*. studied the kinetics of P(3HB-*co*-3HV) copolymer production by *Cupriavidus necator* using VFAs as sole carbon and energy source. They reported that the specific growth rates of microorganism were negatively affected by high initial VFA concentrations. VFA consumption was found to be dependent on pH of the medium and their concentration in the medium, which therefore required stringent regulation in feeding strategies for copolymer production using VFAs as substrates.[92] Various mathematical models for fed-batch production of P(3HB) and its copolymer P(3HB-*co*-3HV) by *C. necator* using glycerol in combination with glucose and valeric acid were developed. The developed model was then used to design various carbon and nitrogen feeds for varying PHA content and monomer composition. Controlled inflow of valeric acid and maintenance of its appropriate concentration was found to be the most crucial factor for regulating HV content in the P(3HB-*co*-3HV) copolymer.[93]

Interesting results have been achieved by application of model-based PHA process optimization. However, it is important to remember that the use of this approach depends on careful and deliberate selection of only those feeding profiles and/or strategies, which could be easily and successfully implemented on industrial scale.

## Concluding remarks

PHAs are exciting materials with interesting applications in diverse fields. The possibility of tailoring their composition and hence properties by regulation of appropriate growth and environmental conditions for PHA producers is truly fascinating. Increasing global interest in production and use of bio-based materials and production protocols is definitely going to increase research and development efforts in PHA production in times to come. Although there are few examples of commercialization of PHA processes, the reduction of production costs still remains the focus of R&D efforts in this area. In addition, it is also being largely discussed that more applications of PHAs should be developed including high-value added ones. This would provide significant market penetration to large-scale producers and ensure profitable business once high amounts of PHAs are available.[19]

This indeed has been demonstrated by the case of Imperial Chemical Industries Ltd. (ICI), UK, which is a classic example of large-scale production of PHAs since the 1980s. The idea of biopolymer production from bacteria was conceived by ICI expecting a burgeoning increase in petroleum prices in the 1970s, which thus reduced availability of petro-chemically derived polyester products.[19,94] ICI sold its polymer under the trade name Biopol which was indeed an scl copolymer P(3HB-*co*-3HV) and used for packaging applications. The process was robust with the strain of *Ralstonia eutropha* growing to significantly high cell densities of 100 g $L^{-1}$ after 72 h of cultivation, making Biopol a huge success. However, petroleum prices did not rise to the extent anticipated by ICI, thus endangering the growing popularity of Biopol over petro-based plastics.[19] ICI was reluctant to let the project die, as Biopol exhibited very interesting properties. Thus, in 1983, a separate entity called Marlborough Biopolymers was created to spin off Biopol and other related research activities. Since then, the patents have been transferred from one company to another and several additional companies have been created from the original Biopol producer.[95] More applications besides packaging, such as medical implants, raw materials for other products, drug delivery, blending with other polymers, e.g., Ecoflex, have been developed by these producers, which continue to produce PHAs profitably at a large scale.[19]

Thus, an amalgamation of appropriate process design, inexpensive substrates, and market applications of PHAs should support their large-scale production. With respect to process development, the fact that only few reports are available on continuous cultivation in the last 5 years is not a good finding, as these systems are significantly useful for obtaining high PHA productivities. This is indeed important for successful large-scale production, and thus requires further investigation. Multi-stage fermentation in fed-batch or continuous mode, sequential batch fermentation, and continuous cell recycle fermentation are some interesting configurations that offer considerably high productivities and should be further explored for enhanced PHA production.

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### List of symbols and abbreviations

CCD – Central Composite Design
DO – Dissolved oxygen
DoE – Design-of-Experiment
Dcw – Dry cell weight
ICI – Imperial Chemical Industries Ltd.
PHA – Polyhydroxyalkanoate
lcl-PHA – Long-chain length PHA
LPS – Lipopolysaccharide
mcl-PHA – Medium-chain length PHA
P(3HB) – Poly(-3-hydroxybutyrate)
P(3HO) – Poly(-3-hydroxyoctanoate)
P(3H4MV) – 3-hydroxy-4-methylvalerate
P(3HB-*co*-3HV) – Poly(-3-hydroxybutyrate-*co*-3--hydroxyvalerate)
P(3HB-*co*-4HB) – Poly(-3-hydroxybutyare-*co*-4--hydroxybutyrate)
P(3HB-*co*-3HHx) – Poly(-3-hydroxybutyrate-*co*-3--hydroxyhexanoate)
P(3HP-*co*-4HB) – Poly(-3-hydroxypropionate-*co*-4--hydroxybutyrate)
P(3HB-*co*-3HV-*co*-4HB) – Poly(-3-hydroxybutyrate--*co*-3-hydroxyvalerate--*co*-4-hydroxybutyrate)
P(3HP)-*b*-P(4HB) – Poly(-3-hydroxypropionate--*block*-poly-4-hydroxybutyrate)
P(3HB)-*co*-P(3HV)-*co*-P(3HO)-*co*-P(3HDD) – Poly(-3-hydroxybutyrate--*co*-3-hydroxyvalerate--*co*-3-hydroxyoctanoate--*co*-3-hydroxydodecanoate)
P(3HN) – Poly(-3-hydroxynonanoate)
P(3HB)-*b*-P(3HHx) – Poly(-3-hydroxybutyrate--*block*-poly-3-hydroxyhexanoate)
PLLA – Poly-L-Lactic acid
scl-PHA – Short-chain length PHA
VFA – Volatile fatty acid
1,3-PD – 1,3-propanediol

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