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REVIEW

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Polyhydroxyalkanoate copolymers from forest biomass

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Abstract The potential for the use of woody biomass in poly-β-hydroxyalkanoate (PHA) biosynthesis is reviewed. Based on previously cited work indicating incorporation of xylose or levulinic acid (LA) into PHAs by several bacterial strains, we have initiated a study for exploring bioconversion of forest resources to technically relevant copolymers. Initially, PHA was synthesized in shake-flask cultures of *Burkholderia cepacia* grown on 2.2% (w/v) xylose, periodically amended with varying concentrations of levulinic acid [0.07–0.67% (w/v)]. Yields of poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) [P(3HB-*co*-3HV)] from 1.3 to 4.2 g/l were obtained and could be modulated to contain from 1.0 to 61 mol% 3-hydroxyvalerate (3HV), as determined by ^{1}H and ^{13}C NMR analyses. No evidence for either the 3HB or 4HV monomers was found. Characterization of these P(3HB-*co*-3HV) samples, which ranged in molecular mass (viscometric, M_v) from 511–919 kDa, by differential scanning calorimetry and thermogravimetric analyses (TGA) provided data which were in agreement for previously reported P(3HB-*co*-3HV) copolymers. For these samples, it was noted that melting temperature (T_m) and glass transition temperature (T_g) decreased as a function of 3HVcontent, with T_m demonstrating a pseudoeutectic profile as a function of mol% 3HV content. In order to extend these findings to the use of hemicellulosic process streams as an inexpensive carbon source, a detoxification procedure involving sequential overliming and activated charcoal treatments was developed. Two such detoxified process hydrolysates (NREL CF: aspen and CESF: maple) were each fermented with appropriate LA supplementation. For the NREL CF hydrolysate-based cultures amended with 0.25–0.5% LA, P(3HB-*co*-3HV) yields, PHA contents (PHA as percent of dry biomass), and mol% 3HV compositions of 2.0 g/l, 40% (w/w), and 16–52 mol% were obtained, respectively. Similarly, the CESF hydrolysate-based shake-flask cultures yielded 1.6 g/l PHA, 39% (w/w) PHA contents, and 4–67 mol% 3HV compositions. These data are comparable to copolymer yields and cellular contents reported for hexose plus levulinic acid-based shake-flask cultures, as reported using *Alcaligenes eutrophus* and *Pseudomonas putida*. However, our findings presage a conceivable alternative, forestry-based biorefinery approach for the production of value-added biodegradable PHA polymers. Specifically, this review describes the current and potential utilization of lignocellulosic process streams as platform precursors to PHA polymers including hemicellulosic hydrolysates, residual cellulose-derived levulinic acid, tall oil fatty acids (Kraft pulping residual), and lignin-derived aromatics.

Keywords *Burkholderia cepacia* · Hemicellulosic hydrolysates · Levulinic acid · Lignocellulosic biomass · Poly-β-hydroxybutyrate-*co*-β-hydroxyvalerate [P(3HB-*co*-3HV)]

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Introduction

Forest-based biorefinery approach to PHA production

Forest biomass represents an enormous reservoir of renewable carbon-rich material, which has the potential to be utilized as a feedstock for the production of a wide variety of industrial and commodity products ranging from paper, lumber, and platform chemicals to a variety

of fuels and advanced materials, including biodegradable polymers [48]. Globally, approximately 80 billion tons of woody biomass is generated per annum, with the production of total plant matter estimated at roughly 180 billion tons annually [82]. Lignocellulosic biomass is by far the most abundant renewable organic resource on earth [21] and is comprised of 30–50% cellulose, 20–50% hemicellulose, and 15–35% lignin, dependent upon the tree species and environmental conditions.

By analogy with modern petroleum refineries, a biorefinery is a technological facility, which may be employed to produce solvents, fuels, power, hydrogen, or specialty chemicals from renewable (bio-based) resources. Flexible avenues for the production of such end products from sustainable and renewable biomass, under benign environmental conditions, will be the hallmark of such manufacturing sites [29]. The critical components of such envisioned factories are specially-adapted microorganisms (or enzymes there-from), which will effect the transformation of waste or of low-cost inputs into more desirable, marketable entities under so-called 'green chemistry' conditions. Generally, the choice of the latter commodities, will be dictated by the structural nature and availability of the starting materials, the location of the facility, and regional economic considerations. Forest biorefinery-related technologies are in development to process and resolve lignocellulosic biomass into its component process streams such as the 'Clean Fractionation' process (NREL CF), developed by the National Renewable Energy Laboratory (Golden, CO, USA) [37], the Lignol process [3, 55], and hydrothermal autohydrolysis of wood chips [72, 81] with subsequent membrane-diafiltration (CESF) (T. Amidon, personal communication, SUNY-ESF) (Fig. 1). The majority of the world's chemical pulp is still produced by the Kraft method, although organosolv processes such as those listed above are receiving increased attention as they become more cost-effective, especially in light of their relatively benign environmental consequences [27]. While the bulk of the cellulosic component is efficiently exploited by the paper/pulp industry, the hemicellulosic and lignin fractions are vastly underutilized process streams, which hold potential as platform intermediates in the production of value-added, bio-based polyhydroxyalkanoate (PHA) polymers.

Polyhydroxyalkanoates (PHAs) as biopolymer alternatives to conventional plastics

As part of an ever-expanding spectrum of biobased polymers, polyhydroxyalkanoates (PHAs) represent a unique class of biodegradable polymers, synthesized as an intracellular carbon and energy reserve by a variety of microorganisms when carbon sources are provided in excess and growth is limited by the lack of at least one other nutrient. Due to the wide range of thermoplastic and elastomeric properties, which can be regulated as a function of polymeric composition, these environmentally biodegradable [78, 83, 86] and biocompatible [10, 63] microbial polyesters are receiving increased attention as alternatives to conventional commodity plastics. Over 125 different hydroxyalkanoates have been identified as constituents of PHAs [64]. These PHA subunits can be broadly subdivided into short-chain length (3–5 carbon atoms; scl) or medium-chain length (6–14 carbon atoms; mcl) monomers, with most as 3-hydroxy-substituted fatty acids [47, 71]. PHAs composed of different molar proportions of monomers within these two classes differ markedly in their physical and mechanical properties; with mcl constituents acting to decrease the crystallinity, melting- (T_m), and glass-transition temperatures (T_g), making the polymers softer, more flexible, and elastomeric [19, 74]. Likewise, the incorporation of the 3-hydroxyvalerate (3HV) monomer into the poly-β-hydroxybutyrate-*co*-β-hydroxyvalerate [P(3HB-*co*-3HV)] copolymer (scl) improves the industrially relevant physical and mechanical properties (Fig. 2), with the T_m, T_g, and Young's modulus (degree of stiffness; GPa) decreasing as a function of the mol% 3HV content [7, 30, 74]. Thus, with the appropriate choice of microorganism, carbon source(s), cosubstrates, and culture conditions, a variety of homopolymers, copolymers, ter-polyesters, etc. can be produced. These biopolymers possess properties similar to those of commodity plastics, such as polypropylene and polyethylene [54], while avoiding many of the environmentally negative characteristics of petroleum-based plastics.

P(3HB-*co*-3HV) from renewable forest-based resources

A major limiting factor in the development of biodegradable polyesters is the expense associated with the carbon substrate used in the fermentation, which can account for up to 50% of the overall production cost of PHAs [12, 15, 44, 84]. Coupled with cost-efficient downstream recovery and purification processes, PHA production schemes based on relatively inexpensive agricultural sources may contribute significantly to lower manufacturing costs, as reviewed by Braunegg et al. [11]. In efforts

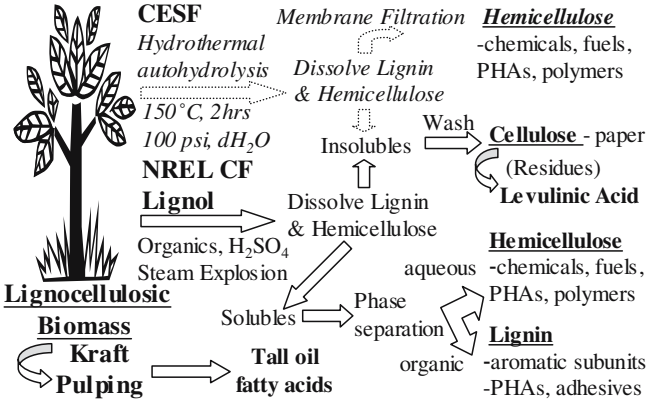

Fig. 1 Selected current depolymerization/resolution methodologies for obtaining lignocellulosic streams from woody biomass, with the associated platform chemicals and cosubstrates available for forest-based PHA production schemes

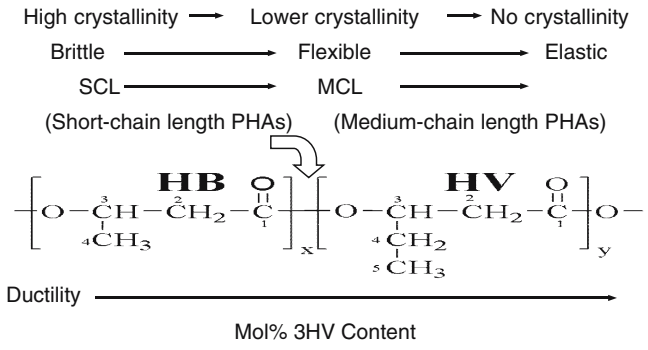

Fig. 2 Thermo-mechanical properties of PHAs as a function of their monomeric composition. Molar ratio of the β-hydroxybutyrate (*HB*) and β-hydroxyvalerate (*HV*) monomers in the P(3HB-*co*-3HV) copolymer (scl) also influence industrially relevant physical and mechanical properties

to evaluate pentose sugars as low-cost carbon sources for microbial PHA production, Bertrand et al. [4] demonstrated the batch production of poly-β-hydroxybutyrate [P(3HB)] at yields of 1.0–1.2 g/l from xylose and arabinose using *Pseudomonas pseudoflava*. These observations were extended by Ramsay et al. [62] and Young et al. [85] who detailed the ability of *Burkholderia* (formerly *Pseudomonas*) *cepacia* ATCC 17759 to accumulate P(3HB) at yields of 1.6 and 3.7 g/l, respectively, in shake-flask cultures containing xylose as the primary carbon source. In these studies, the xylose-specific yield (0.11 g/g) was calculated to reduce substrate cost by more than half compared to glucose [62].

The ability of *B. cepacia* to produce the industrially more attractive P(3HB-*co*-3HV) copolymer [18, 74], has been shown with fructose or glucose and propionic acid in shake-flask cultures [60 61]. Jang and Rogers [26] demonstrated the use of levulinic acid as a less-expensive alternative 3HV-providing cosubstrate (relative to propionic and valeric acids) for the production of P(3HB-*co*-3HV) by *Alcaligenes* sp. SH-69 on glucose as the principal carbon source. Within the rubric of a forestry-based biorefinery [29], we have delineated the bench-scale production of P(3HB-*co*-3HV) by *B. cepacia* ATCC 17759 using xylose, detoxified aspen- or maple-derived hemicellulosic hydrolysates, and levulinic acid as completely renewable, forest-based platform substrates [30 32].

Levulinic acid (4-keto-valeric acid) used in these studies was produced by Biofine Technologies LLC (S. Glens Falls, NY, USA), through a patented two-stage/reactor process based on a dilute mineral acid hydrolysis of hexose sugars [20]. This platform chemical can be produced cost-effectively from a vast array of carbohydrate-containing renewable biomaterials, including cellulose-containing forest and agricultural waste residues, paper mill sludge, and cellulose fines from paper production processes [8, 14] (Fig. 1). Our work has shown that periodic amendment of xylose-based shake flask cultures with levulinic acid enhances growth and P(3HB-*co*-3HV) accumulation by *B. cepacia* [30 32]. Yields of P(3HB-*co*-3HV) obtained with *B. cepacia* in such shake flask experiments ranged from 1.2–4.2 and 0.4–2.0 g/l using xylose and detoxified hemicellulosic hydrolysates as primary carbon sources, respectively, with levulinic acid as a cosubstrate.

The potentially fermentable monosaccharides present in the hemicellulosic fractions of lignocellulosic biomass represent an inexpensive and readily available carbon source for PHA production (Fig. 1). Current fractionation procedures form aqueous process streams containing variable proportions of pentoses (e.g. D-xylose and L-arabinose), hexoses (e.g. D-glucose, D-galactose, and D-mannose), and acetic acid, dependent on the tree species. Hardwood species are distinguished by a relatively high content of a partially acetylated, acidic xylan (i.e. *O*-acetyl-4-*O*-methylglucuronoxylan, comprising 20–35% of the hardwood biomass) and a small quantity of the glucomannan type of hemicellulose [17]. In addition, hardwood hemicellulose is rather highly acetylated, with an average of 7 acetyl moieties per 10 xylose residues, as compared to 2–3 acetyl groups per ten-residue glucomannan molecule in softwood hemicellulose [77]. Following hydrolysis of hardwood biomass, this high degree of acetylation can contribute to inhibitory acetic acid/acetate loads for subsequent fermentation processes [13, 38, 39].

As an inexpensive primary carbon source for P(3HB-*co*-3HV) production, our initial studies began with aspen-based hemicellulosic hydrolysates from the National Renewable Energy Laboratory (Golden, CO, USA) produced according to the 'Clean Fractionation' process (NREL CF) [37]. The organic solvent-based NREL CF process disrupts and solubilizes lignin and hemicellulose in a mixture of water, methyl-isobutyl-ketone (MIBK), ethanol, and sulfuric acid (H_2SO_4), following an acid-catalyzed steam explosion treatment (Fig. 1). Subsequently, maple-based hemicellulosic hydrolysates were also obtained from collaborators within the Paper Science and Engineering Department at SUNY-ESF (CESF). These CESF hydrolysates were prepared by pressurized (100 psi), hydrothermal treatment of maple wood chips with distilled water [1:4 (w/w)], whereby an autocatalytic hydrolysis of hemicellulosic glycans occurs due to liberation of endogenous acetic acid throughout the high temperature procedure (140–160°C, 2 h) [72]. Such CESF hydrolysates were then processed according to a membrane diafiltration procedure, designed both to reduce concentrations of acetic acid in the hydrolysate and concurrently concentrate the xylose fraction in the retentate (Fig. 1). This post-hydrolytic membrane filtration step was found to improve the fermentability of the CESF maple-hydrolysates. Organosolv-derived NREL CF hydrolysates were first processed by rotary evaporation to reduce levels of volatile organics to microbially sub-toxic levels. Subsequent detoxification procedures for the NREL CF and CESF hemicellulosic hydrolysates were based on the detoxification procedures of Strickland and Beck [73], Perego et al. [58], Martinez et al. [51], and Mussatto and Roberto [53]. Overliming with $Ca(OH)_2$ (to remove phenolics, furfuraldehydes,

and sulfates) was followed by pH adjustment to 5.5 with H_2SO_4. Activated charcoal was then added to complete removal of phenolics and to decolorize the growth medium prior to fermentation [32].

Results

P(3HB-*co*-3HV) copolymer accumulation by *Burkholderia cepacia*

In xylose-based (2.2% w/v) shake-flask cultures of *B. cepacia*, levulinic acid appeared to exhibit growth and PHA-accumulation enhancing effects over cosubstrate concentrations increasing to 0.52% (w/v), as can be observed by the associated dry biomass and P(3HB-*co*-3HV) yield maxima of 9.5 and 4.2 g/l, respectively (Fig. 3). Concentrations of levulinic acid exceeding this level increased the 3HV content of the copolymer to 61 mol%; however, this resulted in dramatic declines in both cell and PHA yields to 3.9 and 1.3 g/l, respectively [30]. Maximum intracellular contents of polymer obtained from xylose-based flask cultures were found to comprise 51–56% (w/w) of the dry biomass yield.

Samples of unprocessed NREL CF (aspen-derived) hydrolysate contained 1.8% (w/v) reducing sugar (initial pH 3.5–3.7), which following rotary-evaporation and volume reduction, was increased to 2.3–2.8% (w/v) reducing sugar. After the standard, three step detoxification procedure described above, the reducing sugar content averaged 1.4% (w/v) in the final, filter-sterilized hydrolysate [32]. Similar changes in reducing sugar content as a function of the detoxification procedure were noted for CESF hydrolysate samples. Shake-flask cultures of *B. cepacia* containing the detoxified NREL CF hydrolysate-based medium and 0.45% (w/v) levulinic acid reached maximum dry biomass and associated PHA yields of 5.1 and 2.0 g/l, respectively, when harvested at 112 h post-inoculation (Fig. 4). Corresponding levels of PHA accumulation over this harvest period (82–135 h post-inoculation) ranged from 40 to 18% (w/w) of the dry cell pellet weights [32]. Prior to 82 h of fermentation, yields of copolymer obtained were generally below 1 g/l and these data were therefore not included in Fig. 4. Supplementing other NREL CF-based shake-flask cultures with 0.25–0.5% (w/v) levulinic acid produced copolymers with a wide-range of 3HV compositions (16–52 mol% 3HV) at yields $\leq$2 g/l.

Similarly, shake-flask cultures containing detoxified CESF hemicellulosic hydrolysates and 0.08–0.6% (w/v) levulinic acid produced PHA yields of 0.4–1.6 g/l. Maximum dry biomass yields and levels of intracellular PHA accumulation (contents) for these CESF-based experiments were 4.1 g/l and 39% (w/w), respectively. Overall, the yields and intracellular contents of copolymer obtained from shake-flask cultures of *B. cepacia* containing xylose (or detoxified NREL CF/CESF hemicellulosic hydrolysates) and levulinic acid, are comparable to those reported in other investigations utilizing hexose carbohydrates and this cosubstrate for P(3HB-*co*-3HV) production (Table 1).

P(3HB-*co*-3HV) copolymer characterization

Composition of the P(3HB-*co*-3HV) copolymer can be controlled by regulating the ratio of levulinic acid to xylose in the fermentation medium. Adding cosubstrate doses from 0.07 to 0.67% (w/v) levulinic acid to xylose-based shake flask cultures, the mol% 3HV content of the copolymer was varied between 1 and 61 mol%. Proton resonance peak locations (300 MHz ^{1}H NMR) and splitting patterns obtained for xylose- and xylan hydrolysate-derived PHA samples (data not shown) were characteristic of those reported for other bacterial P(3HB-*co*-3HV) samples [6, 68]. Further, a comparison of the fully

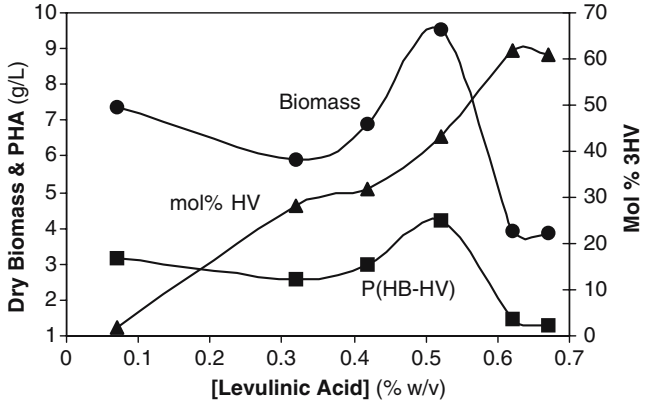

Fig. 3 Biomass and P(3HB-*co*-3HV) yields for shake-flask cultures of *Burkholderia cepacia* grown on 2.2% (w/v) xylose and various concentrations of levulinic acid. Composition of the copolymers, expressed as the mol% 3HV, is also plotted as a function of the levulinic acid cosubstrate concentration [30]

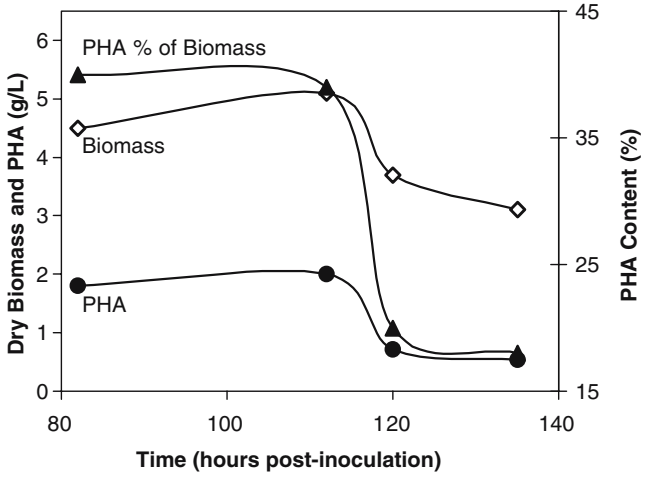

Fig. 4 Time profiles of dry biomass, P(3HB-*co*-3HV) yields, and PHA contents for shake-flask cultures of *Burkholderia cepacia* grown on detoxified NREL CF (aspen-derived) hemicellulosic hydrolysate and 0.45% (w/v) levulinic acid [32]

Table 1 Comparison of P(3HB-*co*-3HV) yields and levels of accumulation (contents) for shake-flask fermentations employing different bacterial systems with carbohydrates and levulinic acid as principal carbon source and cosubstrate, respectively

Microbial P(3HB-*co*-3HV) production system examined (carbohydrate substrate + LA cosubstrate)	PHA content (% w/w)	P(HB–HV) yield (g/l)
B. cepacia: xylose (2.2%) + LA (0.52–0.6%)[a]	56	4.2
B. cepacia: detoxified NREL hydrolysate + LA (0.45%)[b]	40	2.0
B. cepacia: detoxified CESF hydrolysate + LA (0.6%)[c]	39	1.6
W. eutropha KHB-8862: fructose (2.0%) + LA (0.4%)[d]	66	2.0
W. eutropha sp. SH-69: glucose (2.0%) + LA (0.05%)[e]	24	1.1
P. putida GPp104: glucose (1.6%) + LA (1.2%)[f]	51	NA

[a] Commercial grade xylose + LA: maximum PHA content attained (56%) and maximum P(3HB-*co*-3HV) yield (4.2 g/l) from separate experiments utilizing 0.6 or 0.52% LA, respectively [30]
[b] Keenan et al. [32]
[c] Keenan et al. [31]. Production and characterization of poly-β-hydroxyalkanoate copolymers from xylose and levulinic acid using *Burkholderia cepacia*. Ph.D. dissertation
[d] Chung et al. [16]; *Wautersia* (formerly *Ralstonia*) *eutropha*
[e] Jang and Rogers [26]
[f] Gorenflo et al. [23]

relaxed, 150 MHz ^{13}C NMR spectrum of these PHA samples (Fig. 5) to a 125 MHz spectrum of a bacterially-derived poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxyvalerate) [P(3HB-*co*-3HV-*co*-4HV)] terpolyester characterized by Valentin et al. [79], illustrates the lack of the chemical shifts for the 4HV monomer in the former at 31 ppm (corresponding to the 4-HV methylene carbons), 70 ppm (4-HV methine carbon), and 172 ppm (4-HV carbonyl carbon). Combined with the peak area integrations determined for the three carbonyl diad sequences (Fig. 5, expanded inset), these data confirm that the PHAs produced in our shake-flask experiments are random P(3HB-*co*-3HV) copolymers [30 32].

Thermal characterization by differential scanning calorimetry (DSC) of thin solvent-cast film sections of the xylose and LA-based P(3HB-*co*-3HV) samples described above revealed the T_m and T_g to vary as a function of the mol% 3HV incorporated into the copolymers (Table 2). The decrease in T_m from a maximum of 174.3°C (0.8 mol% 3HV) to the 154.2°C minimum at 25 mol% 3HV and subsequent increase back to 171.8°C at 61 mol% 3HV, creates a profile consistent with the pseudoeutectic behavior of the isodimorphic P(3HB-*co*-3HV) copolymer [5–7]. Change in T_g as a function of mol% 3HV exhibits a more linear decline from 2°C at 0.8 mol% 3HV to −11.9°C at 61 mol% 3HV. For all P(3HB-*co*-3HV) samples tested by TGA, the onset temperature for thermal degradation of the polymer chains occurred 54–100°C above the respective T_m [30]. For these samples, intrinsic viscosity-derived molecular mass (M_v) values (via Mark–Houwink constants determined for PHB, $K = 1.18 \times 10^{-4}$ dL/g, $\alpha = 0.78$) were determined to vary from 511 to 919 kDa. Similar results were obtained in the thermal and M_v characterizations of detoxified xylan-hydrolysate and LA-based PHAs (data not reported).

Discussion

Hemicellulosic hydrolysates as carbon sources for P(3HB-*co*-3HV) production

The utilization of low-value forest-based feedstocks as principal carbon sources and cosubstrates offers potential for significant reduction in the raw material component of P(3HB-*co*-3HV) production costs. Such substrate-oriented strategies for PHA cost reduction, in conjunction with optimized downstream polymer recovery and purification schemes, should further the commercialization of PHAs such as P(3HB-*co*-3HV), which has been hindered over the past 20 years due to high manufacturing costs compared to petroleum-based polymers [52]. Results of our studies showed that the maximum PHA yields obtained from commercial-grade

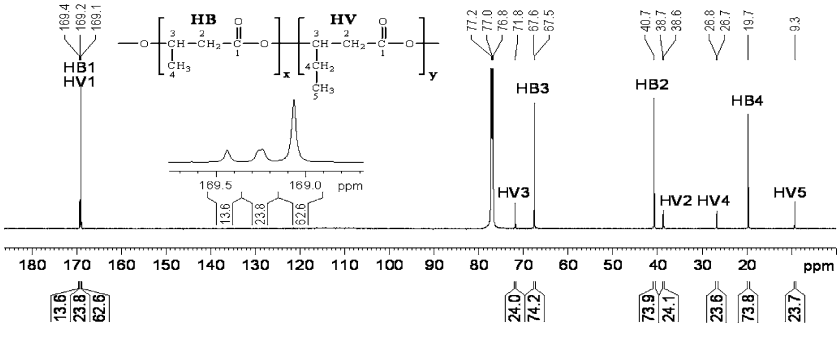

Fig. 5 Fully relaxed, 150 MHz ^{13}C NMR spectrum of a P(3HB-*co*-24 mol% 3HV) sample derived from *B. cepacia* using detoxified NREL CF hydrolysate and levulinic acid. *Expanded inset* displays the field locations and relative intensities of the three carbonyl diad sequences (*3HV–3HV*, *3HB–3HV*, *3HB–3HB*, *left to right*) [32]

Table 2 Physical characteristics of P(3HB-*co*-3HV) copolymers produced from xylose and levulinic acid including melting temperature (T_m), glass transition temperature (T_g), and viscosity average molecular mass (M_v) values (data extrapolated from [30])

Mol% 3HV	T_m (°C)[a]	T_g (°C)	T_{decomp} (°C)[b]	M_v (kDa)[c]
0.8	174.3	2	273.4	511
1.4	173.7	1.6	270.4	919
15	166.1	0.8	254.7	627
20	157.2	−0.6	249.2	683
25	154.2	−0.9	257.3	671
28	154.5	−1.1	259.3	700
56	159.5	−10.6	251.2	721
61	171.8	−11.9	225.5	622

[a] T_m determined to be the peak of the second, higher temperature endotherm in DSC cycles with multiple melting peaks

[b] T_{decomp} represents the onset temperature for thermogravimetric decomposition, arbitrarily defined as the loss of 0.032% of the original sample weight via analysis of the first derivative weight (%/°C) curve

[c] M_v calculations based on the corresponding (η) (intrinsic viscosity) values and the Mark–Houwink constants (K, α) published for the PHB homopolymer

xylose-based shake-flask cultures supplemented with levulinic acid were approximately twice those determined for similar cultures using detoxified xylan hydrolysates as the primary carbon source (4.2 g/l, 56% content or 2 g/l, 40% content, respectively). Average dry biomass (4 g/l) and P(3HB-*co*-3HV) (1.2 g/l) yields for the CESF and NREL CF hydrolysate-based cultures were similar, which makes these different sources of hemicellulose appear equally fermentable for PHA production (after appropriate detoxification). The lower PHA yields obtained for the hemicellulosic hydrolysate-based cultures may be attributed to residual toxins or inhibitors and/or limitation of biologically available xylose monosaccharide (e.g. as xylan oligomers) in the fermentation medium. Overall the results obtained for the shake flask-based cultures of *B. cepacia* reported in this study compare favorably to previous work by Bertrand et al. [4], Ramsay et al. [62], and Young et al. [85] for P(3HB) accumulation from xylose in shake flask cultures. Ramsay et al. [62] postulated that the relatively low xylose-specific (0.11 g P(3HB)/g) and biomass yields obtained with *B. cepacia* were due to a high maintenance energy conferred by its large plasmid load and that reducing this plasmid burden (e.g. through metabolic engineering) could potentially improve the efficiency of substrate utilization and associated P(3HB) yields.

In some instances, detoxified hemicellulosic hydrolysates have been found to confer a stimulating effect on P(3HB) production, when used as a supplement to or compared with xylose-based cultures [41, 70]. Lee [41] found that shake-flask cultures of recombinant *Escherichia coli* strain TG1 (pSYL107) (i.e. containing the *Wautersia* (formerly *Ralstonia*) *eutropha* PHA biosynthesis genes), utilizing 2.0% (w/v) xylose as the sole carbon source, produced P(3HB) at a maximum yield of 1.7 g/l and 36% (w/w) intracellular content. Supplementing these xylose-containing shake-flasks with 10 g/l of cotton seed- or soybean-hydrolysate increased the P(3HB) yield and content to 4.4 g/l and 74% (w/w), or to 2.1 g/l and 55% (w/w), respectively. Silva et al. [70] used shake-flask cultures of *B. cepacia* strain IPT 048 or *B. sacchari* strain IPT 101 (selected soil-isolates) containing detoxified sugarcane bagasse hydrolysate to obtain dry biomass yields and P(3HB) contents of 3.4 g/l, 15% (w/w) or 6.1 g/l, 23% (w/w), respectively. In bioreactor studies, the level of P(3HB) accumulation by *B. cepacia* strain IPT 048 increased from 37% of the cellular dry weight on xylose and glucose to 53% (w/w) on sugarcane bagasse hydrolysate (which contained approximately 12 g/l glucose and 4 g/l xylose). The 53 and 58% (w/w) P(3HB) contents obtained for the bioreactor cultures of *B. cepacia* strain IPT 048 and *B. sacchari* strain IPT 101, utilizing the detoxified hydrolysate, equated to substrate-specific yields of 0.29 and 0.39 g P(3HB)/g substrate, respectively. These relatively high substrate-specific yields and contents for P(3HB) production were 35–44% greater than those obtained with the same strains in an analytical-grade xylose and glucose medium (0.19 and 0.22 g/g for strains IPT 048 and 101, respectively). The authors concluded that the increases in residual biomass and substrate-specific P(3HB) yields, as well as improved levels of P(3HB) accumulation (content) observed in the hydrolysate-based experiments could be attributed to additional, metabolizable carbon sources present in the hydrolysate that were not identified (such as low concentrations of acetic acid and/or phenolics) [70]. Thus, the favorable yields of biomass and PHA obtained from shake-flask cultures of *B. cepacia* in this study may be attributable to stimulatory effects conferred by the use of both hemicellulosic hydrolysates and levulinic acid as substrates. Such observations highlight the possibilities for improvement of fermentation parameters and associated PHA yields/contents in the *B. cepacia*-based experiments by medium engineering techniques including supplementation with complex nitrogen sources, optimization of C/N ratios in hydrolysate-based fermentations, etc.

Levulinic acid as an alternative cosubstrate for P(3HB-*co*-3HV) production

In addition to economic advantages and derivation from forest-based feedstocks, the use of levulinic acid as a cosubstrate in PHA fermentations has also been shown to exhibit growth and PHA enhancing effects in shake-flask cultures of *Alcaligenes* sp. SH-69 and *W. eutropha* KHB-8862, with glucose or fructose as primary carbon sources, respectively [16, 26]. The results obtained with *B. cepacia* using commercial-grade xylose and levulinic acid (Fig. 3) support these conclusions and demonstrate that this microorganism tolerates concentrations of levulinic acid up to 0.52% (w/v) within this growth- and PHA-enhancing range. Yields of dry biomass and P(3HB-*co*-3HV) increased with cosubstrate concentrations from 0.07 to 0.52% (w/v), with maximum yields of 9.5 and 4.2 g/l, respectively (and maximum PHA contents of 56% w/w) (Table 1). Compositions as high as

61 mol% 3HV were obtained, although cell growth (3.9 g/l dry biomass) and PHA accumulation [1.2–1.3 g/l P(3HB-*co*-3HV)] declined dramatically at the higher concentrations of cosubstrate required for these 3HV contents [i.e. 0.62 and 0.67% (w/v)] (Fig. 3). The physiological basis of the biomass/PHA decline as a function of LA cosubstrate concentration relates to the effects of levulinic acid on cellular metabolism and thus differs among microorganisms and fermentation conditions. Levulinic acid is known to inhibit biosynthesis of tetrapyrrols, including cytochromes [66] and may therefore act to suppress growth and polymer production at these concentrations by disruption of general metabolic processes. Overall, the P(3HB-*co*-3HV) yields and contents obtained in our studies using pentoses as primary carbon sources compare favorably to those reported for *Alcaligenes* sp. SH-69 [26], *W. eutropha* KHB-8862 [16], and *P. putida* GPp104 [23] using hexose substrates and similar concentrations of levulinic acid (Table 1).

Physical–chemical characterizations of forest-based P(3HB-*co*-3HV) samples

Control of monomeric composition is important for PHA copolymers, since the comonomer content significantly influences the industrially relevant physical and chemical properties (Fig. 2). The ratios of levulinic acid [0–0.67% (w/v)] to xylose [2.2% (w/v)] present in shake-flask cultures of *B. cepacia* did prove to regulate the mol% 3HV content of these copolymers with a relatively high degree of control, as shown in Fig. 3. Likewise, relatively predictable mol% 3HV compositions were obtained by varying the ratio of reducing sugar to levulinic acid concentrations in CESF and NREL CF hydrolysate-based fermentations. The chemical structure of levulinic acid (4-keto-valeric acid) suggests that the 4-hydroxyvalerate (4HV) monomer would easily be incorporated into PHA synthesized in the presence of this organic acid. Schmack et al. [67] and Gorenflo et al. [23] used levulinic acid in recombinant *P. putida* and *W. eutropha*-based fermentations to produce and characterize PHAs containing 4HV. In contrast to these findings, the copolymers accumulated by *B. cepacia* utilizing xylose (or detoxified hemicellulosic hydrolysate) and levulinic acid, contain only the 3HB and 3HV subunits as a random copolymer, with no detectable molar fraction of the 4HV monomer. These structural determinations are confirmed by the direct correlation of all ^{1}H and ^{13}C chemical shifts, carbonyl diad sequences, and splitting patterns to those determined for other bacterial and synthetic P(3HB-*co*-3HV) samples as described by Keenan et al. [30]. Conversion of levulinic acid to 4HV (and its use as a 4HV precursor cosubstrate) by the genetically engineered strains reported above [23, 67], thus relates to fundamental differences in metabolic carbon fluxes and expression of the PHA biosynthetic genes.

Physical–chemical characterizations of xylose- (Table 2) and CESF/NREL CF-derived P(3HB-*co*-3HV) samples revealed a relatively wide thermal processing range for industrial application of these copolymers, with a 92°C average differential separating the respective melting and thermal decomposition temperatures. Additionally, viscosity-derived molecular mass determinations were relatively high compared to other microbially derived PHAs [47] and support the potential of these polyesters for commercial processing, with M_v values averaging 687 kDa.

Tall oils and lignin as putative PHA precursors

In addition to hemicellulosic hydrolysates, other paper/pulp industry-derived process streams have already been identified as potential precursors for forest-based PHA production. Tall oils, produced as a byproduct of the Kraft pulping process (Fig. 1) consist of 90–99% fatty acids with the bulk as C_{18} (i.e. 52% oleic and 45% linoleic acids). Brandl et al. [10] have shown that selected microorganisms can process long-chain fatty acid substrates, incorporate the respective mcl monomers into PHAs (through C-12), and thus reduce the crystallinity of the resultant polymers. Such compositional changes are manifest with lower T_g and T_m values and more flexible mechanical properties (Fig. 2), which may better suit particular commercial applications where more elastomeric polymers are desirable [24]. Van der Waal et al. [80] demonstrated the production of unsaturated mcl–PHAs by *P. putida* KT2442 from linseed oil- and tall oil-fatty acids. The mcl–PHAs produced from the tall oil substrates were composed of C_6–C_{16} monomers (40% mono- or di-unsaturated) with weight average molecular weights (M_w) of 56,000 Da and polydispersity indices of 1.7. The highly amorphous resins (T_g -60°C) were subsequently applied as environmentally friendly binders in high solid alkyd-like paints and coatings. Kellerhals et al. [33] used *P. putida* KT2442 to produce mcl-PHAs from oleic acid and reported substrate-specific yields (0.49–0.56 g PHA/g oleic acid) close to the theoretical maximum (0.62 g/g) at laboratory (2 l) and pilot (30 l) scales. In our laboratory we have obtained preliminary results demonstrating the use of oleic and linoleic acids derived from wood-based tall oils, as viable substrates for PHA production by *Pseudomonas* spp. PHA copolymers comprised of both scl and mcl-monomers have been detected in a variety of bacterial strains [9, 43]. Such scl–mcl PHA copolymers have been reported to display superior physical and mechanical properties relative to the respective scl or mcl homopolymers, with poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HX)] found to possess properties resembling those of low-density polyethylene [19].

Incorporating lignin-derived monomers as substrates in PHA biosyntheses can be envisioned as another way of channeling an inexpensive and underutilized forest-based residual into the production of higher-value products (Fig. 1). Generally a small fraction (1–2%) of the lignin stream is recovered from spent pulping liquors, although globally this amounts to over 1 million tons/year of a complex aromatic-laden biopolymer with relatively few industrial applications, such as use in

polyurethane foams and epoxy resins [45]. Techniques such as metal catalyzed oxidation, hydrogenolysis [59], or pyrolysis [2, 65] can be used to disassemble the phenyl-propanoid structure of lignin into a variety of substituted, aromatic phenol congeners. Thus, using hydrogenolysis, Pepper and Steck [57] obtained these lignin-derived aromatic congeners at high yield (~50% w/w) from the lignin in aspen (*Populus tremuloides*) woodmeal and were able to optimize the distribution of these various aromatic congeners by changes to the reaction conditions and/or catalyst. Lignin-derived substituted phenols may readily be reacted, as alkali salts, with selected halo-carboxylates to provide phenoxy-alkanoic acids.

Such phenyl- or phenyl-*o*-alkylated congeners can potentially be utilized as platform synthons for the production of phenyl- and phenoxy-alkanoates to be used as cosubstrates in subsequent PHA fermentations. Microbial strains can then be selected on the basis of those empirically possessing the appropriate PHA synthase enzyme, which will recognize and polymerize the phenyl- and phenoxy-alkanoates with monomers derived from other forest-based process streams to novel PHA polymers. Kim et al. [34] have already demonstrated the ability of *P. putida* and *P. oleovorans* to biosynthesize poly-β-hydroxyalkanoates containing aromatic substitutions using various phenyl- and phenoxy-alkanoic acid substrates (e.g. 5-phenylvaleric acid). These random copolymers were found to display number average molecular weights (M_n) of 50 kDa. In some cases, predictable compositional control was achieved by varying the ratios of aromatic-substituted substrates in the feed. The substituted phenyl- and phenoxy-side-chain functionalities impart desirable physical and mechanical properties to the polymers and can serve as sites for subsequent chemical modifications [35], which may broaden the range of potential industrial and specialized biomedical applications. Indeed, several patents describing technologies to produce aromatic-substituted PHAs have been filed with claims of improved biodegradability and dispersibility, high thermal stability, and high charge amount/stability in the modified polymers [75, 76].

Economic considerations for commercial PHA production

Commodity-scale production of P(3HB-*co*-3HV), utilizing glucose and propionic acid as feedstocks, was hampered in the early 1980s by the prohibitively high production cost of US $16/kg, which was 18-fold more costly than polypropylene [44, 63]. Over the past 20 years, however, significant advances in the upstream fermentation and downstream PHA recovery technologies [28, 36, 42] have been made to improve the efficiency and economy of PHA manufacturing [56]. Production of PHAs from low-value feedstocks such as forest-related process streams, has potential to significantly reduce the substrate expenditure component of the overall manufacturing cost (up to 50%) [15, 44]. Lee [40] determined the cost and substrate-specific yields of various substrates on production cost of P(3HB). Based on glucose as the principal carbon source (at US $0.493/kg, 25], with an estimated 38% yield of P(3HB)/g substrate (0.38 g/g), the substrate cost was calculated to be US $1.30/kg of P(3HB) produced. Using hydrolyzed corn-starch as the glucose source (roughly US $0.22/kg), the overall substrate cost was found to be US $0.58/kg of P(3HB). If hemicellulosic hydrolysates were applied as a carbon source (at US $0.07/kg), the final substrate cost was reduced to US $0.34/kg P(3HB), despite a lower substrate-specific yield from xylose (0.2 g P(3HB)/g).

In addition to a primary substrate, production of P(3HB-*co*-3HV) requires a 3HV precursor feedstock. Thus, the production economics calculations for this copolymer must also include expenditures and specific yields associated with this cosubstrate. Since the price of the more conventional 3HV-precursor, propionic acid [63] was approximately twice that of glucose, production cost of P(3HB-*co*-3HV) was found to increase linearly with an increase in the 3HV mol fraction [44]. The degree of this expense increase was found to be most influenced by the 3HV yield per gram of propionic acid consumed, with the rate of increase lowered when the substrate-specific yield was hypothetically raised from 0.2 to 0.5 g 3HV/g propionic acid consumed. Using fermentor-based cultures of *W. eutropha* KHB-8862, Chung et al. [16] determined 3HV yields of 0.5 g/g of levulinic acid consumed, which are relatively high compared to those obtained from propionic acid (0.06–0.13 g/g) in other *W. eutropha*-based studies [22, 49]. In addition to favorable substrate-specific yields reported for levulinic acid, its production economics also favor its use as a PHA platform chemical. Based on the Biofine process, Bozell et al. [8] estimated the production cost of levulinic acid to potentially fall as low as US $0.09–0.22/kg, and recent selling prices for refined levulinic acid in chemical applications have been projected to range from US $0.99–1.21/kg based on a potential 4.5 million kg scale of operation (S. Fitzpatrick, personal communication).

The substrate-cost calculations reported above by Lee [40] did not consider the expenses associated with downstream processing, including polymer recovery and purification, which can contribute up to 26% of the overall production cost [15]. Efficiency of the product recovery and purification steps is strongly influenced by the degree of intracellular PHA accumulation relative to the biomass produced, with higher polymer contents leading to lower recovery costs. For example, Choi and Lee [15] calculated a recovery cost of US $4.80/kg for a microbial system accumulating P(3HB) to a level comprising 50% (w/w) of the biomass and compared this to a much lower cost of US $0.92/kg for a similar process producing an 88% (w/w) accumulation of polymer. The dependency of recovery costs on the efficiency of polymer accumulation is based primarily on the reagents (solvents, enzymes, etc.) and procedures required to extract PHA from cellular biomass and debris [44]. The maximum yields and intracellular contents of P(3HB-*co*-3HV) accumulated

by *B. cepacia* in our shake-flask studies (40–56% w/w) must be improved in fermentor-based systems to reach levels required for commercial application (i.e. 80–90% w/w).

Salient features of PHAs, including biocompatibility and biodegradability [78] continue to unveil a variety of diverse biomaterial applications [50, 69] for these polymers. Utilization of inexpensive, renewable carbon sources such as forest-based residual streams, coupled with optimization of intracellular PHA content and downstream polymer recovery processes, offer potential for significant reduction of production costs for these microbial biopolymers.

Conclusions

The results from the initial phase of our previous work [30] established the ability of *B. cepacia* to produce moderate yields (i.e. up to 4.2 g/l) of P(3HB-*co*-3HV) from xylose and levulinic acid in shake-flask cultures and served as preliminary evidence supporting the use of xylose-rich hemicellulosic hydrolysates as inexpensive and renewable feedstocks. The capacity to control monomeric composition from 0.8 to 61 mol% 3HV, by regulation of cosubstrate concentration was demonstrated and provided a representative series of copolymers with predictable physical–chemical properties. Thermal characterization of the P(3HB-*co*-3HV) samples illustrated the T_m values to vary in a pseudoeutectic fashion as a function of mol% 3HV and to be sufficiently lower than the corresponding T_{decomp} values, providing for a margin of safety in terms of molecular mass integrity following potential melt-processing. In addition, the viscosity average molecular masses for these copolymers were determined to vary from 511 to 919 kDa, well within the 50–1,000 kDa range recommended for adequate material properties and subsequent commercial applications.

Subsequent work in our laboratory, including the development of an effective detoxification procedure, demonstrated the ability of *B. cepacia* to convert (detoxified) NREL CF- (aspen-) and CESF-(maple-) derived hemicellulosic hydrolysates and levulinic acid to P(3HB-*co*-3HV). Specifically, shake-flask cultures of *B. cepacia* utilizing detoxified NREL CF-based hydrolysate and 0.25–0.5% (w/v) levulinic acid were found to produce maximum P(3HB-*co*-3HV) yields of 2.0 g/l (40% w/w, cellular content) and a wide-range of 3HV compositions (16–52 mol% 3HV). Similarly, shake-flask cultures containing detoxified CESF hemicellulosic hydrolysate and 0.08–0.62% (w/v) levulinic acid were found to produce a 1.6 g/l maximum yield of copolymer, with an associated intracellular content of 39% (w/w). The T_m and T_{decomp} profiles of the hemicellulose-based polymers also appeared industrially favorable from a melt-processing perspective, because of an approximately 100°C temperature differential separating these values. Additionally, the relatively high M_v molecular masses and favorable thermal properties determined for these completely forest-based P(3HB-*co*-3HV) samples support the biotechnological potential for these copolymers to substitute for conventional petroleum-derived resins in a variety of commodity [1] and expanding specialty applications [46] markets.

Indicated follow-ups for these studies include medium optimization/engineering and operational simplification of the detoxification scheme used to prepare the hemicellulosic hydrolysates for fermentation. In addition, improved scale-up fermentation strategies, coordinated with optimal cosubstrate feeding regimes, culture conditions, and nutrient concentrations, may further the polymer yield obtained with wild-type and transgenic PHA producers. It is also suggested that exploration of the use of lignin-derived aryl precursors and tall oils will yield PHAs with presumed novel physical and mechanical properties. In comparison to agriculturally based feedstocks, technological advances in forest biomass-based PHA production, in conjunction with optimized downstream polymer recovery processes, hold alternative potential for the manufacture of economically viable biopolymers.

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