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Process Parameters for Influencing Polyhydroxyalkanoate Producing Bacterial Factories: An Overview

Amandeep Girdhar[1]*, Mayuri Bhatia[1], Sunil Nagpal[2], Amol Kanampalliwar[1] and Archana Tiwari[1]

[1]School of Biotechnology, Rajiv Gandhi Proudyogiki Vishwavidhyalaya, State Technological University, Airport Bypass Road, Bhopal 426033, Madhya Pradesh, India
[2]Department of Biochemical Engineering, Indian Institute of Technology, Safdarganj Development Area, Hauz Khas, IIT Campus, New Delhi, Delhi 110016, India

Abstract

The ever increasing potentialities of petroleum plastics with respect to lack of degradation, inability to recycle and the toxic effects of incineration, has urged to design biodegradable polymers, often called Green Plastics. These biodegradable plastics are promiscuous due to their analogous properties and environmental friendliness. Bacterial factories and Plants being their natural sources for production made them a promiscuous solution. Fermentation is the procedural technology used with certain fillers that are known to enhance the chemo-mechanical properties. The process at the industrial level is not well accepted due to the certain lacunas. The review mainly focuses to assimilate a few researches that implicate the best known process parameters for Batch, Fed-batch, Continuous and Two stage modes of fermentation without compromising the downstream processing at commercial level.

Keywords: Polyhydroxyalkanoate; Bacteria; Carbon sources; Batch culture; Economy

Introduction

In the world of advancements today, almost every product is constituted of some kind of polymer. There is no doubt that the need for polymers and thus the products they constitute is ever increasing. Till date, these needs are being fulfilled by synthetic polymers (often called plastics), which are produced from petrochemicals [1] which makes them eco- 'unfriendly'. The inherent nature of petroleum derived products, calls for a serving approach, in the form of Biopolymers-polymers derived from living organisms/renewable resources [2]. To satisfy the consumers and get acceptability substitute needs to exhibit similar (if not identical) characteristics to the product being replaced and so it is for biopolymers while replacing the synthetic polymers properties, ranging from molecular weight, density, melting point, crystallinity, glass transition temperature to O_2-permeability, UV-resistance, resistance to solvents, tensile strength and elongation to break [3]. Fortunately, a special class of biopolymers called PHAs shows some of the extraordinary similarities to the well known synthetic polymers like polypropylene, polyethylene etc. [4], moreover, their biodegradability has made them renowned as biopolymers of today.

History and chemical nature

In 1920, a French microbiologist Maurice Lemoigne discovered a gram positive bacterium *Bacillus megaterium* [5] that accumulated intracellular granules of polyester called poly(3-hydroxybutyrate) [6]. Table 1 enlists different types of PHA.

PHAs are polymeric compounds biosynthesized by a variety of gram positive and gram negative bacteria [7], as carbon and energy reserves (often called carbonosomes) [8]. Structurally, R-hydroxyalkonic acids act as the monomeric form of PHAs.

Natural sources of production

Polyhydroxyalkanoates are produced in microbes. Although efforts have been made from plant cells through transgenics, but has not achieved much success because low yields of less than 10 % (w/w) of dry cell weight can be sustained whereas, high yield limits growth and development of plants [9,10]. On the other hand, PHAs can be accumulated upto 90% (w/w) in bacterial cells [10] and are thus a priority because of the ease in culturing and economical, in contrast to the complex plant system [10].

Bacterial Polymer Production

PHAs accumulation is an inherent response to the stress conditions faced by bacterial cells [11,12], these are generated *in vitro* by exposing bacteria to nutrient limitations, due to which they switch their metabolic pathways and cause PHA production as their carbon and energy reserves [13], to name a few of these substrates are bagasse [14], molasses [15,16], corn cob [14] and other agricultural wastes [4,17]. In fact significant PHA production has been reported among various bacterial strains when growing on kitchen waste [18], industrial wastes [7,14,19], crude and edible oils as carbon sources [20-23]. The costing of these substrates is low or null, making the process cost effective at the upstream level. The Figures 1 and 2 depicts the biopolymer synthesis from bacteria.

PHA production has been reported in wide variety of bacterial

PHAs	Number of Carbon Atoms in Monomers of PHA	Examples
Short chain length PHAs (scl-PHAs)	3 – 5	P(3HB) P(4HB)
Medium chain length PHAs (mcl-PHAs)	6 – 14	P(3HHx) P(3HO) P(3HHx-co-3HO)

Table 1: Types of PHAs [2].

***Corresponding author:** Amandeep Girdhar, School of Biotechnology, Rajiv Gandhi Proudyogiki Vishwavidhyalaya, State Technological University, Airport Bypass Road, Bhopal 426033, Madhya Pradesh, India, Tel: +918989206617; E-mail: amangirdher@gmail.com

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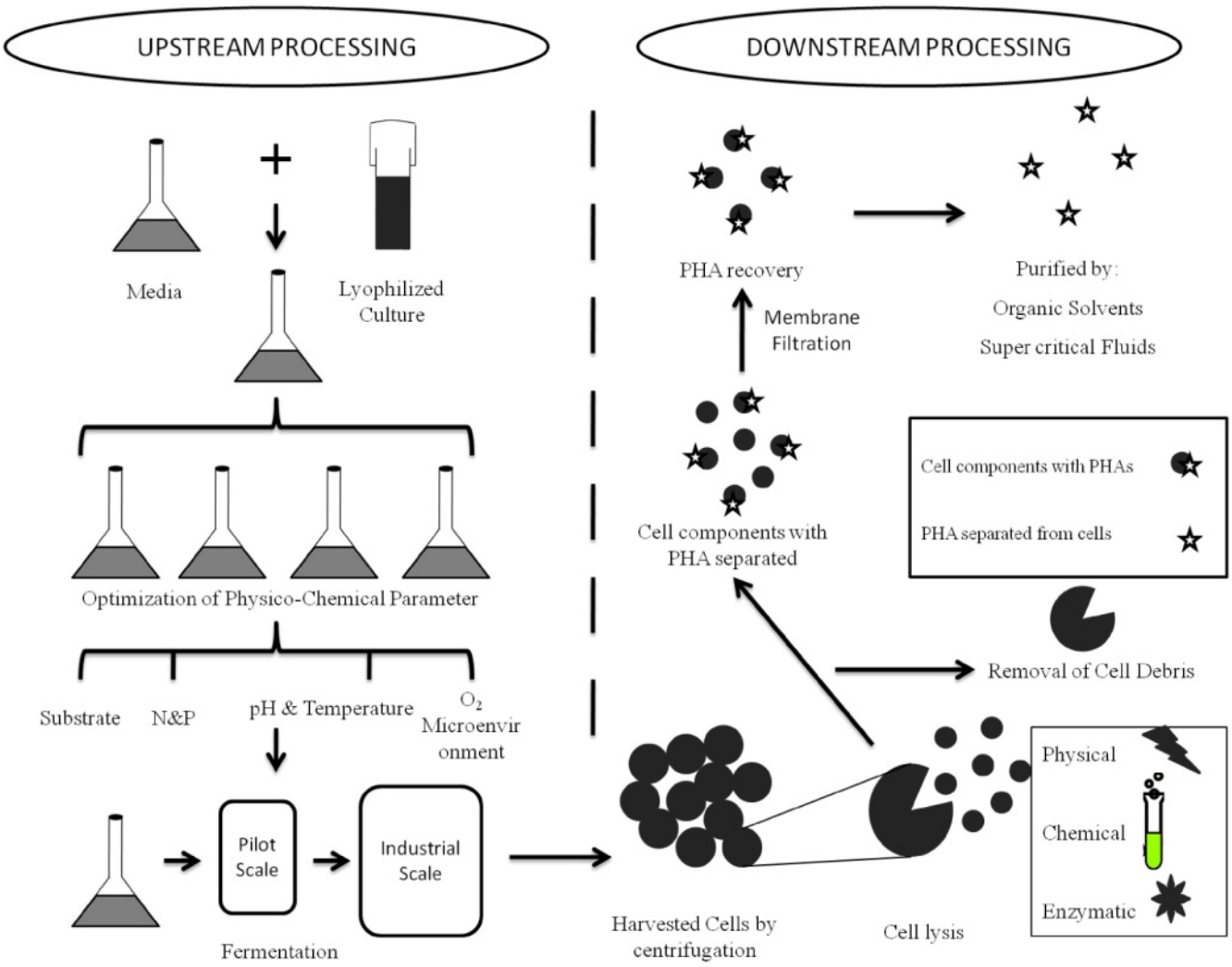

Figure 1: Biopolymer synthesis.

strains, the most commonly studied genera are *Bacillus sp*, *Alcaligenes sp*, *Pseudomonas sp, Aeromonas sp, Rhodopseudomonas sp*, *Halomonas sp*, Transgenic *Escherichia coli, and Burkholderia sp* [7,9]. Table 2 and Table 3 show carbon sources based feeding regimes used by different strains with the PHA Content. The various stress conditions trigger PHA production [11,12]; thus, variation in process parameters is required [24-26] to induce the biosynthesis.

Process parameters

Substrate: Carbon source is an important requisite but is not involved in induction of PHA biosynthesis, although is required for polymer production maximization [54,55]. Glucose is the preferred substrate at industrial scale, many alternatives have also been employed, like sucrose as stated in the research by Azhar et al. of Ain Shams University, Egypt with highest growth and polymer production by *Alcaligenes latus* [56]. Even a broad spectrum of substrates, namely, starch, sucrose, lactose, maltose, galactose, mannose, mannitol, fructose, glycerol, ethanol, lactic acid, malic acid, acetic acid and butyric acid have been used to compare with glucose [56]. In another report by Poonsuk Prasertsan, the halotolerant bacterial strains *Rhodobacter sphaeroides* has exhibited maximum growth and polymer production on acetate as a carbon source compared to glucose and fructose [25]. The conclusion deduced glucose, fructose and acetate as good substrates for cellular growth and polymer formation, but a combination of these substrates did not give good results [25].

Nitrogen and phosphate: A research aimed at the optimal requirement of phosphate and nitrogen in the media (specific or complex) to increase the production tested the growth of *Aeromonas hydrophila* on gluconate enriched MS media containing different phosphate concentrations as (Table 4). It was clearly inferred that a concentration of 11.66 mM phosphate in the media provided a good nutrient deficiency to the bacteria leading to a high PHA concentration [44]. This concentration of phosphate was 1/3 of the normal phosphate concentration used in the media for the bacterial growth. Table 4 illustrates the effect of limiting phosphate concentrations, with nitrogen deficiency in media, on PHA production [44].

Although the nutrient limitation is a prerequisite for PHA production, but, in a recent report on *Cupriavidus necator* in several nutrition media (Mineral medium, Bonnarme's medium, Mandels' medium and Luria broth Mineral medium, Bonnarme's medium, Mandels' medium and Luria broth) with 10g/l sodium glutamate not

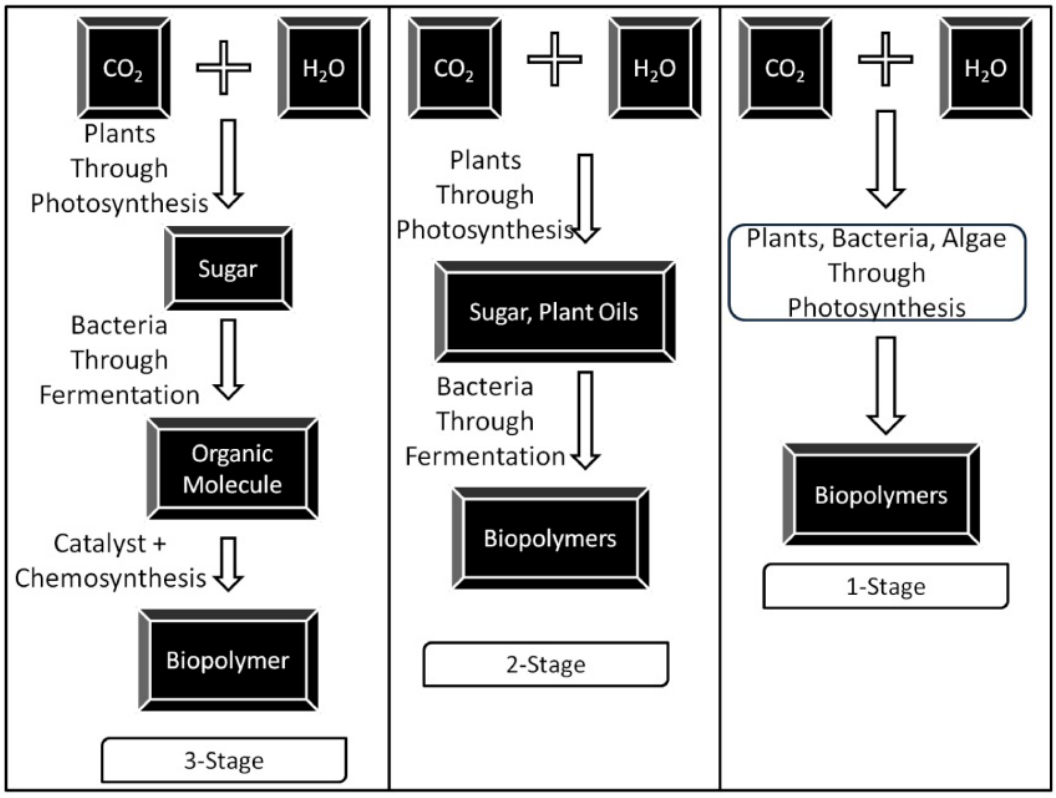

Figure 2: Systems for Biopolymer Synthesis.

only enhanced the overall productivity by 33 times but also decreased the need for limiting nutrients in media [57].

pH and temperature regimes: The pH and Temperature optima completely depend on the nature of organisms. According to Palleroni et al. [58] and recent studies done by Wei et al. [26], the optimal pH range for the growth of bacteria involved in the production of PHAs is 6.0 to 7.5 [26,58]. The temperature requirement for the optimal production is 30 to 37°C as reported by Yu-Hong Wei et al. [26]. These are the generalized range for fine bacterial growth.

Oxygen microenvironment: Oxygen limitation is regarded amongst the effective way of enhancing PHA accumulation, involves volumetric oxygen transfer coefficient (k_La), reducing (k_La) of the system significantly enhance the PHA yield of the bacterial cell, even at agitation speed of 300 rpm, the percentage PHA derivate was 29.8% whereas it was 50.7 % at 50 rpm [24]. Since KL_a is lower at low agitation rate, indicates that increasing the oxygen stress enhances PHA production.

Modes of culture

Four modes of operations are used, these are Batch, Fed batch, Two stage and continuous; although Batch process has been the mode of choice for most of the research and commercial production.

Batch: The batch cultivation is performed from range starting from 100ml working volume at laboratory scale [16,28] to 15000L working volume at industrial scale [59]. *Alcaligenes latus* (BIOPOL, the first commercially produced biopolymer from *Alcaligenes latus* [60]), *Ralstonia eutropha*, *Aeromonas hydrophila*, *Burkholderia* sp. and *Pseudomonas putida* have been given equal importance for polymer production [9]. Maximum PHA production is reported between 96 to 120 hours in almost all species**, w**here glucose and sucrose have been the substrate of choice (in shake flask batch mode *Alcaligenes latus* exhibited 45.96 % and 40.14 % PHB content, and in 2 L reactor it exhibited 56.59 % and 47.53% PHB content using sucrose and glucose as substrate, respectively), with ammonium sulfate as best nitrogen source [56,61]. A 2L batch operation yielded maximum PHB content after 80 hours of operation, after which the content decreased (probably due to hydrolysis during stationary phase), suggesting the critical importance of harvesting time [61].

Fed batch: Basic aim of using the fed-batch mode is to increase the biomass with respect to batch mode and is achieved by controlling the intermittent feeding to reduce the substrate inhibition without affecting the growth of the microbe(s). The basic causative agents of inhibition are the organic acids which in high concentration lead to the collapse of the transmembrane pH gradient of the bacterial cells and a low concentration supports the cell growth and hence PHA accumulation [62]. Two feeding rates for organic acid concentration variation were studied with constant and varying rate on *Ralstonia eutropha*. Three constant feeding rates of 1.2 g/l, 1.8 g/l and 2.5 g/l achieved greater biomass(RBM) and PHA content in case of slow feeding rate with zero residual acids, while it ceased at 24th and 36th hours respectively at intermediate feeding, with a residual acid of 2.02g/l and at 18th and 36th hour at fast feeding rate, with a residual acid of 4.02g/l suggesting that cell growth and production could only continue under a low level of residual acids in the medium, while PHAs accumulate at higher acid concentration. Thus it may be considered essential to feed acids during

Strain	Carbon Source Used	Polymer Yield	References
Aeromonas sp.	Municipal waste water	11.11%	[27]
	Palm oil mill effluent	22.97%	
	Glycerol	19.82%	
	Molasses	20.09%	
Alcaligenes sp.	Municipal waste water	41.11%	[27]
	Palm oil mill effluent	11.69%	
	Glycerol	2.33%	
	Molasses	0.00%	
Bacillus sp.	1:1 mixture of WBH and RBH	59%	[28]
	Municipal waste water	43.95%	[27]
	Palm oil mill effluent	58.925%	
	Glycerol	25.00%	
	Molasses	48.01%	
Bacillus cereus	Municipal waste water	38.07%	[27]
	Palm oil mill effluent	64.09%	
	Glycerol	0.00%	
	Molasses	23.94%	
Bacillus licheniformis	Municipal waste water	40.97%	[27]
	Palm oil mill effluent	62.96%	
	Glycerol	1.69%	
	Molasses	43%	
B. subtilis	Municipal waste water	46.71%	[27]
	Palm oil mill effluent	50.00%	
	Glycerol	18.92%	
	Molasses	16.13%	
Bacillus megaterium	Sugarcane molasses	43%	[15]
Burkholderia cepaca	Glycerol	31.35%	[29]
Chromobacterium sp.	Municipal waste water	25.00%	[27]
	Palm oil mill effluent	40.89%	
	Glycerol	11.86%	
	Molasses	27.85%	
Cupriavidus taiwanensis	Gluconic acid	72%	[26]
Escherichia coli	Xylose	59 ± 4%	[30]
	Sodium butyrate	7.81 ± 0.21%	[31]
Hydrogenophaga pseudoflava	Lactose	29.8 ± 3.0%	[13]
Haloarcula hispanica (previously deposited as Halobacterium hispanicum)	YE/Glucose	2.4%	[32]
Haloarcula marismortui	YE/Glucose	21%	[33]
***Haloarcula* sp.**	Glucose	63%	[34]
Haloarcula japonica	Glucose	0.5%	[35]
Halobiforma haloterrestri	Butyric acid	40%	[36]
H. haloterrestris	YE	15%	[36]
Haloferax mediterranei	YE/Glucose	17%	[32]
H. mediterranei	Starch	6.48g/L	[37]
Haloferax gibbonsii	YE/Glucose	1.2%	[32]
Haloferax volcanii	YE/Glucose	7%	[32]
Haloterrigena hispanica	YE/Casamino acids	0.14%	[38,39]
Halopiger aswanensis	YE/Na acetate/Butyric acid	34%	[40,41]
Pseudomona oleovorans	Gluconic acid	0.9±0.1%	[26]
Pseudomonas putrefaciens	Corn cob	66.67%	[14]
Pseudomonas sp.	Municipal Waste Water	61.05%	[27]
	Palm oil mill effluent	60.08%	
	Glycerol	62.08%	
	Molasses	60.04%	
Proteus mirabilis	Municipal Waste Water	0.00%	[27]
	Palm oil mill effluent	0.00%	
	Glycerol	0.00%	
	Molasses	0.00%	
Ralstonia eutropha	Glucose	0.058	[42]
	Sodium gluconate	87.03%	[43]

Table 2: Data for carbon sources used by microorganisms for scl-PHA production.

Strain	Carbon Source Used	Polymer Yield	References
Aeromonas hydrophila	Gluconate	15%	[44]
Cupriavidus necator	Crude palm kernel oil (5g/L)	77 ± 3%	[22]
	Crude palm kernel oil (10g/L)	82 ± 2%	
	Jatropha oil	62 ± 3%	
	Crude palm oil	69 ± 6%	
	Palm olein	61 ± 4%	
	Soybean oil	65 ± 1%	
	Corn oil	63 ± 2%	
	Coconut oil	70 ± 3%	
Escherichia coli	Xylose	70 ± 1%	[30]
	Sodium octanoate	47.3 ± 5.0 %	[31]
	Decanoic acid	25.7 ± 0.9 %	
	10-Undecenoic acid	41.0 ± 8.0 %	
	Dodecanoic acid	28.6 ± 8.0 %	
Haloarcula hispanica	YE/Glucose	17.33 ± 0.04%	[45]
Halobacteriumnoricense	Triptone	0.08/0.03%	[46]
Halococcusdombrowskii	YE/HyCase	0.15/0.01%	[46]
Halococcussalifodinae	YE/HyCase	0.05/0.01%	[46]
Haloferax mediterranei	Glucose	27%	[47]
	ECS (Extruded Corn Starch)	38.7%	[47]
	ERB (Extruded Rice Bran) :ECS (1:8)	55.6%	[47]
	Bacto Casa amino acids/YE	18.21 ± 1.88%	[45]
	YE/Starch	24.88 ± 1.27 g/L	[45]
Hydrogenophaga pseudoflava	Whey (copolymer)	10.1 ± 0.9%	[13]
Pseudomonas putida	Octanoic acid	49.7 %	[48]
	Octanoate	21%	[49]
	Glucose	ND	
	Acetate	ND	
	Pyruvate	ND	
	Citrate	ND	
	Succinate	ND	
	Glucanoate	ND	
	fructose	ND	
	Dodecanoic acid	54.5 %	[48]
	Oleic acid	68.9 %	
	Xylose	20 %	[50]
	Nananoic acid	0.15g/g	[51]
	NanoicAcid:Glucose (0.8:1)	50.85%	
	Corn oil	17.5 ± 1.4%	[52]
	Glucose	19%	[44]

Pseudomonas oleovorans	Corn oil	15.7 ± 2.5 %	[52]
Pseudomonas chlororaphis	Corn oil	39.5 ± 0.8	[52]
Pseudomonas G101	Waste frying rapseed oil	20%	[53]
Natronobacterium gregoryi	YE/Casamino acids	0.1/0.3%	[46]
Sinorhizobiummeliloti	Rice bran hydrolysate	48.32%	[4]

Table 3: Data for carbon sources used by microorganisms for mcl-PHA production.

Concentration	Amount $Na_2HPO_4.12H_2O$ (g/l)	Amount KH_2PO_4 (g/l)	PHA concentration (%, w/w)
Normal phosphate (35mM)	9	1.5	4.6 ± 0.6
2/3 phosphate (23.35 mM)	6	1	5.5 ± 0.5
1/3 phosphate (11.66 mM)	3	0.5	10.2 ± 0.5

Table 4: Effect of limiting P and N concentrations on PHA production [44].

the cell growth phase, followed by gradually decreasing the flow rate to maintain a lower residual concentration of about 2 g/l. This strategy came out to be successful with the Dry Cell Weight (DCW) and PHA contents as 14.35g/L and 6.89g/L respectively at the end of 42nd hour [62]. pH-stat mode provide randomly variable rate of organic acids, which worked on the principle of acid adjustment/pH control and is successful if the pH of the system varies with the growth of the bacteria. The pH variation is adjusted by organic acids rather than HCl/NaOH leading to a random feeding of organic acids, which caused random feeding leading to less effective PHA production and DCW. In reference, a more careful feed rate control can further modulated for improvement in cell growth and PHA production.

Continuous: Continuous fermentation technology has got advantage of maintaining constant nutrient environment and is useful to study the effect of nutrient limitation on the bacterial growth and productivity in a quick and real time manner [63]. In fact, the documented reports are aimed at studying the effect of limiting the concentration of various nutrients. In a recent example, the metabolic response of *P. putida* KT2442 producing high levels of PHA under single- and multiple-nutrient-limited growth, with chemostat mode has been reported [64]. At the industrial level, continuous mode could prove very useful owing to continuous harvesting of the product, coupled with the ability to tweak the nutrient concentration as per the requirement; although the stringency of sterile environment and a check on mutability of the producing strain becomes obligatory under continuous mode, when compared to the ease of batch mode.

Two – stage batch culture as the best method for PHB production: The two-stage fermentation involves supply of nutrients to the culture in two different stages and composition. Thus, becomes handy for possessing the nutrient limitation, a prerequisite for PHA production [9]. In the first stage, culture is fed with simple carbon sources such as glucose or fructose and other essential nutrients to make sure that cells grow at high specific growth rate and lead to a higher cell biomass yield, whereas in the second stage, supply of essential nutrient such as nitrogen is limited as a trigger to initiate the biosynthesis [56]. A volumetric PHA productivity of 1.06 g L^{-1} h^{-1} was obtained by Two-stage continuous mode of *Pseudomonas oleovorans* culture [65].

Downstream Processing

All the bacterial species reported to produce polyhydroxyalkanoates, accumulate them intracellularly in the form of granules, as carbon and energy reserves [44,59]. It is thus apparent, that the downstream processing chain of PHAs begins from cell lysis of the biomass separated from the medium by centrifugation, so as to expose the cell trapped product [66]. There are many techniques used for the purpose, Table 5 describes a few of these.

Recovery

The PHAs are present in the cell debris after the cell lysis and centrifugation, which can be recovered with a good efficiency using different solvents like cold methanol, chloroform etc [77]. This leaves a dull white precipitate behind; further purification of the product is done by membrane filtration, which helps in segregating PHAs from other small biomolecules still present in the extract. A study with non-halogenated solvents for recovery from *R. eutropha* has shown to give high purity (upto 99%) and have proved potentiality of ethyl acetate and methyl isobutyl ketone to replace halogenated solvent, chloroform, with cost reduction benefits [78]. In a research, 0.1 µm ceramic tubular membranes have shown high separation efficiency owing to their chemical inertness and resistance to pH, temperature and concentration changes [77]. But polyethersulfone polymeric flat plate membrane modules are becoming the choices of the day because of their 5-10 times less cost than ceramic membranes and ability to retain even the smallest PHA granule. However, membranes like polyethersulfone come with an inherent disadvantage of limited life span and need to be replaced once in a year or two. It must be noted that the ultra-filtration carried out to separate the PHAs is operated in a cross flow regime, which avoids the blocking of the membranes and ensures continuous operation for long time [77]. The amount of product recovered in form of filtrate is then determined using Gas Chromatography to confirm

Classes	Techniques	Acting Principle	Microorganism	Reference
Physical/ MechanicalDisruption	Thermolysis	Disruption of Cell wall by the affect of ionic strength, pH and temperature with chelating agent		[67,68]
	Ultrasonication	Ultrasonic waves, followed by centrifugation	*Bacillus flexus*	[68]
	Bead Mill	Grinding cylinder containing beads made of wear resistant materials like glass, alumina, titanium carbide, zirconium oxide and zirconium silicate is driven by motor	*Alcaligenes latus*	[60,67,69]
	High Pressure Homogenizer	Disrupter fitted with a displacement pump monitors the pressure and a discharge valve to homogenize the solution pushed through pump	Gram Negative Bacteria	[70]
Chemical Disruption	Alkali Treatment	Exposure to basic pH (mild alkaline hydrolysis)	*Bacillus flexus*	[69]
	Detergent Solubilization	Detergents like SDS, CTAB, Triton X 100, Saponins, Tween 20 and Tween 80 etc, are used	*Ralstonia eutropha*	[67,71,72]
	Cell Wall Permeabilisation	Organic solvents like toluene, acetone, chloroform and ethylene carbonate are used, followed by non-solvent precipitation	*Bacillus cereus* SPV and *Cupriavidus necator*, respectively.	[73,74]
Enzymatic Disruption		Lytic enzymes in medium with detergent or chelating agent	*Cupriavidus necator*	[75,76]

Table 5: Cell Lysis techniques and acting principles.

Parameters	Synthetic Plastics	Biodegradable Plastics
Synthesis	Crude oil	Microorganism
	Flash distillation	Waste carbon sources
	Column distillation	Culturing
	Cracking (use of catalyst)	Biomass separation
	Polymerization	Polymer recovery
	Heat required for every step	Heat required for film preparation and drying
Degradation	Ethylene, CO_2, CH_4 production	Biological compounds are produced
	Toxic	Non-toxic
	Never degrade	Take days to months

Table 6: Factors deciding cost.

the percentage of recovery [43]. Quantification can be done by NMR [79], Flow Cytometry [80] etc.

Cost

The major factor of concern is the cost of the produced biomaterial i.e. PHA. The production cost is contributed by various factors. The process parameters are involved in upstream cost whereas the down streaming involves the cost of solvents used and the other purification strategies. The Table 6 illustrates parameters involved in cost of production and degradation through biological means. Processing cost in generally 20% of the total cost.

Conclusion

The growing environmental pollution is becoming a global concern, where synthetic plastics contribute to a great extent due to their non-degradability. The bioplastics can be regarded as a working alternative to conquer the conundrum. From all the classes of bioplastics, PHA has shown to have analogous properties with biodegradability. Thus, PHA can be looked forward as an alternate to synthetic plastics as they require natural sources for synthesis like Bacteria, that have come as an effective way for commercial production, *Ralstonia eutropha* is amongst the best producers. Being promiscuous in nature, their production involves certain process parameters to be optimized both at laboratory and industrial scale, where the optimum pH and temperature range depends on the microorganism used in the synthesis. Further the O_2 stress enhances the production. The mode of fermentation is responsible for influencing the biomass yield and accumulation of the product. Not to overlook the Cost factor, which can be reduced by various alternatives to novel carbon sources, but to attain a good yield and commercialization with these alternatives still remain a challenge.

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