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Review

Polyhydroxyalkanoates: Next generation natural biomolecules and a solution for the world's future economy

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ABSTRACT

Petrochemical plastics have become a cause of pollution for decades and finding alternative plastics that are environmental friendly. Polyhydroxyalkanoate (PHA), a biopolyester produced by microbial cells, has characteristics (biocompatible, biodegradable, non-toxic) that make it appropriate as a biodegradable plastic substance. The different forms of PHA make it suitable to a wide choice of products, from packaging materials to biomedical applications. The major challenge in commercialization of PHA is the cost of manufacturing. There are a lot of factors that could affect the efficiency of a development method. The development of new strategic parameters for better synthesis, including consumption of low cost carbon substrates, genetic modification of PHA-producing strains, and fermentational strategies are discussed. Recently, many efforts have been made to develop a method for the cost-effective production of PHAs. The isolation, analysis as well as characterization of PHAs are significant factors for any developmental process. Due to the biodegradable and biocompatible properties of PHAs, they are majorly used in biomedical applications such as vascular grafting, heart tissue engineering, skin tissue repairing, liver tissue engineering, nerve tissue engineering, bone tissue engineering, cartilage tissue engineering and therapeutic carrier. The emerging and interesting area of research is the development of self-healing biopolymer that could significantly broaden the operational life and protection of the polymeric materials for a broad range of uses. Biodegradable and biocompatible polymers are considered as the green materials in place of petroleum-based plastics in the future.

Contents

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1. General presentation, historical outline and properties of PHA

1.1. Polyhydroxyalkanoates (PHA)

Petrochemical plastics are manipulated in various utilizations like plastic pots, vessels, things for refreshment wrapping and as biomaterials [1]. They characterize a major part of wastes and their perseverance in the environment [2]. Many societies are responsive to the negative outcome of these products, including hazardous ones, e.g. pollution on animal life and on natural surroundings of ecosystems, human diseases, etc. In distinction to these conventional plastics, polyhydroxyalkanoates are biopolymers which are produced and catabolised via a variety of microorganisms. These biopolymers can be accumulated in microbial cells under nutrient-unbalanced conditions [3]. The most broadly synthesized bacterial bioplastics are majorly polyhydroxyalkanoates (PHA) and their types. PHA are a class of biopolyesters, made up of short chain length like; 3-hydroxybutyrate as well as medium chain length monomeric constituents like; 3-hydroxyoctanoate along with 3-hydroxydecanoate, synthesized from a huge number of strains as an energy and carbon storage compound as well as the enhancement of stress robustness of the bacterial cells [4]. Recently, PHA have received significant awareness due to opportunity of production from carbon substrates, like; vegetable oils and sugars [5]. These compounds do not produce harmful results in host body and have definite benefits such as biocompatibility and biodegradability over petroleum-derived plastics. A general molecular composition of PHA is given in outline Fig. 1.1. Several substituents can also be incorporated into the side chains [6].

Biopolymers (bioplastics) have gained significance since the decade of 1940. A great number of polylactic acids (PLA), open chain polyethylene terephthalate, polysaccharides as well as fusions of these substrates have also been established effectively until [8] today. These have been used as a replacement of conventional materials, e.g. metals, wood, glass and other tools of construction in various household, industrial and basic environmental uses [2]. Out of all these types of decomposable/biodegradeable plastics, PHA have been illustrating great interest due of their component characteristics which are comparable to the petroleum based plastics [9]. Their durability, stability, mechanical and thermal properties have played a significant role for their use on industrial scale [10]. In instead of these properties of PHA over the petroleum-based plastics, their use is now restricted because of maximum charges of formation [11]. The production of PHA from easily accessible carbon sources like; agricultural wastes, corn, cassava etc. would also be complimentary with respect to both cost-effective and environmental point of view. PHA are degraded at a high rate (duration about 90–270 days) converted into CO_2 along with H_2O by many microorganisms by their own secreted PHA hydrolases [12]. The improved properties of PHA that blends with natural raw materials or other biodegradable polymers, including starch, cellulose derivatives, lignin, poly (lactic acid), polycaprolactone and different PHA-type blends have significant role in biomedical applications [13]. A natural blend of P(3HB)/mcl-PHA that can be processed into films suitable for applications ranging from commodity packaging products to high-value biomaterials. The films showed no significant change in mass or volume during immersion in deionized water, exhibiting only negligible swelling degrees (below 5%), which is in agreement with their hydrophobic nature [14].

Like PHB, PLA also belongs to the family of biodegradable polyesters and finds its application in different areas due to its biodegradability, biocompatibility and sustainability. It is reported that both PLA and PHB are brittle at room temperature and process poor processing

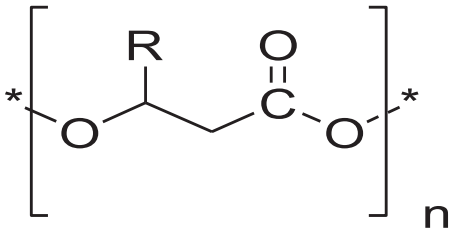

Fig. 1.1. General structure of polyhydroxyalkanoate [7] n: degree of polymerization.

properties [15]. As part of improving their mechanical properties and processing characteristics, several strategies have been tried by researchers, and blending PLA with PHB is one among them. These studies proved that the miscibility between PLA and PHB depends on the molecular weight of the minor component in the blend system. In case of high molecular weight blend components, PLA is immiscible with PHB in all compositions. Also it was concluded that the mechanical properties of the blends are intermediate between those of the individual blend components. The effect of processing conditions on the miscibility, crystallization and melting behavior and morphology of blends of PHB and PLA with and without poly (vinyl acetate) (PVAc) was studied by El-Hadi [16]. The results showed that blending PHB with PLA is a cost effective method to improve the mechanical properties of PLA as the 75/25 blend of PLA/PHB showed significant improvement in mechanical properties compared with pure PLA.

Another economic assessment revealed that a wood-PHA composite as a drop-in alternative WPC product could cost as little as 37% of the cost of its neat PHA counterpart. Thus, wood plastic composites (WPCs) with PHA offer a means to access benefits of PHA in engineering applications at reduced costs; however, further developments are required to improve strain at failure. The WPC global market was as high as 3.0 million tonnes/year in 2014, and is forecast to double by 2020 [17]. The successful adoption of wood-PHA composites into the market is furthermore reliant on support from public sector to encourage biodegradable products where recycling is not a ready solution.

Blending of biodegradable polymers as well as using low-price organic fillers aiming to produce cost-effective composites, besides being an alternative to solve pollution issues, might be a method of developing products with superior mechanical properties. Moreover, the natural and cost-effective Babassu filler was added to the polyhydroxybutyrate and poly(butylene adipate-*co*-terephatalate) PHB/PBAT blends. Babassu is a palm tree naturally found in the North, Northeast and Midwest regions of Brazil. Its fruits are composed by a protective shell rich in lignin and cellulosic material. Usually, the shell is discarded and burnt, i.e., it is a material with no added value. PBAT/PHB/Babassu composites are biodegradable, environmental friendly, and cost effective, products based on these compounds have a great potential since their mechanical properties such as ductility, stiffness, and tensile strength are still suitable for several applications even at lower temperatures (−40 °C). Therefore, its use in polymer composites, beyond scientific and technological improvements can yield economic benefits [18].

1.2. Polyhydroxyalkanoate (PHA) market

Estimated at USD 57 million in 2019, the global polyhydroxyalkanoate (PHA) market size is projected to reach USD 98 by 2024, growing at a CAGR of 11.2%. Europe is the key market for PHA, globally, followed by North America and APAC. The short-chain length type is estimated to lead the PHA market in terms of value and volume. The growing awareness regarding plastic waste creation resulting in an ecological imbalance has changed the market sentiments positively towards sustainable products, hence creating a surge in demand for biodegradable plastics. This factor plays a crucial role in increasing the demand for short-chain length and medium-chain length PHA. Europe is expected to account for the largest market share in PHA during the forecast period, in terms of value. The presence of various PHA players, such as Bio-on (Italy), Biomer (Germany), Natureplast (France), ColorFabb (Netherlands), and EarthBi (Germany), has a positive impact the market [19]. Additionally, the growing use of biodegradable plastic in packaging & food service, bio-medical, and agriculture applications in the region is augmenting the demand for PHAs [20].

1.3. Historical outline of PHA

A brief historical outline of PHA production is shown in Fig. 1.2. First stage of research comprised of discovery of short chain length PHA (scl-PHA). Lemoigne [21] from 1926 to 1944 confirmed the occurrence of the biopolymer (PHB, polyhydroxybutyrate) in the genus *Bacillus* comprising *B. megaterium, B. anthracis and B. mycoides* as well as *Azotobacter chroococcum* [22–24] In the following years, the granules of PHB had been detached from protoplasm in 1942 [25] and their role because of an intracellular alternative for energy and carbon was determined during 1958 [26]. In 1973, discovered to facilitate PHB which could be accumulated up to 70% of the dry mass of bacterial cells, under different nutritional and ecological conditions [27]. De Smet et al [28] in 1983 shown the production of another monomer of PHA, 3-hydroxyoctanoate (3HO) from *Pseudomonas oleovorans*.

Moreover, some other hydroxyalkanoate (HA) units including 4-hydroxyalkanoate (4HA) [29] and 5-hydroxyalkamoate (5HA) [30] were revealed. These results were therefore marked as the commencement of the second developmental stage [31] of research in the field of PHA (Fig. 1.2).

The third phase of research concerned the cloning of genes and classification of enzymes involved in the biosynthesis of PHA. Gerngross confirmed that PHA synthase enzyme was situated on exterior body of PHB particle during its development first time in 1993 [32].

The 4th stage comprises the advances in the science and technological improvement of PHA. In this stage, researchers entered into the era of biotechnology, protein engineering, analytical and organic chemistry (Fig. 1.2). Efforts have been prepared to produce PHA industrially from those strains which were not able to produce PHA naturally. For this function, genes accountable for the synthesis of these polyesters were introduced to these strains. A recombinant *Escherichia coli* strain was found to develop speedily and to produce high yields of PHB and mcl PHA in different cultural conditions [33].

The 5TH stage comprises the advancement in optimized upstream processing, efficient, sustainable product and recovery from microbial biomass. Efforts have been prepared for the bioreactor development, bioprocess engineering, process regime, and fermentation mode. Next is the formation of PHA-based composites and blends with other fillers or polymers also bioprocess analysis in silicon approaches. More studies on genetic engineering will lead to the generation of new products produced by recombinant halophiles. The PHA granules synthesized by *Halomonas* spp. can also be produced in large size for convenient separation and drying processes [34]. Halophilic bacteria were found able to grow in open and continuous fermentation processes in unsterile seawater medium without contamination for at least two months [35]. This will promote the emerging "Next Generation Industrial Biotechnology" or NGIB for bio-production with reduced cost and thus improved competitiveness.

1.4. Monomer composition and types of PHA

PHA can be normally composed of a linear/Straight, head-to-tail polyester comprising of hydroxy fatty acids [36]. It is accumulated as intracellular granules (0.2 μm – 0.5 μm) in the cytoplasm by various microorganisms. Each granule contains 10^3 to 10^4 polymer chains [37]. Methyl-carbonyl group connections in PHA make a firm and stable helix. Majorly they are optically active having a chiral centre but 4-hydroxybutyrate is not optically active (Fig. 1.3a).PHA differ in structure, physical and chemical characteristics, monomeric constitution, number and mass of particles depending on type of microorganism and culture conditions.

1.5. Types of PHA

Because of carbon chain length, PHA can be classified in two different kinds:

Scl-PHA those contain C_3 or C_5 monomers (outline Fig. 1.3b) e.g. polyhydroxybutyrate (PHB) and PHV poly (3-hydroxyvalerate). Their properties are correlated to traditional petro-chemical plastics.

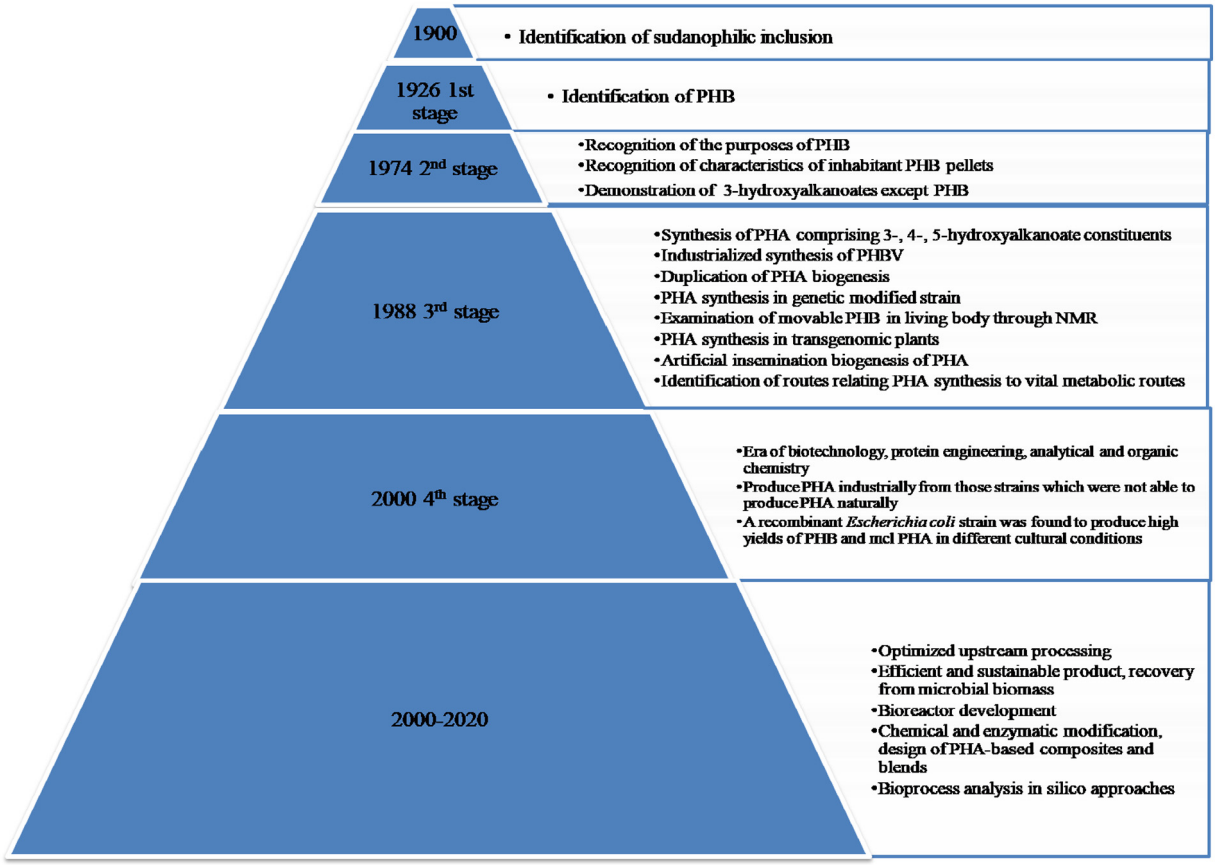

Fig. 1.2. Advancement of PHA in science and technology through 20th and 21th century [31].

PHB is the commonly used participant of PHA family. It is an carbon and energy storing compound generally stored when a fundamental nutriting element like; phosphorous, nitrogen, oxygen and sulphur are restricted during the existence of maximum carbon substrate [8].Their physical and mechanical properties were found related to polypropylene, although, its percentages of elongation to break is less than polypropylene as well as tailoring the composition of PHA on the level of the monomeric constituents [38] (Table 1.1). Thus, PHB is a breakable and crystalline material [39]. Copolymers of PHB are produced using different substrates together. When valeric acid and glucose are used together as substrates, PHBV copolymer is synthesized (Fig. 1.3a). This polymer shows isodimorphism with HB and 3 HV monomers [40]. In addition, high level of 3 HV monomer as compared to HB monomer content decreases the affinity to break during elongation.

Mcl-PHA contains C_6 to C_{14} monomeric units (Fig. 1.3c), e.g. PHHx, PHO, PHD. Some mcl-PHAs are semi crystalline and some are completely amorphous hetropolymers. They have a typically low glass transition temperature and comprising elastoplastic characteristics which differentiates them from scl-PHA.

Copolymers of PHA comprise of 3-hydroxybutyrate, 3-hydroxyvalerate, 3-hydroxyhexanoate as well as 4-hydroxybutyrate monomeric constituents are also reported in the literature [41]. Blends of scl-PHA as well as mcl-PHA are favoured for the industrial uses because of their developed physical properties e.g. crystallinity, and elongation to break, etc. (Table 1.1). For a carbonated substrate having an odd number of carbon atom, PHA copolymers with only C-odd monomers are produced. On the other hand, only C-even monomers are found in the copolymers if carbonated substrate has even digital value of carbon atoms. Low substrate specificity of PHA synthases (from various bacteria) supports the variety of biosynthetic PHA. This results in the formation of monomers having unusually branched, unsaturated or aromatic side groups [42]. By adding supplement compounds or substituted side chain carbon sources, functional groups (for example hydroxyl, carboxylic, epoxy, phenoxy groups, and halogens) could be included into the side chain.

1.6. Structure of PHA granules

In general, microorganisms start to accumulate PHA under circumstances of metabolic pressure/stress like; limited accessibility of vital nutrient component (e.g. oxygen, phosphorus and nitrogen). PHA is stored in the shape of grains in a microbial cell as a carbon as well as energy storage components. As given in Fig. 1.4, PHA pellets are bounded through a phospholipids uni layer incorporated, various enzymes (like PHA polymerizing enzyme along with PHA depolymerising enzyme) as well as structural proteins known as phasins [31]. Carbon starvation or alteration in the environmental pH stimulates PHA depolymerases to discharge 3-hydroxyalkanoic acids [43].

The abundant proteins in grains are phasins, few of them performing as transcriptional controllers/regulators. Phasins are thought to be non-catalytic proteins consisting of hydrophobic and hydrophilic domains. Hydrophobic domain is bounded by PHA surface body of pellets as well as hydrophilic zone is visibled in cytoplasm of bacteria. The amphiphilic coating of phasins balances PHA pellets and stops them from merging. PHA pellets can be detected intracellular because of light-twisting pellets through phase contrast microscopy as well as electron-clearness drops through TEM (outline Fig. 1.4a).

1.7. Properties of PHA

PHA may be rigid and highly crystalline, flexible, amorphous or elastomeric [46]. Their melting temperatures range as of 30–180 °C (Table 1.1). PHA monomeric units with linear, branched, aromatic side chains, saturated and unsaturated have been recognized [42]. Side

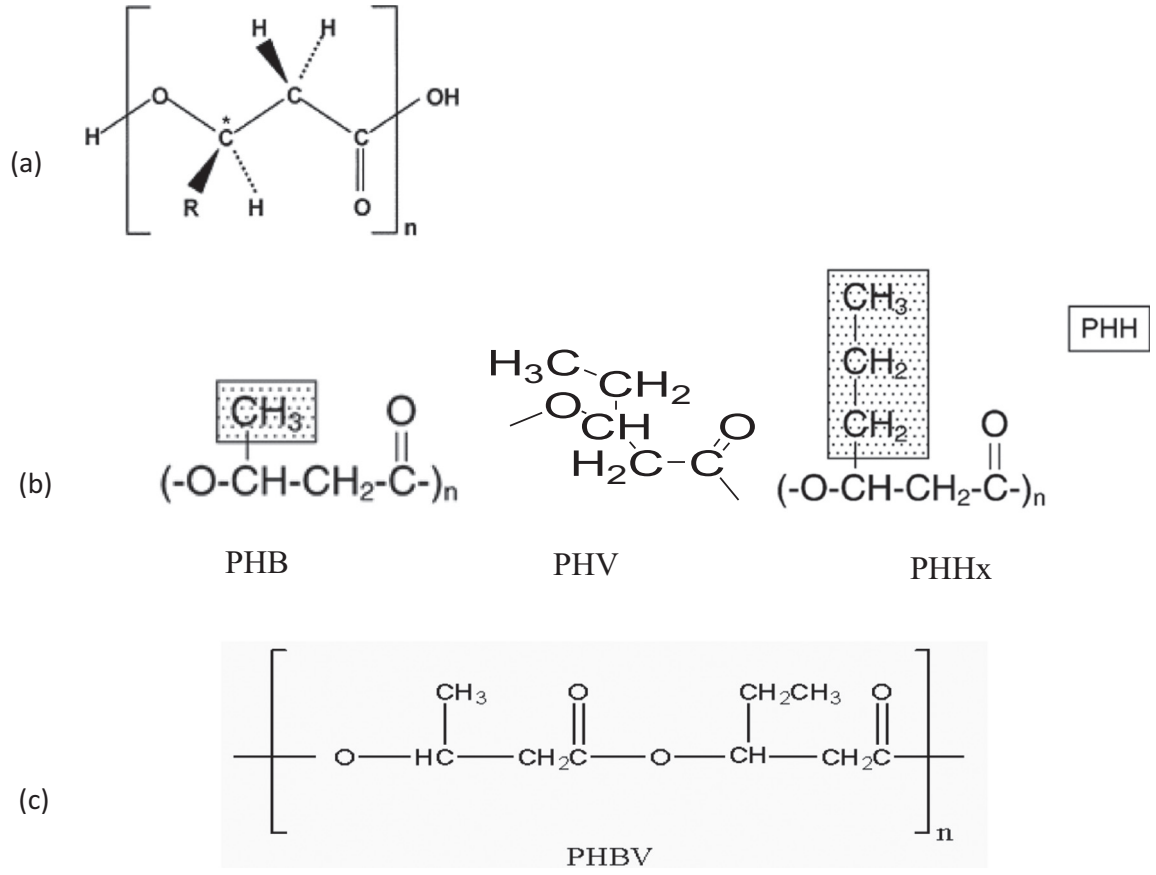

Fig. 1.3. Chemical structures of polyhydroxyalkanoate (PHA) monomers. (a). Common formula of PHA containing asymmetric carbons. All these monomeric units comprise one chiral midpoint (*) in R arrangement. R = CH_3; R = C_2H_5; mcl-PHA: R = C_3H_7–$C_{11}H_{25}$, n = 100–30,000 monomeric constituents, (b). Chemical structure of scl-PHA (PHB, PHV and their copolymer PHBV), (c). Mcl-PHA (3-hydroxyhexanoate).

chain extent of the monomeric unit along with functional unit significantly affects component characteristics like; crystallinity, glass-transition temperatures and melting etc. So, find their ultimate use. The mechanical and non-breakable characteristics of PHA can also be altered through mixing and transforming their body surface. This can also be done by uniting PHA with other type of polymers, inorganic compounds and enzymes [41]. Modified PHA composition allows good mechanical, non-breakable characteristics, biocompatibility and degradation rates under particular physiological conditions. Due to these physical properties, they are considered as environment friendly bioplastics.

PHB is harder and more fragile/brittle than polypropylene. It is 100% stereo-specific along with asymmetric carbon atoms comprising D(−) configuration which makes it extremely crystalline [47]. Its melting point (177 °C) is little bit lesser than its humiliating temperature which is 185 °C. This assembles complicated the fabrication of a plastic part by inoculation. PHB structure is very analogous to that of polypropylene, as both have same degree of crystallinity, melting point, glass-rubber transition temperature and a compact helical configuration. On the basis of these qualities they have attracted the interest of industries like ICI (Imperial Chemical Industries, UK) for biodegradable shampoo bottles made up of BIOPO™.

PHB has a number of beneficial characteristics like; optical purity, moisture proof and insolubility of water. This characterises PHB from recently existing decomposable plastics those are water loving and wetness effective. PHB displays no excellent penetrability of oxygen [48]. PHB also possess far lesser liquid resistance and good natural barrier to UV weathering [49].

Mcl-PHA is elastomers and adhesive compounds which can be improved to formulate gums/rubbers. The characteristics of mcl-PHA are appropriate for several uses, e.g. agriculture, food, chemistry, and medical applications, because of their innate biodegradable along with biocompatible characteristics [50]. Sophisticated bio-methods and suitable choice of carbon source to promote the development of ideal

Table 1.1
Relationship of materially characteristics of several PHA with polypropylene [39].

Properties	SCL-PHA	MCL-PHA	PHBV	PHBHHx	Polypropylene
Melting point (°C)	80–180	30–80	102–180	110–130	170
Glass transition temperature (°C)	−5 to −20	−25 to −49	−8–4	−1 to −2	−15 to −45
Crystallinity (%)	30–90	20–40	30–70	20–35	50–70
Elongation to break (%)	2–8	300–555	2.8–100	400–900	400
Density (g.cm^3)	1.18–1.26	1–1.05	1.16–1.25	N/a	0.905–0.94
Molecular weight	1×10^4 - 3×10^6	$1.35–5 \times 10^5$	$1.7–9.8 \times 10^5$	$1.9–7 \times 10^5$	$2–7 \times 10^5$

N/A: Data not available.

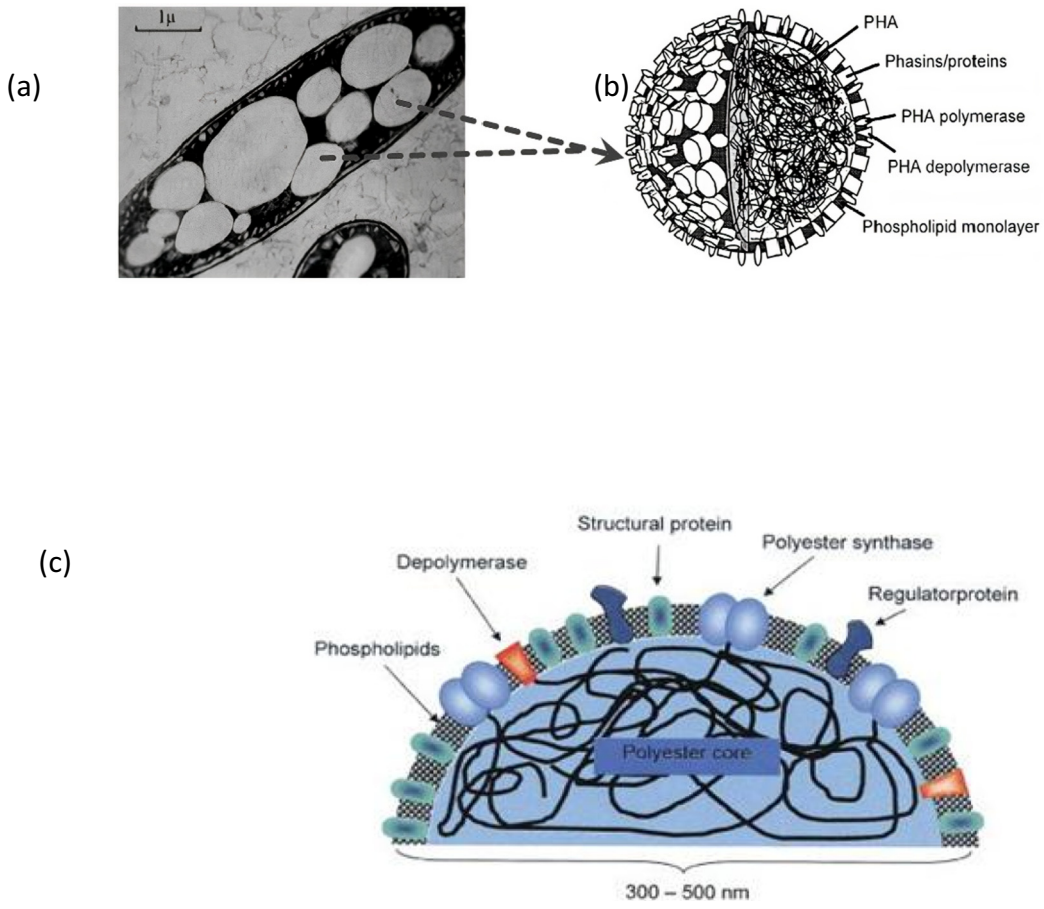

Fig. 1.4. Polyhydroxyalkanoates (PHA) structural formula and constitution (a) Transmission electron micrograph of *Azotobacter chroococcum,* (b) and (c) schemes of a PHA granule [44,45].

mcl-PHA. Biggest prospective of mcl-PHA has to exist into hydro-repellent layering of body tops. PHA copolymers comprised of mainly HB along with a segment of larger chain monomeric units (like; HV, HH along with HO) are relatively better elastic as well as tough bioplastics as compared to pure PHA.

Wenkuan and Qiang [51] studied that extension in side chain had an important effects on qualities of mcl-PHA such as flexibility and amorphous properties as compared to short chain which are more rigid. Poly (3HO-*co*-3HHx) is general kind of mcl-PHA established in *pseudomonas.* The crystallinity of this copolymer is between 20 and 40% (Table 1.1), concluding in less liquefying endothermic, that is representative for elastomeric components.

2. Producers of PHA, biosynthesis pathways and physiology

2.1. PHA accumulating microorganisms

Based on the culture circumstances, PHA forming strain are generally classified into two classes/groups. The first group accumulates PHA only in existence of an extra carbon substrate, if accessibility of vital nutrient elements is limited. This group includes *Cupriavidus necator* [52], *methylotrophs* [53] and *pseudomonas* strains. Second set does not need nutritional restrictions in favour of PHA production. The microorganisms are able to store PHA throughout normal development. It comprises *Alcaligenes latus*, *Azotobacter vinelandii* as well as recombinant *Escherichia coli* strains [33]. Generally, two kinds of PHA exist, scl and mcl-PHA and their formation depends on nature of bacterial strain and carbon source used in the culture medium. Specific bacterial specie produces only specific kind of PHA, scl-PHA and mcl-PHA. For example, almost all *Pseudomonas* are known for mcl-PHA production, and similarly *Bacillus* is well known for PHB production.

Until 1983, efforts had been made to produce PHB and to clarify the metabolic pathway in different bacterial species including *Bacillus* and *Azotobactor*. In 1983, De Smet et al. [28] observed poly (3-hydroxyoctanoate) PHO in intracellular granules of *P. oleovorans* ATCC 29347 developed in two-stage media consisting octane. Later, a number of studies were carried out on mcl-PHA. Not only *P. oleovorans* but also a huge number of further species of *Pseudomonas* (*P. putida, P. fluorescens as well as P. testosteroni*) accumulated mcl-PHA by n-alkanoic acids along with a diversity of other carbon sources [54].

Due to endotoxin (pyrogen) development in Gram negative strain like; *Methylobacterium organophillum, Cupriavidus necator* and *Pseudomonas oleovorans,* their use on industrial scale was effectively replaced by Gram-positive bacteria which were good bacterial sources of PHA for biomedical applications [55]. Among these microorganisms, *Bacillus* spp. [56], *Clostridium* spp., *Corynebacterium* spp. [57], *Nocardia* spp., *Rhodococcus* species [58]., *Streptomyces* species [59] and *Staphylococcus* species accumulated PHA [59]. In Gram negative bacteria, endotoxin is a lipid portion of lipopolysaccharides released through outermost cell wall and may triggers fever if enter into blood- stream of human beings and animals [55]. Endotoxin could be separated during PHA purification using various methods such as NaOH digestion and temperature controlled methods [60]. Upto 100% pure mcl-PHA have been recovered using these methods [61]. Cost of these methods recommended the utilization of Gram-positive strain for PHA formation.

Large variety of microorganisms have already been studied in support of biopolymer formation in fed-batch and continuous fermentations. Some bacterial strains were commonly studied: *Bacillus megaterium* [45,62], *Cupriavidus necator* [52,63], *Bacillus cereus* [64],

Pseudomonas putida [65–67] and *Pseudomonas oleovorans* [68]. For example, Kim [68] studied PHA production by fed batch fermentation using *Pseudomonas oleovorans* using octanoic acid and ammonium nitrate as nitrogen and carbon substrates. PHA quantity of 62, 75 and 67% of DCW, and yields of 1, 0.63 as well as 0.16 g l^{-1} h^{-1} correspondingly were gained using 10, 20 and 100C/N ratios (g of octanoic acid/g of ammonium nitrate) in the culture. Furthermore, the pulse feed strategy turned out as a reliable and relatively easy strategy for quick optimization of fed-batch processes, especially in the case of rather toxic substrates like octanoic acid [65]. Kulpreecha [62] used *B. megaterium* BA-019 strain for gaining maximum cell density along with PHA synthesis by batch cultivation utilizing urea and sugarcane molasses as nitrogen and carbon substrates. Consumption of sugar content and urea, cell dry weight, PHB contents, PHB concentration and optical density are presented as function of time in Fig. 1.5. Maximum obtained cell growth (72.6 g.l^{-1} DW) with PHB quantity (42% DCW.) obtained during 24H fermentation period, succeeding to enhanced PHB yield (1.27 g.l^{-1}. h^{-1}) using 400 g/L sugar concentration and C/N mole fraction was 10 (Fig. 1.5).

When *Pseudomonas putida* was grown lying on either glucose, nonanoic acid, or a mixture of both under nitrogen limitation conditions, PHA produced contained mcl-PHA monomers, $C_7 + C_9$, using nonanoic acid and $C_8 + C_{10}$ from glucose [67]. When both substrates were provided together in the medium, all these monomers were produced. The yield of $C_7 + C_9$ became double i.e. 0.450 molmol^{-1} from 0.254 molmol^{-1}, whereas, yield of $C_8 + C_{10}$ decreased to 0.011 molmol^{-1} from 0.057 molmol^{-1}. This change in the PHA quantity demonstrated that substantial savings can be gained using co-substrates. Mixed bacterial cultures have been used to produce PHA instead of single media. Merged bacterial mediums are generally utilized in waste water cure. Activated mud, a famous diverse medium, is capable in the direction of accumulation of PHA like carbon as well as power storing compounds. Microorganisms can adapt rapidly to changing conditions of nutrients and substrates. In a study conducted by Salehizadeh, activated sludge stored PHA under anaerobic conditions. The produced polymer was 20% of cell dry weight while, in pure culture PHA production was greater than 88% of DCW [69]. It was also found that PHA quantity of activated mud/sludge could be improved to 62% in a microaerophilic-aerobic mud/sludge method.

2.2. PHA biosynthesis pathways in microorganisms

The biosynthetic pathway of scl-PHA is the simple as compared to other mcl-PHA monomers. The process comprising of three types of enzymes, and their coding genes (Fig. 1.6) [70,71], which allocate the production of PHB and copolymer PHBV using simple substrates. Firstly, carbon substrate like glucose is entirely changed into acetyl-CoA. Then alteration of the acetyl unit of acetyl-CoA to large repeating linear polyester PHB is gained in a short metabolic pathway of three steps:

(a) Claisen compression of two acetyl-CoA compounds into acetoacetyl-CoA from a 3-ketothiolase enzyme, programmed by *phaA*.
(b) Reduction of acetoacetyl-CoA compound into (R) 3-hydroxybutyryl-CoA or (R) 3-hydroxyvaleryl-CoA from NADP-specific acetoacetyl-CoA enzyme (reductase) encoded as the *phaB* gene [47].
(c) (*R*)-3-hydroxybutyryl-CoA monomeric unit's polymerization catalyzed via PHA synthase enzyme that is programmed as *phaC* gene [45,72]. All these types of enzymes in support of PHB production be positioned into cytosol, where PHB storage occurred [3]. Production of other scl-PHA is feasible by accumulation of several carbon substrates to the culture (propionic or valeric acids), which results to synthesis of a by chance copolymer made up of 3HB and 3 HV (PHBV). In this way, reaction of propionyl-CoA in the company of acetyl-CoA is facilitated through a distinctive ketothiolase [73] (Fig. 1.6). Reduction of 3-ketovaleryl-CoA compound into (*R*)-3-hydroxyvaleryl-CoA compound also succeeding polymerization to make (PHBV) was synthesized via similar enzymes implicated during PHB production [74].

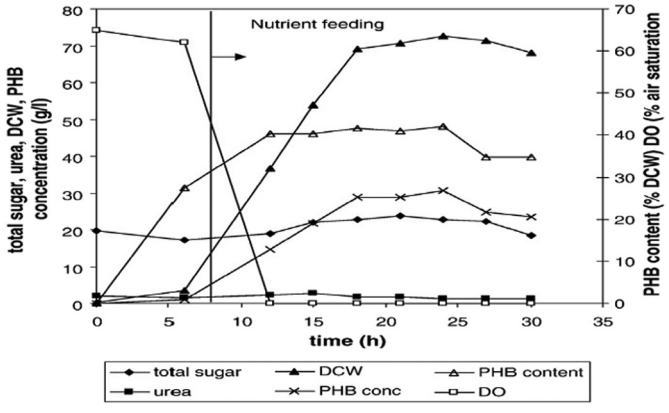

Fig. 1.5. PHB and cell biomass production by *B. megaterium* BA-019 in fed-batch cultivation. Feeding nutrient was used with a C/N molar fraction of 10. *B. megaterium* was cultured in a pH-stat controlled fed-batch cultivation system [62].

PHA polymerases are classified into four most important categories with respect to their basic structures and substrate specificities [76]. These four classes and their genes are presented in Table 1.2. First class of PHA synthase genes was obtained from a *phaCAB* operon in *C. necator, Acinetobacter* spp., *A. latus and P. acidophila*. Second class of PHA synthase, found in all *Pseudomonas* species, composed of two subunits: phaC1 and phaC2 and is associated to the synthesis of mcl-PHA. In few species comprising *Allochromatium vinosum* and *Thiocystis violacea*, the third type of PHA enzyme (synthase) comprises of two subconstituents: PhaC as well as PhaE (Table 1.2). This kind of synthase mostly catalyzes production of scl-PHA, however catalyzes polymerization of mcl-monomeric unit [47,77,78]. This type of enzyme catalyzes hydration of 2-enoyl-CoA compound in support of giving 3-hydroxyacyl-CoA monomeric constituents in support of mcl-PHA development [79]. In some species of *Bacillus* such as *Bacillus megaterium*, fourth type of PHA enzyme (synthase) is also contained two subunits: PhaC as well as PhaR (Table 1.2). Here these sub-units are linked with the formation of scl-PHA. Sources selectivity of PHA enzyme (synthase) has been a significant field on behalf of researchers and the monomeric constitution of PHA produced was found reliant on it [45].

In bacteria which produce mcl-PHA, such as *P. putida*,β-oxidation as well as fatty acid biogenesis routes are used in the direction of supply of sources in support of polymer production [31]. Mostly, mcl-PHA precursors are gained via β-oxidation route, a pathway firstly recommended in beginning of 1970s for microorganisms exist in activated sludge [80]. This route is used to change fatty acid into (*R*)-3-hydroxyacyl- CoAs, whereas, fatty acid fresh synthesis route is utilized to alter carbohydrate intermediates into (*R*)-3-hydroxyacyl-CoAs [81].These metabolites (*R*)-3-hydroxyacyl-CoA compounds are utilized same as the substrate from PHA enzyme (synthases), that catalyzed devoted phase of mcl-PHA biosynthesis and ultimately terminated in PHA biopolymer. The precursors of β-oxidation routes containing enoyl-CoA compound, 3-ketoacyl-CoA compound as well as (*S*)-3-hydroxyacyl-CoA compound, work like originators of mcl-(*R*)-3-hydroxyacyl-CoA compound, that is utilized openly during mcl-PHA production.

Most *pseudomonads* (for example *P.oleovorans*), relating towards rRNA homology class I, generate mcl-PHA by utilizing β-oxidation cycle of PHA biosynthetic pathway (Fig. 1.7). These bacteria accumulate mcl-PHA when cells are cultivated on associated carbon substrates, like; alkanes, alkanols and alkanoic acids. In addition, *Pseudomonads* utilize

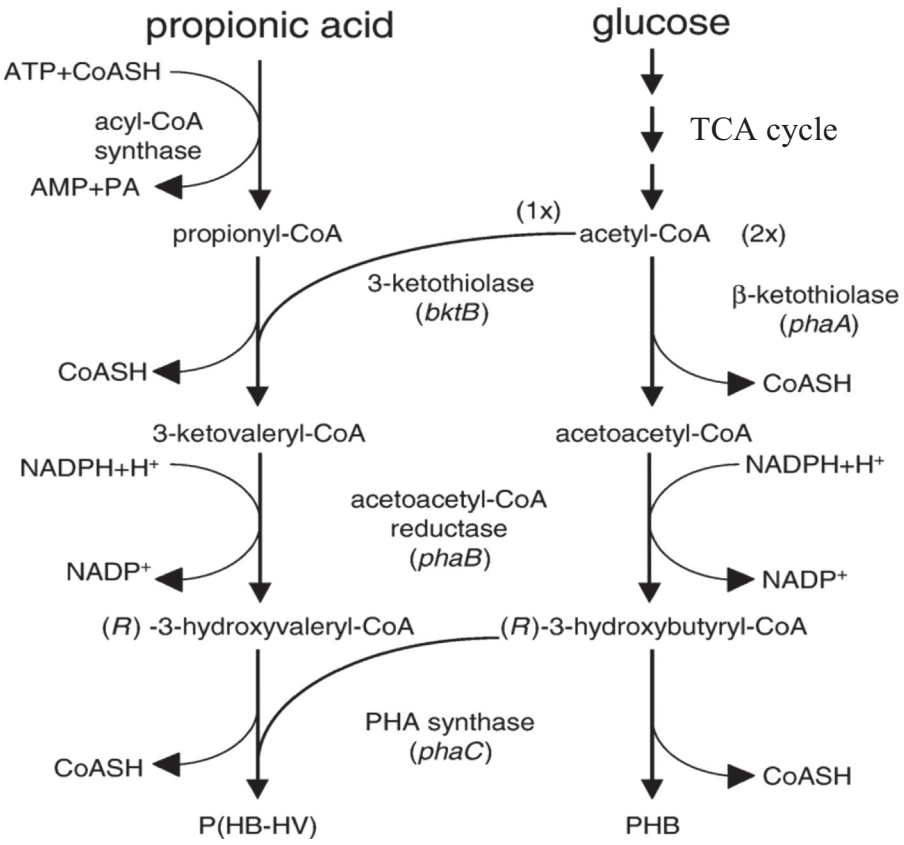

Fig. 1.6. PHB and PHBV biosynthetic routes in *Cupriavidus necator* [75].

fatty acid synthesis route for biosynthesis of mcl-PHA by Acetyl-CoA (Fig. 1.7). This enables the use of distinct carbon sources, such as carbohydrates [82]. *P. citronellolis, P. oleovorans, P. putida as well as P. mendocina* are kinds of *pseudomonads* so as to possess this route. *Pseudomonas* sp. 61–3 is an exception as it is capable to store PHB from a route same to *C. necator* presented in Fig. 1.7, although a heteropolymer comprising a combination of monomeric units [83].

β-oxidation cycle in different strains of *Pseudomonas putida* that provides hydroxyalkanoate monomeric units for mcl-PHA bio-formation is given. β-oxidation route includes the contribution of acyl-CoA synthetase enzyme (FadD) that activates stimulation of *n*-phenylalkanoic along with n-alkanoic acid compound ($n > 4$) in the direction of acyl-CoA types. After that, an acyl-CoA dehydrogenase (FadF), that needs contribution of an electron transporting flavoprotein (FadE), activates insertion of a double bond into β-configuration. Conclusively, a compound (FadBa), enoyl-CoA-hydratase, activates elimination of two carbon constituents of acyl-chain managed [86].

Acetyl-CoA stimulated originators of PHA are either produced in the path of anabolism through de novo fatty acid production or in pathway of breakdown by fatty acid β-oxidation which are provided towards unit cells.

Production of SCL and MCL monomers from fatty acid biosynthesis via the overproduction of genetically engineered FabH proteins and PHA synthase. 1. Fatty acid biosynthesis pathway (1). FabH catalyzes the condensation of malonyl-ACP and acetyl-CoA to form acetoacetyl-ACP (2). FabH mediates the transacylase reaction. 5. PhaC catalyzes the PHA polymerization reaction (3,4) given in Fig. 1.8. The β-oxidation pathway of *E. coli* (1). PhaJ catalyzes the conversion of enoyl-CoA to (*R*)-3-hydroxyacyl-CoA (2). FabG catalyzes the reduction of 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA (3). PhaC catalyzes the PHA polymerization reaction (4) given in Fig. 1.9 [87].

Enzymes which catalyze both mcl-PHA formation pathways (β-oxidation as well as fresh fatty acid production) in microorganisms are presented in Fig. 1.7. The chief enzymes involve in PHA bio-formation are: PhaA that encodes 3-ketothiolase enzyme, PhaB that encodes (*R*)-3-ketoacyl-CoA reductase enzyme (in support of PHB biogenesis, this specific enzyme is acetoacetyl-CoA reductase), and PhaC that is PHA synthase, PhaG that encodes (*R*)-3-hydroxyacyl-CoA transacylase enzyme, and PhaJ that is for enoyl-CoA hydratase enzyme [88]. Whereas, PhaC is particular on behalf of enantiomeric units into (*R*)-position.

2.3. Regulation of gene expression

Management of PHA metabolism is a difficult process, (i) at enzymatic stage, and (ii) at transcriptional stage [89]. In bacteria, such as *R. eutropha*, scl-PHA production is motivated by good intra cellular quantities of acetyl-CoA and free CoA [75]. NADPH and high ratios of

Table 1.2
Four categories of PHA synthases [45].

Class	PHA synthase genes	Subunits	Species	Substrates
1	PhaC	~60-73KDa	*Cupriavidus necator*	$3HA_{SCL}$-CoA(C_3-C_5), $4HA_{SCL}$-CoA, $5HA_{SCL}$-CoA
2	PhaC1, PhaC2	~60-65KDa	*Pseudomonas aeroginosa*	$3HA_{MCL}$-CoA(~ ≥ C_5)
3	PhaC, PhaE	~40KDa, ~40KDa	*Allochromatium vinosum*	$3HA_{SCL}$-CoA, $3HA_{MCL}$-CoA(~C_6-C_8), 4HA-CoA, 5HA-CoA
4	PhaR, PhaC	~40KDa, ~22KDa	*Bacillus megaterium*	$3HA_{SCL}$-CoA

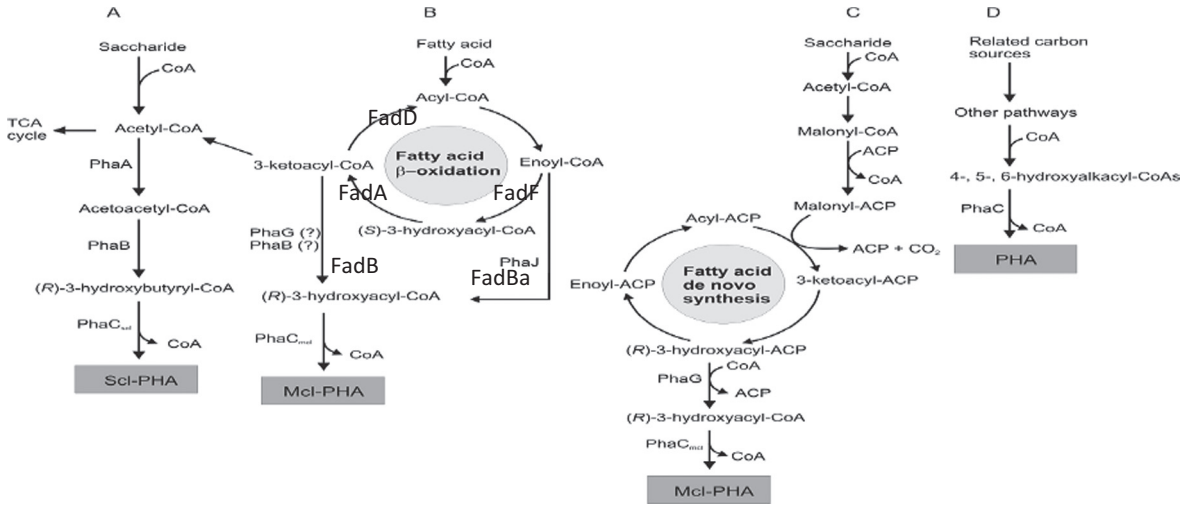

Fig. 1.7. Metabolic routes of PHA biosynthesis [84,85].

NADPH/NADP are also important in management of polymer production. It facilitated metabolic change of acetyl-CoA compound Figure PHB formation route. In strain like; *P. oleovorans* as well as *P. fragii* that generate mcl-PHA, one of most general routes to provide hydroxyalkanoate monomeric unit is metabolism of fatty acids (β-oxidation and production routes) as shown in Fig. 1.7. Therefore, same regulatory factors have an effect on the action of the enzymes of these metabolic routes and the management of PHA bio production. It was assumed that PHA synthases fight with β-oxidation compound for sources. As PHA accumulation by alginate over generating mutant of *P. oleovorans*, cultured on gluconate, decreased in contrast to that of wild type bacteria [90]. Whereas, *phaC1* transcription was found equal

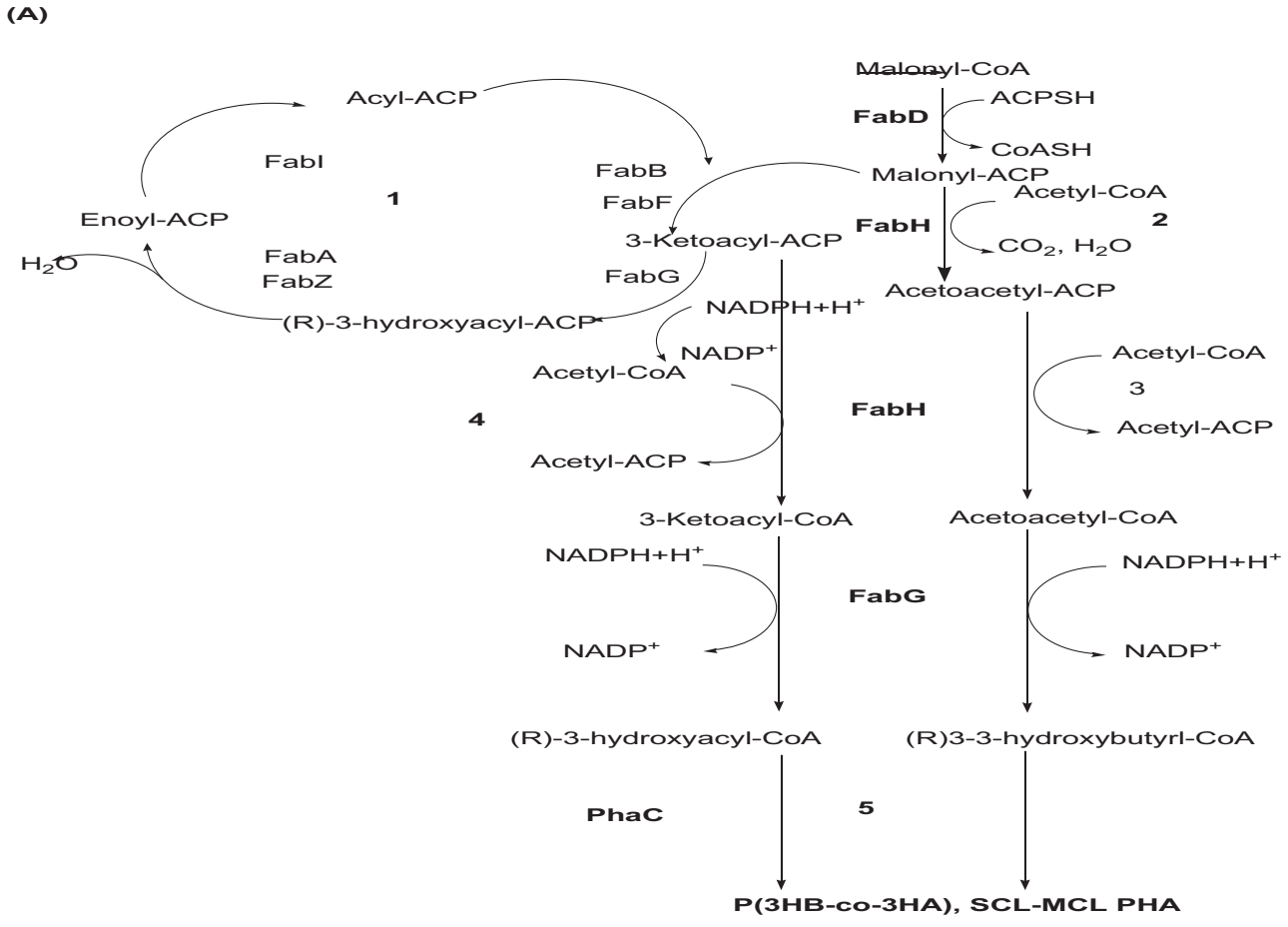

Fig. 1.8. Metabolic pathways for monomers derived from carbon sources. (A) Production of SCL and MCL monomers from fatty acid biosynthesis via the overproduction of genetically engineered FabH proteins and PHA synthase [87].

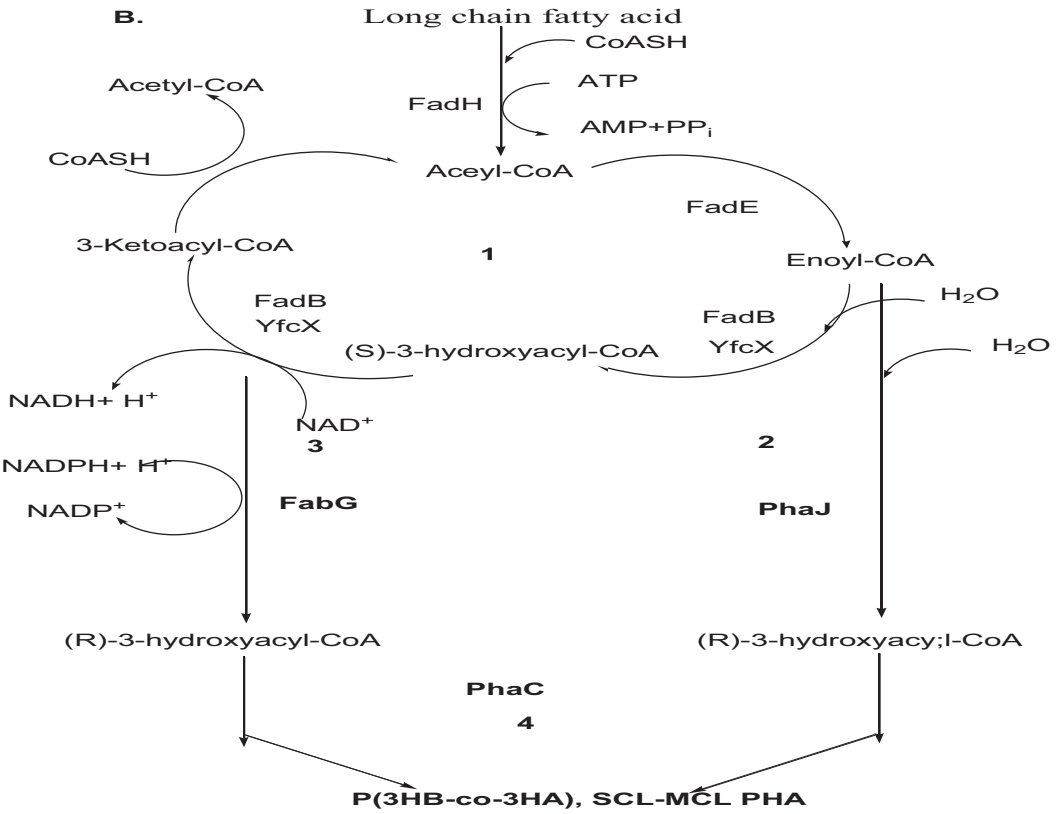

Fig. 1.9. (B) The production of SCL and MCL monomers from the β-oxidation pathway in *E. coli* [87].

in both these strains, they indicated a competition between alginate PHA biosynthesis and PHA biosynthesis of wild type bacteria. Sandoval et al., observed to over synthesize of PhaC1 synthase within *P. putida* affected number and mass of grains. Wild-type phenotype was re-established through the over expression of both *PhaC1* and *PhaF*. To differentiate transcriptional regulation in microbial metabolic routes, there are two ways. First, particular regulatory mechanism reaction drive the expression of related genes, in the presence of a source, a precursor metabolite or also a route manufactured goods.

Superimposed authoritarian systems manage the appearance of route gene group to common biological position of unit. This second process is determined from universal controllers [91]. Gene *phaD* of pha group relates with transcriptional regulators. In *P. putida phaD* acts a significant function during mcl-PHA bio-formation. Mutation of that gene affected mcl-PHA production such that it was reduced to less than 20% as related in the direction of production with wild type. Since *PhaD* is unrelated along with PHA particles, result has also been recognized with a changed appearance of phasins. Recently, *PhaD* was found to drive appearance of *PhaI* as well as *PhaF* present in proteins into reaction with substrates in support of development. Because of this *phaD* reliant appearance of phasins, *PhaI* and *PhaF* functions as a pleiotropic transcriptional regulator; it determines the accessibility of nucleosome DNA to the transcription machinery in *P. putida*, phenotype of *phaD* mutant can be explained [92]. The elimination of *phaD* decreases the production of PHA, whereas, a *phaD* mutant with a plasmid-over expressing *phaF* did not show this effect. Therefore, in this strain, *PhaD* could be participated in management of *phaF* appearance as a transcriptional stimulator. Strategies for peptide polymer functionalization based on the use of natural intrinsic P. putida PHA granules as scaffold, to immobilize fusion proteins in vivo via the granule binding module of PhaF phasin.

No transcript was found to be larger than 3 kb (by mRNA analysis). Therefore, it was concluded the *phaC1*, *phaZ*, *phaC2*, and *phaD* cannot make component of similar part and mRNA dealing occurrence is participated. Investigation of *phaC1* as well as *phaZ* gene exposed the presence of hybridization bands for *phaC1* and *phaC1Z* transcription units. In *P. oleovorans*, *phaC1* and *phaZ* were reported to be co transcribed (106). Appearance of *phaF* as well as *phaIF* gene is controlled contrarily within *P. putida* as well as in *P. oleovorans* which suggested the possible presence of two promoters [75]. In *P. putida* GPo1, *phaF* gene disruption resulted in increased expression of *phaC1*. Therefore, it was assumed *phaF* acted the same as a depressing controller of *phaC1* expression into same bacteria. The *phaF* transcript was detected into wild-type bacteria when glucose and citrate was utilized as growth substrates. In the presence of these substrates, *PhaF* could bind to DNA instead of granules and turned off appearance of *phaC1* (*phaC1Z*) as well as *phaIF* (*phaD*) impression components. Expression of *phaIF* transcript was dependant on the fatty acids like; octanoic acid in medium. *PhaD* regulator also stimulated expression of *phaIF* transcript [93]. This controlling system indicates a stable existence of *PhaF* within units. As reported by Sandoval [94], here is no proof of straight binding of *PhaF* towards DNA organizer places of *phaC1* and *phaIF*. In the existence of octanoic acid, also *PhaF* is linked to pellets as well as transcription degrees of *phaC1*, *phaI*, along with *phaF* enhance considerably proceeding to synthesis of innovative pellets. Dissimilar in other strain, pha in *P. putida* looks to be incorporated from six various parts (*phaC1*, *phaZ*, *phaC2*, *phaD*, *phaI*, and *phaF*).

2.4. Aerobic and anaerobic biodegradation

Biodegradation of polymers can take place under aerobic or anaerobic circumstances, leading to various products. Aerobic degradation (in the existence of oxygen) mostly utilizes oxygen as an ultimate electron acceptor, while microorganisms that execute anaerobic degradation (in the absence of oxygen) use CO_2, nitrates, sulphates etc. as an alternating electron acceptor to produce energy for the cell functions [95,96]. Most

biodegradable polymers illustrate confirmation of degradation in both aerobic and anaerobic conditions [97]. For enzymatically degraded polymers, it is the temperature that generally affects whether polymer scission happens. For example, PLA requires a temperature equal to or greater than its Tg (~55 °C) to biodegrade efficiently [98]. The vital chemical equation for aerobic biodegradation is the change of organic carbon into CO_2 by microorganisms, in the existence of oxygen (Eq. (1)). The carbon atom is usually part of a complex structure, and in some cases oxygen can be derived from the polymer itself, like from polyesters. During cellular respiration of a carbon source (i.e. glucose), the liberated oxygen reacts with free hydrogen ions to produce water (Eq. (2)) [99].

$$C + O_2 \rightarrow CO_2 \quad (1)$$

$$H^+ + O_2 \rightarrow 2H_2O \quad (2)$$

Anaerobic biodegradation on an industrial scale following standards is a considerably less studied area as compared to aerobic biodegradation, due to the environment control required for the study, and it does not comprise the majority of natural environments where plastic waste could biodegrade (soil and marine) The stoichiometric mass balance of anaerobic biodegradation of plastics and natural fillers is defined by Eqs. (1), (3) and (4).

$$C + 2H_2 \rightarrow CH_4 \quad (3)$$

$$C_6H_{12}O_6 \rightarrow 3CO_2 + 3CH_4 \quad (4)$$

Under anaerobic conditions, biodegradation results in methane production and some CO2 is produced depending on the residual oxygen in the environment or the type of degraded stuff. Two types of anaerobic environments exist on a large scale due to commercial actions, biogas facilities and landfills. Biogas facilities deal with anaerobic digestion of organic and plastic materials, capturing the released methane for energy conversion. However, landfills are of particular concern, because any uncontrolled biodegradation of organic and plastic materials can result in methane generation into the environment. In 2007, it was estimated that only 10% of the potential methane generated is captured in the United States, which has only increased to approximately 20% by 2017. Methane is a 25 times more potent greenhouse gas compared to CO2 over a 100-year period. Furthermore, in the waste sector, the largest contributors of methane in the atmosphere are the landfills and solid waste treatment facilities which do not collect biogas, indicating uncontrolled management of this greenhouse gas [100].

2.5. Biodegradation of PHA

PHA degradation is achieved in strain from at least two various routes, intracellular and extracellular processes [12]. In the first one, the accumulated PHA is hydrolyzed to meet the needs of carbon source of microorganism. Here degradation of thermo-polymer is taken through depolymerases enzyme (phaZ) attached to PHA particle. During extracellular breakdown, exogenous PHA also used as a carbon as well as energy substrate. Substrate of this polymer from PHA-storing microbes that experience breakdown. These particles are multiply in ecosystems also hydrolyzed using released enzymes within water-loving monomers. Capability to decompose scl-PHA is extensive along with strain. Thus, various scl-PHA depolymerases enzymes have also been considered more than last decade as well as greater than 20 genes have also been detected [12]. Extracellular mcl-PHA degrading microbes are not normally present in surrounding. *Pseudomonas fluorescens* enzyme is mcl-PHA depolymerase (extracellular) [66]. This depolymerase enzyme appears to be mcl-PHA-particular as it hydrolyzes PHO and PHD however it was not capable to decompose PHB and PHV.

In the extracellular process of biodegradation, microbes grown on body of polymer also release enzymes which decompose PHBV into HB as well as HV constituents. Then these components are utilized from these microorganisms as a substrate in support of development. Degree of polymer biodecomposition relies on diversity of causes, comprising body area, bacterial movement of disposal ecosystem, temperature, moisture, pH as well as stress of many other nutritional constituents. Moreover, selectivity and performance of extracellular depolymerases and characteristics of polymer (stereo regularity, crystallinity, molecular mass of the molecules) as well as salinity and acidity of the medium are also important. PHBV is hydrophobic and is unaffected by water, does not breakable under normal environmental circumstances, and is constant for an indefinite period in air [8,101]. The final stuff of PHA deprivation in aerobic atmospheres is CO_2 as well as H_2O, although ethane is synthesized into anaerobic circumstances.

In comparison with extrinsic PHA depolymerisation, intrinsic decomposition of formerly stored PHA is implicated little bit. Though, throughout the previous duration, examination of genomes of *C. necator* and other PHB creator bacteria such as cloning and description of various PHB depolymerases as well as hydrolases enzymes, exposed with the intention of PHB hydrolysis is a extremely complicated procedure. According to intracellular mcl-PHA biodecomposition, a single model defined thus distant is of PHA recruitment in *Pseudomonas*. Firstly mcl-PHA depolymerase enzyme was observed to be programmed from *phaZ*, positioned involving two mcl-PHA enzyme (synthases) genes (*phaC1* as well as *phaC2*) in *P. putida*. It is an intracellular depolymerase positioned in PHA pellets which hydrolyzes particularly mcl-PHA comprising aliphatic and aromatic monomeric units [66]. In another report, PhaZ2 was observed to link with PHB insertion body also because of soluble enzyme in *R. eutropha*, while, PhaZ1 linked absolutely to the insertion bodies. PhaZ2 was a new PHB depolymerase, which contributes in organization of PHB in *C. necator* with PhaZ1 [102].

3. Substrates

PHA production from renewable resource is attractive as an alternative of using petroleum-derived polymers. Due to biodegradability of PHA, its characteristics are harmless to the environment. High cost (5–10 times higher than conventional polymer) of bacterial fermentation is the major hurdle for commercial synthesis of PHA. Therefore, inexpensive carbon substrates (e.g. industrial unwanted products and by-products) have to be used in direction to minimize this problem [59].

Numerous alkanes, alkanoic acids, fructose, glucose and glycerol were utilized the same as substrates for the synthesis of mcl-PHA [103].Glycerol was utilized as substrate in favour of synthesis of economically favourable biopolymer utilizing *Pseudomonas oleovorans* and *Pseudomonas corrugata* [104]. PHB and mcl-PHA (containing primarily of 44 mol% 3HD (C10) and 31 mol% 3HDde (C12:1)) were produced respectively.

After 72 h of batch culture, cellular productivity was 40% in favour of *P. oleovorans* within 5% (*v*/v) glycerol as well as was 20% in favour of *P. corrugata* within 2% (v/v) glycerol. Accumulative glycerol medium quantity from 1 to 5% (v/v) produced a 61 and 72% decrease in molar mass of PHB along with mcl-PHA biopolymers, correspondingly. Development of both bacterial strains on glycerol in a mix culture produced natural fusions of PHB and mcl-PHA. Through changing the period of cultivations, the obtained PHB/mcl-PHA fractions ranges from 34:66 to 96:4. Similarly, Atlic et al [63] used glucose as substrate for growing *Cupriavidus necator* DSM 545 in a multistage (5 stages) bioreactor. For PHB produced from this strain, volumetric and specific productivities ($1.85 gL^{-1}\ h^{-1}$ and $0.100\ gg^{-1}\ h^{-1}$ correspondingly), polymer yield (77% *w*/w) as well as biopolymer characteristics (Mw = 665 kg/mol, PDI = 2.6) were obtained. Structurally distinct carbon substrates, like; gluconate have been utilized for PHA production via *Pseudomonas*, however, this is commonly lesser proficient than constitutionally associated carbon sources [89]. Some strains may store as much as 40% PHA

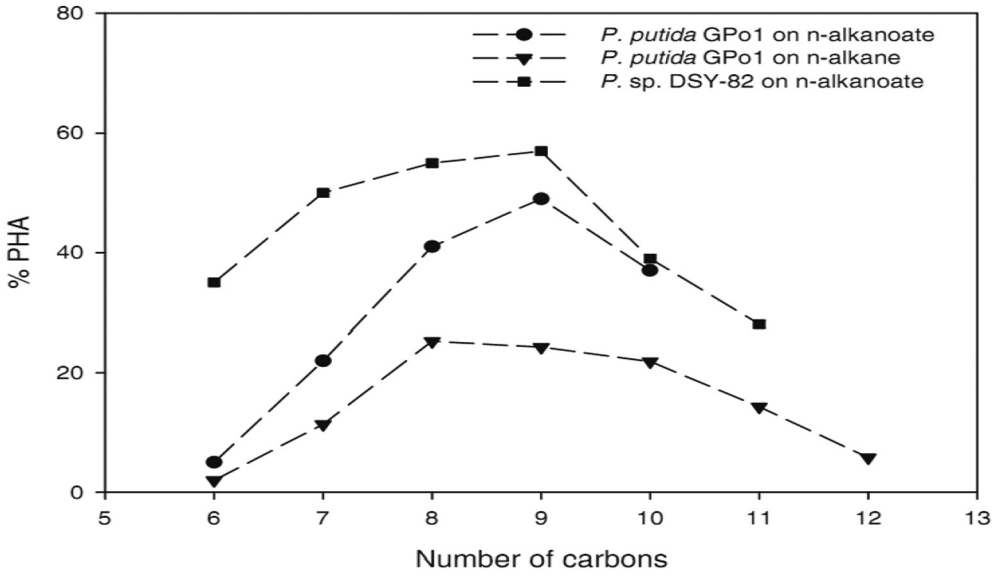

Fig. 1.10. PHA biopolymer quantity of *P. putida* bacteria utilizing alkanes and alkanoate of different extent [67].

utilizing these types of carbon substrates, yields are generally much lesser than pure carboxylic acids.

It is already discussed that for mcl-PHA synthesis substrates are mainly provided by fatty acid β-oxidation route through structurally associated carbon substrates. Carbon substrates like; octane, nonanoate, and octanoate are deliberated to be most proficient sources for mcl-PHA production [67]. In Fig. 1.10, PHA quantity (%) was plotted versus number of carbon atoms of PHA monomers obtained by growing *Pseudomonas* bacteria utilizing alkanoate and alkane as substrates. Generally alkanoate produce larger quantities of PHA (more than 40%) as compared to simple alkanes (approximately 20%).

Durner et al [105] used P. *putida* GPo1 for mcl-PHA production by growing in octanoate as well as nonanoate with nitrogen restriction in a study ($\mu = 0.2\ h^{-1}$). Biopolymer yield was 42 as well as 31%, correspondingly. Throughout PHA storing stage of batch fermentation by *P. putida*, particular biopolymer production speed utilizing nonanoate was significantly greater than by octanoate ($0.082 gL^{-1}\ h^{-1}$ vs 0.036 g. $g^{-1}.h^{-1}$) recommending that nonanoate is a proficient substrate in favour of biopolymer production. Oleic acid, a general carboxylic acid present within vegetable oils, has also been utilized of proficient synthesis of mcl-PHA (biomass concentration of $30gL^{-1}$ containing 23% PHA) from *P. putida* KT2442.

Octanoic acid was utilized the same as substrate during pH-stat methods for growing *Pseudomonas oleovorans* with nitrogen restriction to gain $47gL^{-1}$ biomass comprising 55% biopolymer [106]. Similarly, in another study, from *Pseudomonas oleovorans*, $63gL^{-1}$ biomass containing 62% PHA was obtained using octanoic acid as carbon source [68]. *Pseudomonas putida* KT2440 was cultivated using nonanoic acid in nitrogen restraint environment in two phase fed batch reactor [67]. It accumulated 27% of mcl-PHA. Then this bacterium was developed in substrate limited culture ($1.31gL^{-1}$ of nonanoic acid instead of $3gL^{-1}$) to observe the effect of the substrate instead of nitrogen limitations. Biomass concentrations and PHA contents produced are represented in outline Fig. 1.11 as a utility of period of culture (h).

4. Fermentation process development for PHA production

Fermentation is complete in direction to allow microorganism to use accessible carbon in media as a carbon and energy substrate and to store intracellular PHA polymer within their body, during the fermentation development microbes used different kinds of carbon as well as nitrogen sources for their development and then stored the PHA molecules into their cytoplasm in shape of pellets. To avoid utilization of polymers used for microbial development, unbalance nutrients are provided. To

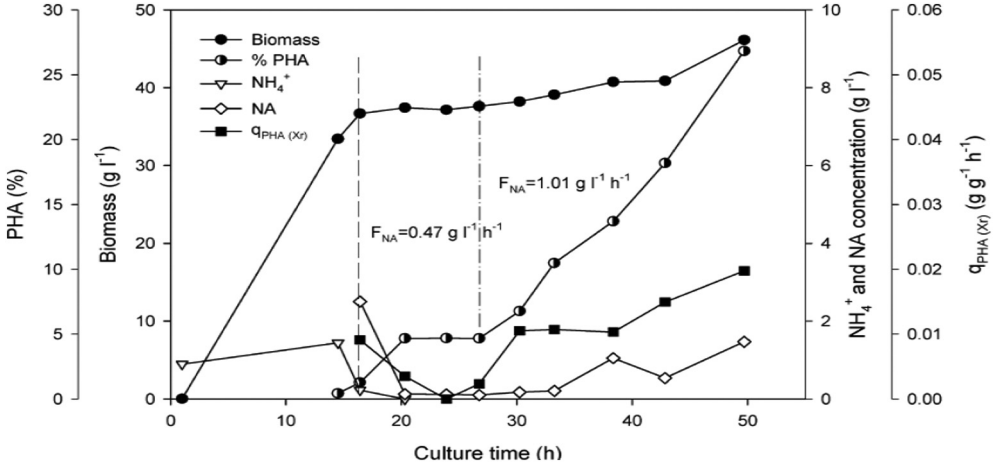

Fig. 1.11. Influence of development speed on biopolymer production in *P. putida* under N-restricted growing in nonanoic acid. The nonanoic acid feeding rate (F_{NA}) was enhanced ranges from 0.47 g $L^{-1}h^{-1}$to 1.01$gL^{-1}h^{-1}$at 27 h throughout the N-restricted stage, as represented with dash-dot line [67].

accomplish high polymer efficiency, the plan of cultivation is to enhance the weight of cells by supplying at first sufficient nutrient sources in the media and then limiting the development nutrients excluding carbon substrate. These approaches have also been attained through fed-batch cultivation.

A fed-batch is a biotechnological batch procedure that is controlled by nourishing a development restrictive nutrient source to a media to gain better cell densities in bioreactor. Generally fed solution is extremely intense to avoid dilution in bioreactor. Substrate limitation allows metabolic regulator, avoids catabolite repression, osmotic effects and run-off metabolism of the side products [107]. Batch and fed-batch cultivations are commonly utilized in industrial cultivation methods. The latter is highly effective to achieve high yield and cell concentration, as culture constitutions can also be organised through source inhibition. So, maximum early intentness of sources can also be diverted. Restrictions of fed-batch are extended among two batches, that consequences during maximum procedure charges [108]. Fed-batch medium is appropriate for strain which needs restriction of vital nutrients like; oxygen, nitrogen as well as existence of extra substrate in favour of proficient formation of biopolymer [61]. Various strategies can also be utilized to regulate the development in a fed-batch method including: PO_2, carbon substrate, pH (acidification is connected to high glucose), ammonia (nitrogen source) and temperature. Fed-batch fermentation can be further divided in two types depending upon volume of medium used.

A) Fixed volume fed-batch

In such kind of fed-batch, source in minimum amount is nourished without diluting media/culture. Feeding of developmental restrictive sources can preserve culture volume constant the same as a precise rigorous liquid and gas (oxygen). A specific kind of comprehensive fed-batch, repeated fed-batch media, implies a periodic removal of a portion of cultural media and use of residual cultural media as beginning point for further fed-batch method [109].

B) Variable volume fed-batch

In variable quantity fed-batch quantity of medium fluctuates with cultivation time because of carbon sources supply. Means this quantity varies is reliant on needs, restrictions as well as aims of microbial culture.

C) Continuous Cultivation

There are mutually input as well as output movements in continuous method, however, reaction quantity is set aside stable. While high substrate level hinders the development and product development or when undesired constituents are formed in analogous with a preferred product, the fed-batch operation is revealed to be superior to both batch cultivation and continuous procedures [110].

5. Extraction of polyhydroxyalkanoates

There are different kinds of method utilized for recovery of biopolymers described in previous studies with the analysis of their benefits and disadvantages. Majorly used techniques for the extraction are discussed below.

5.1. Solvent extraction

Solvent extraction technique is majorly utilized for improving of PHAs. Firstly, pretreated product using some solvent to rupture the granules and then, chloroform, acetone, 1, 2-dichloroethane and ethylene carbonate are used for the solubilization of PHAs. After that chilled ethanol or methonol is used for the precipitation as a non-solvent. The advantages of this method are that it does not decompose polymer as well as remove endotoxins of dry cell mass [111]. 1,2-propylene carbonate preferably used due to its less toxic property as compared to others.

The main purpose of this study is using various types of solvents like; n-hexane, 1,2-propylene, acetone, chloroform, ethylene acetate and methylene chloride. Mostly efficient solvent, methylene chloride showed the rocovery of polymer 86%(*w*/w) at room temperature [60]. Ethyl acetate and acetone showed the impurities smaller than 10% and purity of polymer increased nearly 100% when chilled mehaol is used for the precipitation.

5.2. Floatation method

This is generally modified form of solvent extraction technique in which less number of chemicals are used as related to solvent extraction. This extraction is followed via the self-floatation of pellet remains. In this method pellets are merged in chloroform at 30 °C for 72 h. Then stay it for over-night at room temperature for self-floatation of pellet remains. This method reported purity and efficiency of the PHAs upto 98% and 85%(w/w) respectively [112]. The other benefits of this method are easy handling, less costly and reduction of wastage of extracted polymer.

5.3. Digestion method

In this type of extraction digestion mechanism is involved. Number of chemicals is used for this method instead of chloroform or acetone in solvent extraction. There are two ways of digestion method one is chemical and second is enzymatic digestion. In chemical digestion sodium hypochlorite used for the recovery of PHAs in existence of other chemicals like; sodium dodecyl sulfate, betaine and palmitoyl carnitine as a surfactants [113]. If only sodium hypochlorite is used for the extraction, it may lead the 50% degradation of polymer. Both the surfactant and sodium hypochlorite used together for the better recovery and efficiency of polymer, separately they gave poor performance [114]. Enzymatic digestion consists of three steps, first is heating treatment, second is enzymatic hydrolysis and third one is surfactant washing, gave the high percentage of recovery product [111].

5.4. Supercritical fluid extraction

Supercritical fluid is most preferable method utilized now these days for the recovery because of its low toxic and cost. Carbon dioxide, ammonia and methane are used as supercritical fluids. The advantage of this method is to achieve the dominant recovery of the polymer due to their high speed, low viscosity, and no surface tension. Some studies reported the purity of polymer up to 89% using supercritical fluids for *Cupriavidus necator* [115]. This method is a preferable extraction method used to form the impurities free PHA for the biomedical uses. Supercritical fluid provides the purity of polymer up to 100% as compared to the conventional extraction methods [116]. The limitation of this method is to maintain the high pressure up to 350 bar which requires the specific technology.

5.5. Sonication method

Latest and advanced method of extraction is the use of ultra-sonic waves. Mostly preferable and beneficial method is sonication method in which 1 g of cell will be mixed with 100 ml of hot chloroform in a beaker and will be sonicated in an ultrasound bath of 40 kHz frequency of 200 W at 20 °C for 2 h. After that the high power sonication will be subjected for 30 min. Then released granules will also be entirely liquefied with chloroform. Biopolymer will also be precipitated by using 1 L ice-cold ethanol (99.8%) and it will be dried out in vacuum oven at 40 °C [117]. Summary of extraction of polyhydroxyalkanoates as discussed in Table 1.3.

Table 1.3
Extraction of polyhydroxyalkanoates.

Sr. no recovery methods	Advantages	Recovery agents	Analysis
1. Solvent extraction	High clarity, exclusion of endotoxin, minimum polymer humiliation	Chloroform, methanol, hexane [111]	Create contamination due to the high quantity of chemicals
2. Floatation method	Less use of chemicals, minimum polymer degradation	Chloroform [112]	Highly preferable due to less chemicals, easy to handle and less costly
3. Chemical digestion	High purity, no polymer degradation useful for high volume	NaClO, chloroform/ethanol [121]	Time spending, mixture of chemicals necessary, 50% degradation of polymer occurred if only one chemical is used
4. Enzymatic digestion	High quality polymer recovery, high clarity, no employ of chemicals	SDS, EDTA, Alcalase, Lysosyme [121]	Highly costly, highly complex
5. Supercritical fluid	Low cost, less toxic, High purity	CO_2 [113]	Less costly, but limitation is to maintain the 350 bar pressure during extraction
6. Ultra sonication method	Highly efficient 99.8%	Chloroform, ultrasound frequency 40KHz, power 200 W [117]	Highly effective, high quantity of chloroform is used like 100 ml of solvent required for 1 g of dry cell mass
7. Biological digestion	Purity 94%	*Tenebrio molitor*, water, SDS, diluted HCl [119]	Highly effective but takes long time for recovery

5.6. Biological digestion of non-PHA biomass by animals

More recently, it was successfully confirmed that the non-PHA fraction of biomass could selectively be digested by some animals, most prominently by the meal worm *Tenebrio molitor*. The principles and potential of this process were comprehensively summarized by Chee and colleagues [118].The experiments underlying this review involved the feeding of meal worms with PHA-rich *C. necator* biomass, whereby the animals digested the non-PHA biomass material. The remaining white fecal pellets were simply washed with alkaline water. Purity of PHA obtained by this novel process reached 94%, which is sufficient for various applications of PHA. This biological method does not cause reduction of molecular mass, simple post-treatment of the granules using water, SDS, slightly elevated temperature and strongly diluted HCl results in an almost 100% pure product [119]. This method takes long time for recovery [120].

6. Characterization and quantification of PHA

6.1. Observations of PHA granules

PHA grains can be found intracellular as light-deflecting pellets via phase-contrast microscopy. They can also be observed as electron-free accumulation via TEM of slight segments. Staining methods are currently used to visualize intracellular PHA granules with light or fluorescent microscopy. Sudan Black B was initial dye utilized by microbiologists for the detection of the existence of PHB grains in microbial cells [122]. Ostle and Holt (1982) [123] demonstrated that Nile Blue A, a hydrophilic primary oxazine dye that shows an intense orange fluorescence at wavelength of 460 nm has a higher resemblance as well as greater selectivity than Sudan Black used for PHB. Fig. 1.12 shows PHB accumulation by *Bacillus megaterium* MC1 grown in nutrient culture with 1 $g.L^{-1}$ glucose for 16 h. Inhabitant PHA inclusions can also be marked by Sudan black B and Nile Blue A while Nile Red be able to utilize in the direction of PHA developing in microbial colonies [27].

6.2. Microbial and PHA analysis by Fourier transform infrared spectroscopy (FT-IR)

In infrared spectroscopy, infrared rays are preceded from a sample. Few of these IR are immersed through the sample, whereas, few are transmitted. The concluding band represents molecular incorporation as well as transmission, forming a molecular diagnostic feature of trial. FT-IR constitutes radically several approaches for the detection of microbes since 1980s. It measures the total constitutions of bacterial cells in a non-toxic way, with bands from all cellular components, e.g. membrane and cell wall components, proteins, and nucleic acids. IR spectra of intact bacteria permit bacterial cells to be illustrated at several taxonomic stages, even to the strain level [124,125].

Specific cells composition like; capsules or storing compounds can be detected and identified by FTIR [126]. Various processes have been utilized to detect PHA yield in strain, however they need highly and difficult sample formation, such as extraction, purification, and methanolysis [127]. In contrast to those, FT-IR spectroscopy is fast, easy to implement and cost-effective. This method has a high power for identification of PHA in laboratories and industries, as it permit the determination of minimum quantities of biomass, and needs no reagents [127]. PHB is noticeable by FT-IR spectra in intact strain, grains, capsules or endospores [128]. Mcl-PHA can also be quickly identified via FT-IR spectroscopy within joint units of *Pseudomonas* [129,130]. IR spectra of pure PHA monomers and of bacterial cells of *Azotobacter* and *Pseudomonas*, having PHA and not having PHA, are shown in Fig. 1.13. FT-IR band of uncontaminated PHA comprising scl-HA monomeric units like; hydroxybutyrate (HB), mcl-HA monomeric units involving HO along with HD, in cooperation of HB with mcl-HA monomeric units displayed strong characteristics bands at 1728 cm^{-1}, 1740 cm^{-1} and 1732 cm^{-1}(carbonyl ester band), correspondingly. Other associated bands close to 1280 cm^{-1} and 1165 cm^{-1}(polysaccharides region) for PHB as well as mcl-PHA respectively were helpful in favour of identifying kinds of PHA. Moreover, the strength of band close to 2925 cm^{-1}(region of fatty acids) delivered additional informational materials for PHA characterization [130].

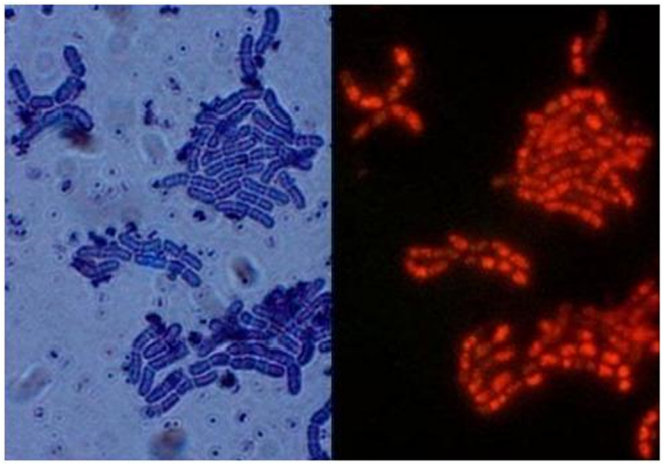

Fig. 1.12. PHA pellets stained using Nile blue-A [27].

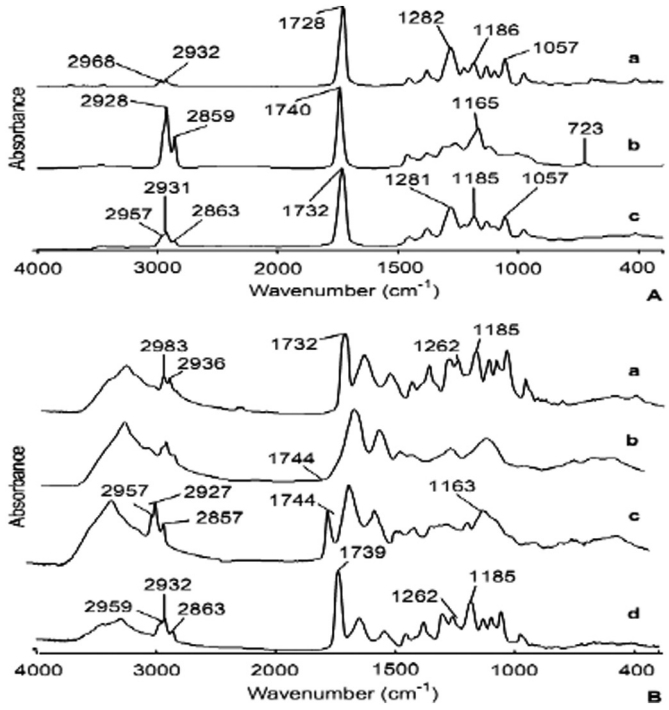

Fig. 1.13. Fourier-transform infrared spectrum of PHA extracted by several cells. [125] a. The PHB using *Azotobacter vinelandii*; b. Mcl-PHA using *Pseudomonas mendocina*; c. PHA comprising HB along with mcl-HA monomeric units using *Pseudomonas pseudo alkali genes*. FT-IR band of (a) PHB forming pellets of bacteria *A. vinelandii*; (b) cells of *P. mendocina* do not comprising PHA; (c) mcl PHA forming pellets of *P. mendocina*; (d) pellets of *P. pseudo alkali genes* generating PHA comprising HB as well as HA monomeric units.

FT-IR is a qualitative method in the direction of determining the type of PHA in intact bacteria and this technique has also been used in literature to determine polymer content (semi quantitative analysis). For example, Randriamahefa [129] used several *Pseudomonas* strains to produce mcl-PHA from sodium octanoate (substrate). Utilizing absorbance of ester band of PHA (1728 $cm^{-1}$for PHB and 1743 $cm^{-1}$for mcl-PHA), a rapid distinguish between PHB and mcl-PHA (PHO) was made. Polymer content in intact bacteria was also determined using intensity of this ester band. Among all bacterial strains polymer contents varied from 0 to 53%. The calibration curve was found to be valid from 3% of polymer content, whereas very small polymer yield (<3%) was complicated to detect. FT-IR spectroscopic process needs little training of sample test rather than gas chromatographic analysis and was found to be very beneficial in favour of showing a huge diversity of *Pseudomonas* [129].For studying spectra of strains that produce PHA, five spectral windows were normally used making their particular information content and their judgement power: (1) window from 3100 and 2800 cm^{-1} (fatty acid area I) is subjected from -CH3, >CH, and = CH stretching ambience of functional units generally found in lipids constituents of different covering amphiphiles; (2) window from 1800 and 1600 cm^{-1} (amide region) is subjected through the ketones/esters, amide I as well as amide I1 spectra of proteins as well as peptides; (3) window from 1500 and 1200 cm^{-1} (diverse area) is a supernatural area consisting instruction through fatty acids, proteins along with phosphate-holding components; (4) window from 1200 and 900 cm^{-1} (polysaccharide area) is subjected through fingerprint-like inclusion spectrums of carbohydrates found inside cell wall; (5) window between 900 and 700 cm^{-1} (true fingerprint) shows few extraordinarily particular supernatural patterns, those are until now unassigned to cellular constituents or headed for functional units [125].

Mustafa Kansiz [131] used FT-IR spectroscopy as a tool for determining crystallinity in PHA. Similarly, with the aid of FTIR, absorbance changes of crystalline as well as formless spectra shown that unfreezing of PHB happened on a slight temperature value. Unfreezing of PHBHHx polymer happened on a extensive temperature value, also it was revealed that copolymer had a greatly broader allocation of lamellar wideness [132]. FT-IR technique was able of distinguishing equally induced mediums of *P. putida*, with 3,5-xylenol and m-cresol as single carbon substrate [133].

6.3. PHA analysis by gas chromatography (GC)

The requirement for rapid analytical techniques for identification of PHA into biomass straightly required to improve the chromatographic methods like; high performance liquid chromatography (HPLC) as well as gas chromatography (GC). HPLC dealings just PHB as well as it is constructed on transformation of PHB in the direction of crotonic acid monitored through UV revealing at 210 nm. This technique states 84% healing of PHB [134].

GC analysis needs a quantitative depolymerisation of polymer, generally joined along with a derivatization. GC used for the purity, identifying and analysing the compounds during the process. The first derivatization method for GC was developed for PHB [126] on foundation of methanolysis of pellets in existence of sulphuric acid along with chloroform for PHA extraction and hydrolytic esterification of the polymer. Afterward, Riis and Mai, developed an improved method, built on hydrolysis and transesterification of PHB along with hydrochloric acid and propanol this method was sensitive, as20μg amount of PHB could be determined [135]. While alkaline hydrolysis resulted in a combination of 3-hydroxyalkanoic acid methyl esters along with 2-alkenoic acid methyl esters [80], acidic trans-esterification concluded in only one methyl ester per component, which made it more favourable. In accumulation, it was significantly quicker as extraction and transesterification continued in one step. But, sulphuric acid is of restricted usage the same as common catalyst in support of trans-esterification reactions, appropriate for more breakdown of 3-hydroxy esters [43,135], e.g. by acid catalyzed removal. Thus type and concentration of acid have considerable effect on the kinetics of depolymerisation [136,137]. One of complications in inquiry of smaller series esters of bacterial fatty acids as well as PHA monomeric units is that they are comparatively polar [138]. Furrer [74] developed a better technique for quantification of mcl-PHA via GC-FID. Boron trifluoride within methanol was used to methanolyse special mcl-PHA (efficiency >94%). This technique is appropriate in support of inquiry of both purified and intracellular mcl-PHA.GC coupled with mass spectrometer (GC–MS) is currently used to characterize PHA monomer units in microbial cells.

During analyses on GC-FID monomeric constitution of PHA formed was originated equal to 7.2 mol% 3-hydroxyhexanoic acid, 50 mol% 3-hydroxyoctanoic acid, 10 mol% 3-hydroxydodecanoic acid, 24 mol% 3-hydroxydecanoic acid, 8.5 mol% of 3-hydroxytetradecenoic acid and 0.3 mol% 3-hydroxytetradecanoic acid [139]. While in another study, GC–MS investigation showed existence of PHBHHx copolymer in *C. necator* using soybean oil. Results of GC-FID analysis revealed the presence of 5 mol% 3HHx in the copolyester after 96 h fermentation. The mole ratio of 3HHx in copolyester reduced from 10 to 5 mol% with time interval. Therefore, it was accomplished that 3HHx ratio in polymer was reliant on fermentation period as well as fermentation approach. Similarly, Arcos-Hernandez used GC-FID to quantify PHA production in 22 bacterial strains. Quantification of these strains showed a range of overall PHA yield from 0.03 to 0.58 *w*/w) in these samples, also fluctuating constitutions 3 HV quantity of 0–63% in mol basis [140]. Throughout batch cultivation of bacteria RA26, 250 $mg.l^{-1}$ of PHA was produced. Granules were filled with 80–90% of cellular size/volume. The monomer constitution of PHA formed (using glucose-enriched medium) was determined by GC–MS and GC-FID. GC analysis found 0.3 mol% 3HB, 1.2 mol% 3HHx, 24.1 mol% 3HO, 64.5 mol% 3HD, 5.9 mol% 3HDDE as well as 3.9 mol% 3HDD in samples [141]. Findlay and White [142] detected 11 short-chain 3-hydroxyacids (major one 3HB and 3 HV) in polymer take out by marine sediments using capillary

gas-chromatographic (GC) from *Bacillus megaterium*. In another study, also conducted on *Bacillus megaterium*, extracted purified polyester was found to contain 95% 3HB, 3% 3-hydroxyheptanoate, 2% 3-hydroxyoctanoate, along with trace quantities of three further 3-hydroxyacids [143].

As far as the solvents used for PHA samples are concerned, chlorinated solutions have develop conditions in favour of derivatization as well as abstraction/removal of PHA using mud in support of GC investigation [126,137,144]. Dichloromethane has also been recommended to increase precision of thermo-polymer inquiry [145]. However, this solvent is correspondingly unwanted through a work-related health and care perceptions [146]. However, chloroform is generally utilized in support of biopolymer detection, additional solvents can also be utilized on behalf of strategy along with treating of biopolymer [147].

Chlorinated solvents are not good for health. Therefore, it was tried to avoid their use in laboratory protocols and is often replaced by propanol/butanol [148]. An environment friendly extraction method for GC analysis was tried to develop by Werker et al [149]. He used activated sludge biomass for PHA production. Instead of chlorinated solvent, acidic alcoholysis of dehydrated bacterial content utilizing 3:1 butanol with rigorous (37%) HCl at 100 °C intended for 8 h was performed. Hexane was used as co-solvent. It hydrolysed the accumulated PHA as well as covering lipids. Esters of hydroxyalkanoates and prolonged chain bacterial fatty acids are dependably taken out in hexane used for GC investigation as well as for its quantity identification. This procedure is striking for predictable assessment as it does not need any chlorinated solutions that are entirely utilized nowadays in favour of PHA investigation via GC.

6.4. PHA analysis by high performance liquid chromatography (HPLC)

This technique is used to separate different constituents of a compound via high pressure to push solvents through the column. HPLC is a most extensively used procedure to identify, quantify and separate the constituents of a mixture [150]. HPLC was used by Karr et al., [134] to quantify poly-β-hydroxybutyrate (PHB) in *Rhizobium Japonicum*. This method can measure crotonic acid in samples comprising 0.01 to 14μg of PHB. Detection and quantification of PHB synthesized in *Cupriavidus necator* done by transforming PHB into crotonic acid via HPLC [151].

Tailor-made PHA was synthesized by *Bacillus cereus* FC11 with the addition of different co-substrates along with glucose in medium. High performance liquid chromatography testing exposed that the retention time of crotonic acid (by-product) gained from digested PHA was approximately 24.97 min which was corresponding to the retention time of crotonic acid (by-product) gained by the digestion of commercially accessible PHB and PHBV (Sigma–Aldrich) [152] (Fig. 1.14).

6.5. PHA analysis by nuclear magnetic resonance (NMR)

NMR techniques have been used effectively for the detection of PHAs. The composition of the hydroxyalkanoate units in a copolymer can be determined by detecting the nuclear magnetic resonance (NMR) spectra [154–157]. The advantage of NMR analysis is that the hydrolysis step of the polymer can be avoided. In NMR analysis, 2-5 mg of the copolymer sample is dissolved in deuteriated chloroform ($CDCl_3$) (1.0 ml) and high resolution H-NMR spectrum is recorded on a NMR spectrometer.

Jacob et al., [158] showed that it was feasible to apply cross polarization magic-angle spinning ^{13}C NMR spectra of lyophilized samples of *Pseudomonas* sp. Strain LBr to examine directly and non-destructively PHB stored and utilized by this microorganism. Same method was utilized by Doi et al., [159] for PHB isolated from *A. eutrophus*. Doi et al., [160] have also analyzed the configuration of PHB in chloroform solution by 500 MHz ^1H NMR spectroscopy. Nuclear Magnetic Resonance spectrum ^{13}C of biogenic PHB (40 mg/ml) was recorded at 100 MHz using $CDCl_3$ as solvent and the ^1H NMR spectrum was recorded at 400 MHz at 24 °C using Bruker Spectrometer. ^{13}C NMR indicating the existence of different types of carbon atoms (C═O, CH, CH_2 and CH_3) in the PHB. The chemical shift signals obtained are almost similar with the chemical shift signals gained for PHB synthesized by *Bacillus megaterium* and *Cupriavidus necator* as reported by Doi et al., [161] and Oliviera et al., [162] respectively. PHB was further confirmed from the ^1H NMR spectrum (Fig. 1.15) which showed major peak at 1.2 ppm that may be recognized to the existence of CH_3 side chain. A chain of peaks at 2.4–2.6 ppm clearly specify the existence of CH_2whereas the peak at 5.2 is due to the existence of CH group.

^1H NMR spectra were gained for PHB and PHBV copolymer standards. These peaks match those observed in previous studies [159,164]. The PHB as well as PHBV ^1H NMR calibration curves were gained utilizing industrial standards. The area of each PHA-specific signal was directly proportional to polymer concentration. From the PHBV data, the average copolymer composition was examined as 12.34% ± 0.52% HV by calculating the intensity ratio of the HB and HV methyl signals at 1.28 and 0.90 ppm respectively. The vendor specified HV content was 12%. The ratio of methine peak areas at 5.2 and 5.16 ppm also gave similar values for HV content [43,159]. The CDCl3 peak at 7.25 ppm served as a reference signal, and the TMS peak at 0.00 ppm was used as an internal standard (Fig. 1.16).

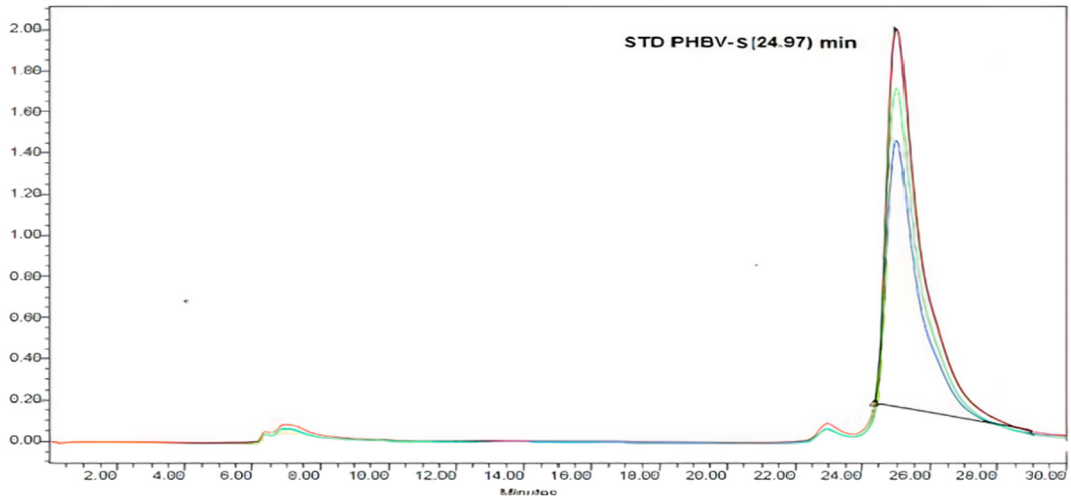

Fig. 1.14. HPLC chromatogram of PHB (upper), PHBV-S (middle) gained from Sigma-Aldrich and PHA gained from *Bacillus cereus* FC11 (lower) [153].

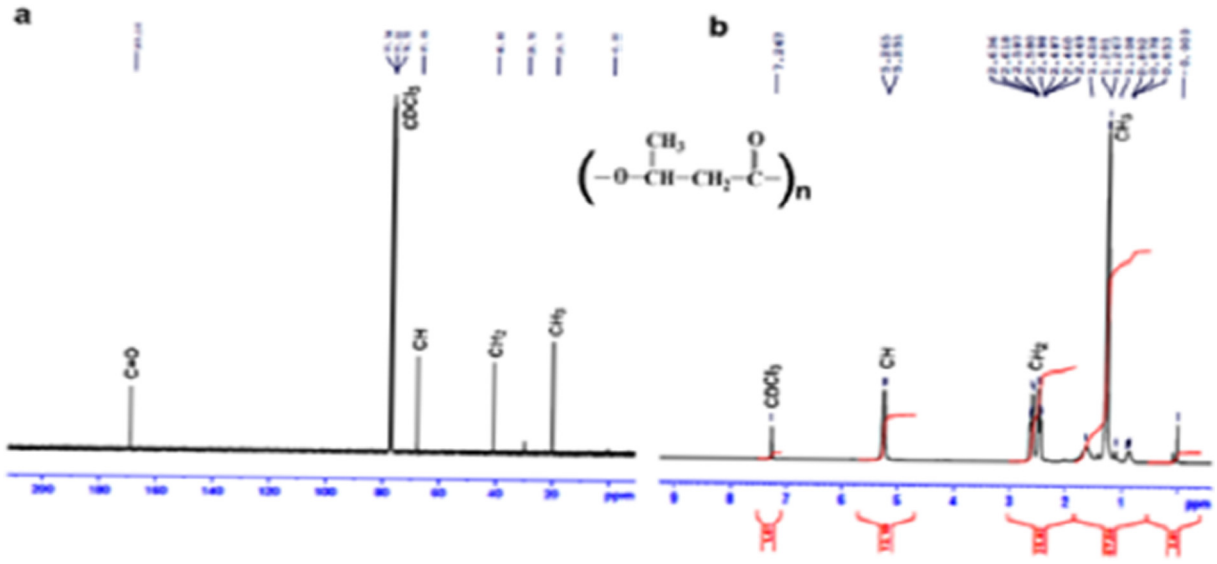

Fig. 1.15. (a) ^{13}C NMR and (b) ^{1}H NMR spectra of PHB produced by *Bacillus cereus* [163].

Furthermore, ^{1}H NMR spectrum of PHB produced is seen as Fig. 1.17. PHB peaks at $\delta = 5.2$, 2.5, and 1.2 which correspond to a —CH doublet, —CH2 multiplet, and –CH3 doublet respectively [37]. Two small additional peaks at $\delta = 0.9$ and $\delta = 1.6$ were found may be due to impurities present.

The resonance signals around 1.25–1.43 and 0.83–0.89 ppm designates the presence of methyl group proton (CH_3) in HB and HV. The existence of methylene proton (CH2) in PHA monomers gives multiple resonances around 1.58, 1.68, and 2.17–2.64 ppm. Multiple resonances around 5.24–5.28 ppm showed the presence of the methane proton in PHA and PHV [166–168] (Fig. 1.18).

7. Applications of PHA

A large number of research articles have been published on fermentation, biosynthesis, and characterization of the PHA. Laboratory research has now motivated towards industrial and commercial field. PHA has changeable properties because of their huge variety of different monomeric components and may also be utilized for an extensive variety of uses including recyclable products.

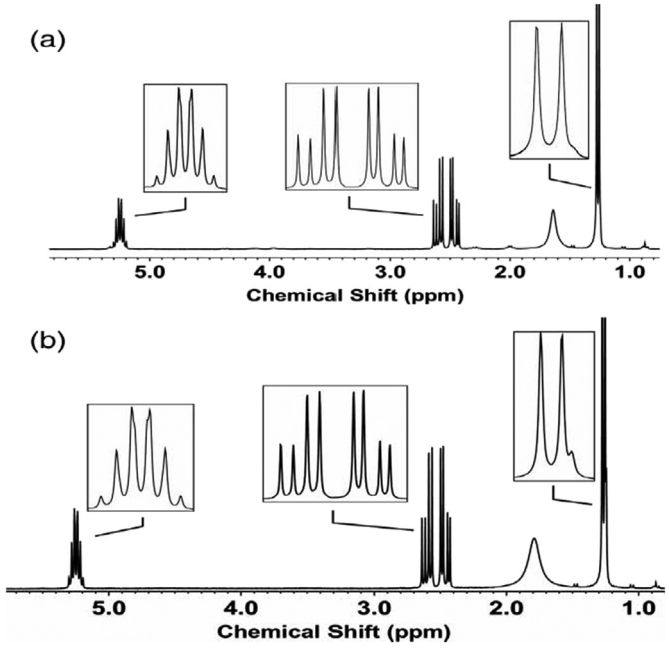

Fig. 1.16. (a) Signals observed in ^{1}H NMR spectra for *C. necator* and (b) signals observed in ^{1}H NMR spectra for *A. vinelandii* [165].

A waterproof coating for film, paper and cardboard can also be formed by latex of PHA (Figure 1.19a) [170]. This is cost operative since a very minor quantity of PHA is necessary for the layer. Similarly, PHA is used to form films, foils and diaphragms. Due to the biodegradability of scl-PHA in soils and home waste cure organisations [49], PHB and its copolymers with HV (PHBV) have been used as biodegradable plastic materials. Characteristics of mcl-PHA are appropriate for several uses, e.g. food, farming, pharmacy, chemistry as well as medicinal uses (Figure 1.19b and c), as of their natural biodegradability and biocompatibility [171]. New handling of mcl-PHA comprises the improvement of composite materials with inorganic, organic or metallic nano-particles or fibers [5] to develop physical characteristics like; tensile strength. Complicated bio-methods along with suitable choice of sources permit formation of ideal mcl-PHA. PHA is also known to generate fibre resources, like; unwoven cloths. Long chain hydroxyacids of PHA have also been utilized in self adherent paste formations [172].

Wella (the German hair-care company) tried to fabricate first time a shampoo bottle (SANARA made of BIOPOL™) in 1992 from the copolymer P(HB–HV) [8]. Biopol™ was the first trade name given to the copolymer PHBV, and was promoted through ICI/Zeneca as well as from Monsanto until 1995. PHB and copolymer PHBV have also been utilized as watertight films at backside of diaper coverings by U.S. manufacturers. In 2003, various disposable items were made from PHA and commercially distributed in Japan [40]. A German company, Biomer, produced PHB from *Alcaligenes latus* on industrial scale and used it to make pens, combs and bullets. They also sold polymer pellets for usage in classical transformation methods. This polymer degraded within two months in the environment. Over the last decade, uses have enlarged both in diversity and specialization. Industrial formation of PHBHHx by *Aeromonas hydrophilahas* was made by Tsinghua University (China) and used the polymer to make flushable, binders, nonwovens, elastic wrapping, synthetic paper, thermoformed articles, and medical devices.

7.1. Biomedical applications of PHA

PHA is also used in many medical applications (Table 1.4), such as bioimplants, cheesecloth, stitch thread, as well as bone fixative components [31,41]. Mcl-PHA are used to produce heart valve gallows [44].

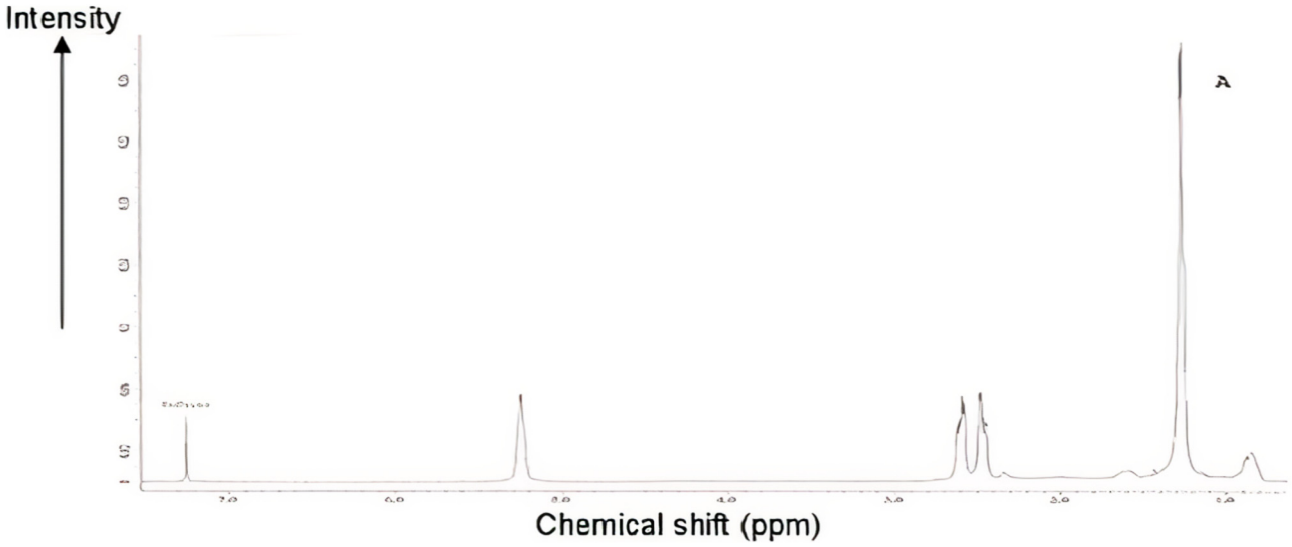

Fig. 1.17. ^{1}H NMR spectrum of PHB in *Cupriavidus necator* [151].

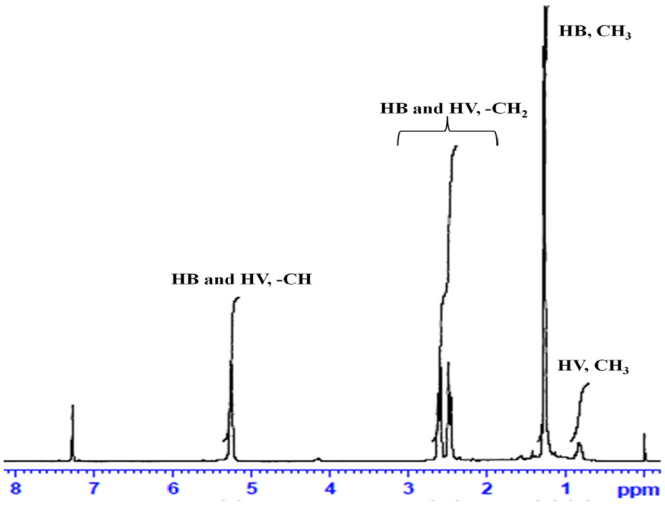

Fig. 1.18. ^{1}H NMR spectrum of PHB in *Cupriavidus necator* [169].

Table 1.4 summarizes medical applications of PHA in detail. Moreover, both types of PHA, scl and mcl-PHA, have applications in orthopedy, dental treatment, and drug [173] delivery and to make skin substitutes during wound management (Table 1.4). Wrapping layers and containers, biodecomposabe transporters for controlled chemical and drug discharge, sutures, and surgical pins, decomposable items, wound bandages and bone replacements also include in their applications in biomedical field.

PHA also have commercial power, e.g. cross linked mcl-PHA were used as biodegradable rubber-like materials [85]. They were applied like; stereo-regular molecules and chiral originators for chemical production of optically stimulated or chiral molecules [40]. PHA can be readily depolymerised to R-3-hydroxyalkanoic acids by acidic hydrolysis, or by enzymatic depolymerisation [171]. These hydroxyalkanoic acids are used to synthesize β- and γ-amino acids or peptides (novel polymers) [171]. They can then serve as novel nutrition sources and mono-functional spacers for peptide-related drugs [171]. PHA have been studied as biodecomposable transporters for long span dose of medicines, narcotic, pesticides as well as weed killer [40].

Characteristics of PHAs like; biodegradation, biocompatibility, non-toxicity, porosity, interference adherence, molecular weight and energy are the essential requirements for choice of polymer as biomedical

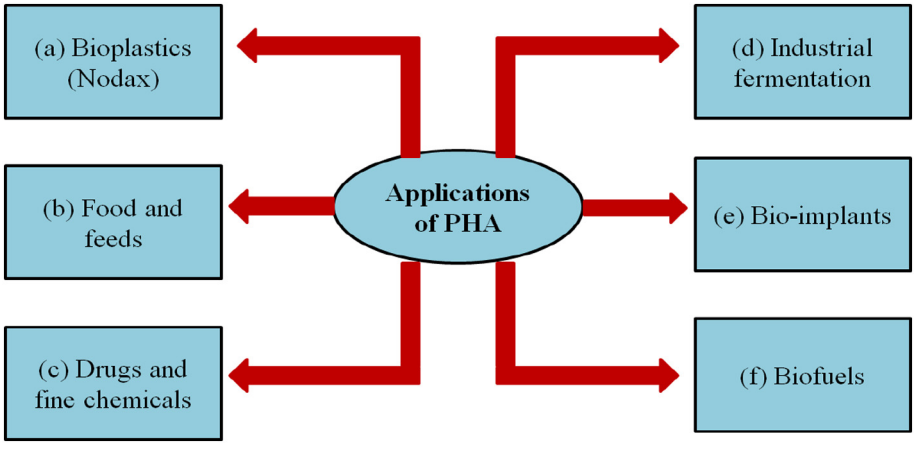

Fig. 1.19. Uses of PHA biopolymer in different fields [41]. (a) bioplastics, (b) foods and seeds, (c) drugs and chemicals, (d) fermentational usage, (e) bio-implants and medical usage, (e) biofuels.

Table 1.4
Possible uses of PHA in medication [174].

Type of application	Products	Type of PHA
Vascular system	Heart valves, pericardial patches, vascular grafts, cardiovascular fabrics	Mcl
Wound management	Nerve cuffs, skin substitutes, sutures, staples, surgical meshes, swabs	Scl, mcl
Urology	Urological stents	Scl, mcl
Orthopedy	Cartilage engineering, bone graft substitutes, spinal cages, internal fixation screws	Scl, mcl
Dental	Barrier material for guided tissue regeneration in periodontosis	Scl, mcl
Computer assisted tom-ography and ultrasound imaging	Micro and nanosphere of anticancer therapy	Scl, mcl
Drug delivery	Micro and nanosphere of anticancer therapy	Scl, mcl

implantations [174,175]. Scl and mcl-PHA have showed the excellent biocompatibility to epithelial cells [176], adreno cortical cells [177], smooth muscle cells [178], chondrocytes [179], bone marrow cells, osteoblast, human keratinocyte cells [50], human adipose derived stem cells [180]. PHA-based implants are considered biological safe due to its properties. They do not show any immune response in the host organism [174]. Firstly reported bio implant materials are poly (3HO-*co*-3HHx) base tubes, films, microspheres as well as tablets in rats the same as animal exemplary as well as revealed to be susceptible and biocompatible [181]. Some studies reported that PHB, PHBV as well as PHBHHx layers are used like blood connection materials [178]. Similarly, PHB based patches are used in gastro intestinal tract of dog did not show any negative response [182]. Bio polymers are majorly used in tissue manufacturing. Tissue engineering is technique of science that targets to redevelop damaged tissue and unhealthy tissue into new tissue by biomaterial implantations [183,184]. There are also two kinds of tissue engineering one is soft as well as other is hard tissue engineering. During soft tissue engineering technique the material is more porous biodegradable scaffold and implant with other cell types followed through the ex-vivo tissue development examples are heart, liver, valve, skin and nerve tissue engineering and hard type scaffold inserted like; the bioactive support for develop new cells examples are bone and cartilage tissue engineering [185].

7.1.1. Vascular grafting

Vascular grafting is the repairing of arteries and veins in cardiovascular pathologies. The end stage of the cure for the cardiovascular diseases is the surgical substitutions of the tissues by auto-graft (same organism), xenography, homograft or synthetic prostheses (natural and synthetic materials used for cure of veins or vascular heart disorders. Composites from PHA are considered as valuable materials for treatment of vascular disorders by cardiovascular patches, stents, vein valves and pericardial patches [174] [186]. Some studies reported that the improvement of PHBHHx scaffold via fibronectin cells covering and plasma treatment for the better outputs. They observed better proliferation of smooth muscle cells (SMCs) by ammonia coated polymer with the combination of plasma coated PHBHHx (PFn-PHBHHx) instead of only using fibronectin coated polymer [187]. PHBV and PHBHHx are considered as good materials for the treatment of cardiovascular disorders. Scaffolds of PHBV with poly caprolactone (PCL) are getting more importance due to their properties [188]. PHBV and PHBV-PCL scaffolds are considered as future biomaterials for the cardiovascular grafting [189]. Blended scaffolds are most beneficial then the pure scaffolds of PHBV and PHBHHx because they show the dual characteristics of biomaterials [190]. Novel amphiphilic materials with good biocompatibility for possible blood contact applications with hydrogel properties were reported [191].

7.1.2. Heart tissue engineering

Polyhydroxyalkanoates play a great role for the better proliferation of heart valve tissue. Scl and Mcl PHAs based scaffolds observed very effective for the heart valve tissue engineering technique from previous days [173]. Fu et al., stated that the fabricated scaffolds consisting of poly glycolic acid and poly 4-hydroxybutyrate showed fast growth of human body pediatric aortic cells [192]. Wu et al., used the coated scaffold of PHBHHx for the aortic valves and implanted into sheep about 16 weeks [193]. Coated scaffolds showed better proliferation as compared to the un-coated scaffolds. The blends of PHO/PHB have many valuable characteristics, they were well penetrated in host cells and made it more permeable and elastic as compared to pure scaffolds, they are considering the best blended polymers for the future biomaterials [194].

7.1.3. Skin tissue repairing

Skin is biggest organ of individual body. Significance researches have been done for the PHA-dependent scaffolds applications on skin tissue repairing [176,195]. Terpolyester poly (3HB-*co*-HV-co-HHx) was very good biomaterial for the development of HaCat because of its mechanical or thermal properties [196]. Now these days 2-D and 3-D PHBV polyesters are used for the proliferation and better cell adhesion in skin cells due to their mechanical properties. PHBV and 2-D PHBV have been studied for the good cell union and propagation like contrast to polystyrene [197]. Composites of PHBV fabricated with Chitosan instead of pure PHBV polymer have been studied especially for the repairing of wound tissue [198]. As the developing of 3-D macroporous chitin/PHBV hydrogel blends are considered good material for the skin tissue engineering [199]. Many studies developed that the blended polyesters of PHBV majorly used for the adhesion and proliferation of skin tissue cells due to their more elastic and adhesion properties.

7.1.4. Liver tissue engineering

In present there are large numbers of improvements in PHA developed for the liver tissue culturing. But they are still not completely explored for the liver tissue engineering. As Zu et al., developed the fabricated scaffolds of PHBV for the liver tissue culturing [200]. Culturing of human hepatoma HepG2 and Hep3B developed by the scaffolds, microspheres and films of PHBV. Some studies demonstrated that the good proliferation property of microspheres as compared to the films because of enhancement of cells through accumulation of hepatic cells of body. The improvement of union and propagation of Hep3B was observed by the fabrication of ECM protein-modified PHBV scaffolds. ECM proteins are the collagen, fibronectin and laminin, they are blended with PHBV microspheres, enhance the activity of polymer. Blended PHBV/PLGA evaluated and developed the polymer as the model of hepatocyte growth factor as compare with uncontaminated PHBV or uncontaminated PLGA [201].

7.1.5. Nerve tissue engineering

Nerve tissue manufacturing technique is repairing peripheral nervous system damages. Natural polyesters are considered as the good and valuable biomaterials for cure of peripheral nervous system disorders. Natural biodegradable or biocompatible materials are considered as good nerve conduit materials. Scl and mcl-PHA are used for the treatment of nerve tissue and for the regeneration of nervous cells [202]. Some studies reported that the PHBHHx used for the Schwann cells regeneration with the PHB [203]. Other studies also reported that the blend of PHBHHx with the poly (DL-lactide) has the good ability for nerve conduits [204]. Blended PHBHHx developed the valuable results due to the reduced crystallinity of PHBHHx/PDLLA as compared to the pure PHBHHx. On other hand blended PHBV microtubules with the PLGA attained the attention of scientists to develop the more elastic

fabricated films for the nerve tissue engineering [205]. The other type of blended composite of PHBV with the Collagen has also been used for the regeneration of nerve tissue. Mostly blended microspheres or films PHBV/Collagen are used instead of pure polymer because they showed high proliferation ability of nerve cells as compared to pure polymer [206]. Masaeli et al., made the Electrospun scaffolds from the mixing of PHB and PHBV nano-fibers for myelinic cell redevelopment [207].

7.1.6. Bone tissue engineering

Bone and cartilage tissue manufacturing technique is difficult type of tissue manufacturing. Tissue engineering technique is great development of damaged and missing bone or cartilage tissues. For this purpose, scaffolds of biopolymers are used due to their high mechanical properties and regulation of osteoblast and chondrocytes. In human bodies the bone composition is 40% collagen and 60% HA. Mainly scl-PHA is good for the repairing of bone tissues due to their toughness and stiffness properties. Many researches have also been done for the development of hard tissue engineering by mcl-PHA due to their high mechanical power and more elasticity as compared to others. Wang et al., established the 3-D scaffolds of PHB, PHBV, PLA, and PHBHHx for the good adhesion and proliferation characteristics [208]. Li et al., stated that compatibility to PHBHHx-dependent scaffolds, enhance the Ca^{+} and collagen deposition for the proliferation of osteoblast as compared to pure PLA and PHB [209]. Cool et al., reported that the PHBV-based scaffolds with nano-sized HA (n-HA) resulted the better mineralization and less inflammatory responses as compared to other biopolymers [210]. Hayati developed the scaffolds of PHB with the blending of n-HA composites (PHB/HA), showed the porosity and mechanical properties equal to the natural bone tissues [211,212]. Sultana also developed the scaffolds of PHBV with the high content of HA resulted the high porous and good mechanical product which is suitable for better proliferation and adhesion of osteoblast and chondrocytes [213]. Misra et al., designed the scaffold of PHB with the blending of bioactive glass BG (PHB/BG). Scaffolds of PHB/BG showed the better proliferation of osteoblast and no immune response after implementation of 1 week in rat [214].

7.1.7. Cartilage tissue engineering

Cartilage tissue manufacturing is also hard type in hard tissue manufacturing technique. Many researches have been done on the development of PHA scaffolds. Many types of PHAs are considered as the good biomaterials for implantation of cartilage tissues. PHB, PHBV, PHBHHx are the most efficient biomaterials for the regeneration of chondrocytes due to their more elastic, good proliferation and better adhesion properties [179]. Wang designed the 3-D scaffolds of PHBHHx along with engineered PHBHHx for the cartilage tissue repairing in the rabbit like as the model animal [186]. Yu et al., grown the blended PHB/PHBHHx for the integration of haSCs to improve proliferation and then inserted to hypodermic coating of cartilage of mouse like as model animal [215]. Li et al., developed the blended polymer by assimilation of poly (L-lactide-*co*-Ɛ-caprolactone) PLCL increase mechanical characteristics of PHB as for the cartilage tissue engineering [216]. PHBV/PLCL scaffolds showed the better proliferation and mechanical properties as compared to the pure PLCL and PHBV microspheres.

7.1.8. Therapeutic carrier

The chief drawbacks of conventional therapeutic systems are the poor oral bioavailability, toxicity, inconsistency and non definite distribution of therapeutics into targeted or non-targeted cells in past few decades [217]. Now these days there are some improvements developed for drug deliverance systems. Biomaterials are valuable polymers used in favour of delivery of drugs, hormones, antibiotics, gene therapy agents, anti-inflammatory, antioxidants and immunogens [218]. The PHA-based biopolymers considered as a good carrier for drug deliveries due to their biocompatible, biodegradable, non-toxic, porosity and more elastic behavior [219]. Scl-PHA matrix is used as the drug delivery carriers. But they are inappropriate for the drug delivery because they release the drug by the PHA matrix because of their crystal-like nature [174,209]. PHA-based nano-particles are considered to have the important factors for the therapeutic carriers of drug delivery [220]. PHB-based nanoparticles are used for the best therapeutic carriers for drug delivery in animals [221]. Functionalized PHBHHx-based water-soluble thermogels have been reported as the drug carrier for docetaxel (DTX) [222]. The use of doxorubicin loaded in the gel was tested with human fibroblast cell lines. A quadriblock system of PHBH/PEG/PPG/urethane where PHBH represented the hydrophobic moiety, while PEG and PPG represented the hydrophilic and thermolabile segments respectively, allowed an efficient docetaxel drug loading and melanoma reduction in xenograft of mouse models [223]. PHB as well as PHBHHx based nano-particles are utilized in favour of effective drug delivery purposes [224]. Poly (3-HB-*co*-5 mol%3-HV) PHBV-S, poly (3-HB-*co*-11 mol%3-HV) PHBV-11 and poly (3-HB-co-15%mol3-HV) PHBV-15 used for anticancer drug ellipticin due to their biodegradable properties [225]. Shah et al., developed the amphiphilic nano-particle of PHA/mPEG by the blending of PHA into monomethoxy poly (Ethylene Glycol) showed the better results for the drug deliveries [226]. Shah also developed the blended polymer poly (3HB-*co*-4HB) they associated along with to reduce toxicity related to drug dosing [227]. The degradation rate of copolymers PHB⁻PHBV⁻PHBHHx was depended on the side chain length [228].

8. Self-healing biopolymers: a new era in the field of biopolymers

Self-healing materials are very useful for high-performance structures and considered as the next generation materials. The specific functions performed by the self healing materials are the repairing of microscopic and macroscopic damages in polymer composites. Polymer and fibre-reinforced polymers (FRPs) are used the same as general structural materials because of their low weight, easy processability and useful for adverse environmental impacts. Low velocity and high velocity impacts are the critical issues for the FRPs. Micro-cracks are the basic reason for degradation of polymer. If micro-cracks are untreated they lead to the macro level cracks which are the main cause of polymer failures. To overcome these failures, next generation self healing polymer is used for the automatic healing of destructive polymer. Diverse kinds of repairing methods are utilized conventionally for the polymer repairing. Similarly, thermoplastics are very useful for the self healing process because of their excellent mechanical as well as thermal characteristics. Personally curing of thermoplastics can also be attained through the diverse process or mechanisms [229]. A detailed description is given below.

8.1. Molecular interdiffusion

Many researches have been done on the self healing of biopolymers via molecular interdiffusion in 1980s. It has been revealed when two fragments of similar polymer come closer to each other at the temperature higher than its crystal transition temperature (T_g), then polymer crossing point slowly decreases and mechanical power of polymer-polymer interface rises. Then fissure heals slowly because of the polymer interface dispersion at atmospheric stress or in space or at temperature greater glass transition temperature (T_g) as described in Fig. 1.20. Glass transition temperature ranges start −50 °C and reaches upto +100 °C. Other studies have been developed for the influence of molecular mass and rate of copolymerization on fissure curing. Jud and Kausch considered fissure curing manners of copolymer of poly (methyl methacrylate) as well as poly (methoxy ethylacrylate) [229]. Self healing depends upon some experimental parameters which are investigated by these researchers. Crack healing depends upon the period among fracturing and linking of fracturing surfaces, curing period (more than 1 min), healing temperature (5 °C more than the glass transition temperature), and atmospheric pressure. These parameters

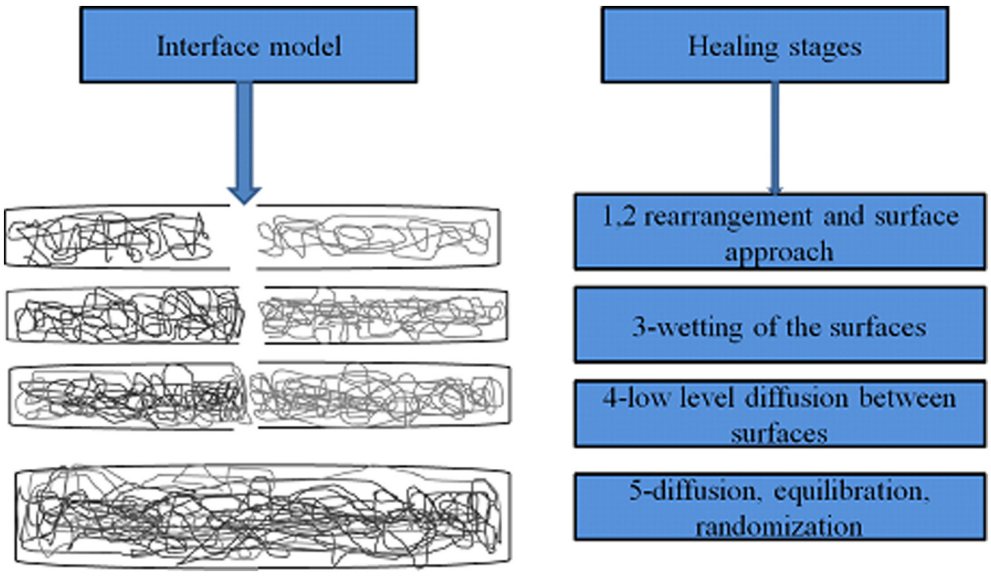

Fig. 1.20. Self healing of biopolymer via molecular interdiffusion [230,234].

required for the significant growth of interface adhesion. Number of models was proposed by many researchers [230–232] for the crack healing of thermoplastics like model of chain dynamics established from de Gennes [232] and later Doi and Edward [233].

8.2. Photo-induced healing

Kind of photo-induced self-healing in PMMA was stated from Chung et al., [235]. In this mechanism photo-addition cyclobutane produced by the photochemical [235] cycloaddition of cinnamoyl [236] and turn-around of cyclobutane to original cinnamoyl happens in solid phase may leads to the crack formation [237]. The mixture of 1,1,1-tris (cinnamoyloxymethyl) ethane (TCE) as well as urethane dimethacrylate (UDME), triethyleneglycol dimethylacrylate (TEGDMA) as well as a sensible light photoinitiator camphorquinone (CQ) was polymerized in a tough as well as clear film after the exclusion with 280 nm light source for 10 min. Healing of fracture polymer developed through re-irradiation for 10 min and light source range greater than 280 nm.

8.3. Recombination of chain ends

Reassociation of chain ending is a considerably new method used for the healing of molecular and structural damages in thermoplastics. Some studies reported that the engineering biopolymer produced by the condensation reactions like polycarbonate (PC), polyether-ketone (PEK), polybutylene terephthalate (PBT), and PEEK can be developed by fundamental reaction that is reversible of chain scission reaction [238,239]. Number of events was recognized primarily throughout healing method. These events are formation of chain breakage because of degradation, then propagation of oxygen into polymer material, then reassociation of cleaved polymer via the redox reaction in existance of copper catalyst and then removal of water during healing process.

8.4. Self-healing via reversible bond formation

Thermoplastic fractures can also be healed by the inclusion of reversible bond at ambient temperature in polymer matrix. This supply an altenative method to UV light and catalyst started curing of covalent bonds because explained in earlier studies along with utilization of hydrogen bonding and ionic linkages to repair breaked polymer networks.

9. Conclusion and future perspective

Thermoplastics are considered as the green materials. Biopolymers are biogedradable candidates in place of petro-chemical plastics. The basic problem for formation of PHAs are maximum formation cost in contrast to the petro-chemical plastics production cost. Main research and developments are still required to minimize cost of PHA production by using suitable extraction methods. Mainly the PHA produced from fermentation is the mixture of different monomers instead of single type monomer. Many developments are needed to be producing the single type of monomer by different strategies. Geneticaly modified strain are specific areas to increase the growth or accumulation rate within cells and to produce single type of mononer instead of mixture of copolymer. These biodegradable biopolymers have great application in medical field for high purity, efficient extraction and recovery method for the development of good yield. Genetically modified gram-negative strain can also be used to enhance purity of polymer and good yield with the effective recovery and extraction methods. These bacteria can produce toxin free polymers which are highly effective for biomedical applications. Modified gram-positive strain can also be used for maximum yield and toxin free biopolymer production. This is highly useful for the biomedical applications. Also thermoplastics have great importance due to their properties; they are used as self healing biopolymer. Many developments are required to modify these self healing capabilities of biopolymers. Estimated at USD 57 million in 2019, the global polyhydroxyalkanoate (PHA) market size is projected to reach USD 98 by 2024, growing at a CAGR of 11.2%. Increasing demand for biodegradable plastics fuelled by stringent government regulations & policies against single-use plastics and trends related to sustainable development and circular economy are driving the PHA industry. Europe is the key market for PHA, globally, followed by North America and APAC. The presence of various PHA players, such as Bioon (Italy), Biomer (Germany), Natureplast (France), ColorFabb (Netherlands), and EarthBi (Germany), has a positive impact the market. The short-chain length type is estimated to lead the PHA market in terms of value and volume. The growing awareness regarding plastic waste creation resulting in an ecological imbalance has changed the market sentiments positively towards sustainable products, hence creating a surge in demand for biodegradable plastics. This factor plays a crucial role in increasing the demand for short-chain length and medium-chain length PHA. Europe is expected to account for the largest market share in PHA during the forecast period, in terms of value. For

future perspectives, to make them cost effective by using inexpensive carbon sources, by manufacturing the biocomposites/blends with other biodegradable polymers and fillers. There should be the advancement developed in sustainability of PHA as well as the consideration of the market acceptance. With respect to develop policy advice, great efforts will be required for the introduction and guidance about PHA significances to the consumers rather than the PHA manufacturers. There are many application fields like; in medical field, in packaging, toys as well as for construction aspects, PHA has great importance than petroleum based plastics. Biodegradable and biocompatible polymers are considered as the green materials in place of petroleum based plastics in the future.

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