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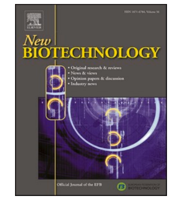

Biosynthesis and properties of polyhydroxyalkanoates synthesized from mixed C_5 and C_6 sugars obtained from hardwood hydrolysis

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A R T I C L E I N F O

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A B S T R A C T

Glucose and xylose are fermentable sugars readily available from lignocellulosic biomass, and are a sustainable carbon substrate supporting industrial biotechnology. Three strains were assessed in this work – *Paraburkholderia sacchari*, *Hydrogenophaga pseudoflava*, and *Bacillus megaterium* – for their ability to uptake both C_5 and C_6 sugars contained in a hardwood hydrolysate produced via a thermomechanical pulping-based process with concomitant production of poly(3-hydroxyalkanoate) (PHA) biopolymers. In batch conditions, *B. megaterium* showed poor growth after 12 h, minimal uptake of xylose throughout the cultivation, and accumulated a maximum of only 25 % of the dry biomass as PHA. The other strains simultaneously utilized both sugars, although glucose uptake was faster than xylose. From hardwood hydrolysate, *P. sacchari* accumulated 57 % of its biomass as PHA within 24 h, whereas *H. pseudoflava* achieved an intracellular PHA content of 84 % by 72 h. The molecular weight of the PHA synthesized by *H. pseudoflava* (520.2 kDa) was higher than that of *P. sacchari* (265.5 kDa). When the medium was supplemented with propionic acid, the latter was rapidly consumed by both strains and incorporated as 3-hydroxyvalerate subunits into the polymer, demonstrating the potential for production of polymers with improved properties and value. *H. pseudoflava* incorporated 3-hydroxyvalerate subunits with at least a 3-fold higher yield, and produced polymers with higher 3-hydroxyvalerate content than *P. sacchari*. Overall, this work has shown that *H. pseudoflava* can be an excellent candidate for bioconversion of lignocellulosic sugars to PHA polymers or copolymers as part of an integrated biorefinery.

Introduction

Microbial polyhydroxyalkanoates (PHAs) are a class of biopolymers that are biodegradable and can be obtained from renewable resources. PHAs may be a promising alternative to some conventional plastics in packaging [1], biomedical [2], and agricultural [3] applications. They are synthesized and polymerized intracellularly by certain species of microbes, particularly upon imbalanced or nutrient-limited growth conditions as a reserve of carbon and energy [4]. Despite promise, the cost of PHA production combined with limitations in their thermomechanical and gas barrier properties, has limited more widespread application. The carbon substrate is estimated to account for about 50 % of production costs [5], so utilizing low-cost substrates is a leverage point in improving the viability of PHAs.

Within this context, lignocellulose-derived sugars may be the only foreseeably sustainable carbon source supporting heterotrophic industrial biotechnology processes. A significant limitation in valorizing lignocellulosic biomass is access to the sugars available in the hemicellulose and cellulose fractions enmeshed in lignin [6]. The well-known PHA-producing species do not possess the enzymes required to

Abbreviations: PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate); GPC, gel permeation chromatography; M_w, weight-average molecular weight; M_n, number-average molecular weight; PDI, polydispersity index; TGA, Thermogravimetric analysis; DSC:, Differential scanning calorimetry; CDM, cell dry mass.

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depolymerize lignocellulose into sugars. However, recent interest in lignin as the world's only sustainable supply of aromatic compounds has put increased emphasis on biomass deconstruction technologies – such as the thermomechanical pulping-based process developed by FPInnovations (Canada) for hardwood biomass – called 'TMP-Bio' [7]. This technology combined mild treatment with enzymatic hydrolysis at high (up to 20 %) solids loading to produce H-lignin as well as an aqueous stream of 120–140 g/L total sugars containing predominantly glucose and xylose at a ratio of approximately 2.5:1 [8]. The presence of both C_5 and C_6 sugars in mixtures is challenging because many organisms do not have the ability to metabolize xylose and an even smaller subset of those have the ability to synthesize PHA [9]. Furthermore, xylose uptake is typically suppressed in the presence of glucose due to a widely-conserved phenomenon known as carbon catabolite repression [10]. Currently, hemicellulose-derived C_5 sugars are under-utilized [6], and complete utilization is key to optimize process economics and reduce downstream waste-treatment costs.

A further challenge is the material properties that result from PHA polymers synthesized directly from sugars. Most wild-type species with the ability to synthesize PHA from glucose-xylose mixtures synthesize homopolymers of poly(3-hydroxybutyrate) (PHB). PHB homopolymers tend to be brittle and have high melting points close to their thermal decomposition temperatures and poor gas barrier properties [11]. In response to this limitation, studies have shown that incorporation of even a small amount of a co-monomer (most typically 3-hydroxyvalerate or 3-HV) results in a poly(3-HB-*co*-3-HV) copolymer (PHBV) with significantly improved properties [12].

The goal of this study was to demonstrate the possible conversion of mixed sugars into PHBV. To reach this goal, several publicly available PHA-synthesizing strains capable of glucose and xylose catabolism were identified. The growth of these strains and the resulting PHA-synthesis characteristics, on both synthetic glucose-xylose mixtures and the TMP-Bio sugars resulting from hardwood hydrolysis, were compared, and the strains' ability to synthesize poly(3-HB-*co*-3-HV) copolymers via the addition of appropriate chemical precursors to the sugar mixtures was investigated. Molecular weights and thermal properties of the polymers synthesized by these strains were also characterized to document their potential commercial applicability.

Materials and methods

Strain and maintenance, medium, and substrates

Three strains were used in this study, namely *Paraburkholderia sacchari* DSM 17165, *Bacillus megaterium* ATCC 14581 and *Hydrogenophaga pseudoflava* ATCC 33688. Glycerol stock cultures were periodically revived in nutrient broth (NB) and maintained on NB-agar plates. The mineral salts medium (MSM) used in this study followed the recipe described in [8]. The phosphate buffers and ammonium source were autoclaved for 20 min at 121 °C and the remaining components were added afterward as filter-sterilized solutions. Prior to starting the experiment, the carbon source was added to each flask as either TMP-Bio sugars (normalized to a final concentration of 15 g/L sugars, roughly a ten-fold dilution) or synthetic mixtures of glucose (10 g/L) and xylose (5 g/L). For cases where TMP-Bio sugars were used as the carbon source, a slightly more concentrated version of the MSM medium was prepared to account for the roughly 10 % dilution resulting from the sugar solution with total sugar concentration of approximately 135 g/L.

Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). The TMP-Bio sugar stream was produced at FPInnovations (Thunder Bay, ON, Canada) as detailed in [7].

Cultivation conditions

To initiate an experiment, a single colony was picked from the aforementioned plates and grown in NB overnight or until an OD_{600} of 5 was reached. Approximately 1 mL of these cultures was used to inoculate 500 mL flasks containing 100 mL MSM supplemented with sugar substrates such that the starting OD_{600} was 0.05. Experiments were monitored up until 72 h, with orbital shaking at 125 rpm. Growth temperatures were 30 °C for *P. sacchari* and *B. megaterium*, and 37 °C for *H. pseudoflava*. Samples (2 mL) were periodically withdrawn for immediate measurement of OD_{600} and pH. Each condition was assessed in biological triplicate.

Synthesis of PHBV copolymers from TMP-Bio sugars supplemented with precursors

For certain experiments, the medium described above was supplemented with propionic acid at concentrations ranging from 5 to 20 mM (0.37–1.48 g/L). Depending on the tolerance of the organism to propionic acid, different strategies attempted included: 1) batch with 20 mM (1.48 g/L) propionate initially; 2) batch with 5 mM (0.37 g/L) propionate; and 3) a 'pulse' fed batch starting with an initial concentration of 5 mM and an additional 5 mM supplemented 24 h post-inoculation. In each case, the medium pH was adjusted to 6.6 using a filter-sterilized solution of 5 N NaOH.

Sample handling and biomass measurement

Following measurement of OD_{600} and pH, the 2 mL samples were centrifuged at 16,000 × *g* for 5 min. The supernatant was separated from the pellet and stored at − 20 °C for subsequent analysis of residual carbon and/or nitrogen source(s). At the end of the cultivation, the entire remaining culture volume was centrifuged at 12,000 × *g* for 20 min. The cell pellets were washed once in phosphate buffered saline (PBS), and then transferred to a pre-weighed aluminum tray. The tray was placed in a 60 °C oven for a minimum of 24 h or until no further loss in mass was detected, and the final mass was taken for determination of the total biomass titer.

Analytical methods

Estimation of intracellular PHA content and monomer composition

Samples (10 mg of dry biomass) and standards of PHBV (Catalog # 403105, PHV content 8 mol%, (Sigma Aldrich, Milwaukee, WI, USA) were subjected to acid-catalyzed methanolysis to convert the PHAs to methyl esters for identification and quantification using gas chromatography as previously described [13]. Following methanolysis, the organic layer was analyzed using an Agilent 6890 N gas chromatograph equipped with a DB-1 capillary column (15 m × 530 µm × 0.15 µm) and a flame ionization detector (GC-FID) (Agilent Technologies, Inc., Santa Clara, CA, USA).

PHA extraction and purification

The PHA was recovered from the biomass by 4-h extraction in chloroform using a Soxhlet apparatus and purified by precipitation with 10 volumes of cold (− 20 °C) methanol. The purity of the extracted samples was confirmed using 500 MHz ^{1}H NMR (Bruker, Billerica, MA, USA).

Estimation of PHA molecular weight

Samples were prepared for analysis of molecular weight by gel permeation chromatography (GPC) by dissolving 1.5 mg of the extracted and purified polymer in 1 mL HPLC-grade chloroform. A Viscotek GPCmax TDA 305 was used for the analysis (Malvern Panalytical Ltd, Malvern, UK). A combination of Waters Styragel Columns (HR2, HR4 and HR5, Waters Corporation, Milford, MA, USA) was used with HPLC grade chloroform as eluent at a flow rate of 1.0 mL/min and triple detection (refractive index, viscosity, and light scattering). A 1-point calibration was performed using a polystyrene standard with a narrow-range polydispersity index (PDI) and validated using a polystyrene standard with a broader PDI (Malvern Panalytical PolyCal PS 105k (narrow, M_w: 104,401; M_n: 101,009) and PS 245k (broad, M_w: 246,874; M_n: 111,534)). All samples and standard were filtered with 0.2-μm PTFE syringe filters prior to injection. OnmiSEC 4.6.1 software was used to generate the molecular weight distribution for samples. The obtained data included the weight-average molecular weight (M_w) as well as the number-average molecular weight (M_n) and the PDI.

Estimation of PHA thermal properties

Thermogravimetric analysis (TGA) was carried out to assess thermal degradation temperatures (T_d) of the polymers using an STA 449 F1 thermoanalyzer instrument (Netzsch Group, Serb, Germany). PHA samples (10 mg) were heated from 30 °C to 450 °C at a heating rating of 10 °C/min under N_2 atmosphere at a constant flow rate of 20.0 mL/min. Differential scanning calorimetry (DSC) was performed on approximately 5 mg of the purified PHA sample using a Q2000 (v.24.10) DSC (TA instruments, New Castle, DE, USA). For each heating cycle in this procedure, the samples were equilibrated at − 60 °C for 1 min and the temperature was subsequently ramped at 10 °C/min to 200 °C and held at that temperature for 1 min prior to being cooled back to − 60 °C at a rate of − 10 °C/min. This procedure was performed twice and the data from the second cycle was used in the analysis. Determination of the melt temperature (T_m), melt enthalpy (ΔH_m), crystallization temperature (T_c), crystallization enthalpy (ΔH_c), and glass transition temperature (T_g) was completed using TA instruments Universal Analysis 2000 (v4.5A) software. Sample crystallinity (X_c) was obtained by expressing the sample melt enthalpy as a percentage of the melting enthalpy previously reported for a 100 % crystalline PHB, i.e., 146 J/g, according to the approach described in [14].

HPLC analyses of the supernatant

Sugars and volatile fatty acids were quantified in the aqueous medium by HPLC using a Waters system consisting of a pump model 600, an autosampler model 717 Plus, a refractive index (RI) detector (model 2414) and a PDA detector (model 2996) (Waters Corporation, Milford, MA, USA). Separation was performed at 40 °C using a Transgenomic ICSep ICE-ION-300 column (300 mm × 7.8 mm OD) (Transgenomic®, Omana, NE, USA) and mobile phase (0.003 N H_2SO_4) at a flow rate of 0.4 mL/min. Ammonium was analyzed by ionic chromatography, using a Waters HPLC (model Alliance) equipped with a Waters model 432 conductivity detector (Waters Corporation, Milford, MA, USA). The separation was achieved at 40 °C using a Hamilton PRP-X200 resin-based ion chromatography column (250 mm × 41 mm OD) (Hamilton, Reno, NE, USA) and 30 % methanol in a 6 mM nitric acid solution as mobile phase, at a flow rate of 1.75 mL/min.

Calculation of rates and yield coefficients

By knowing the intracellular PHA content of the biomass from the GC-FID analysis, the total biomass (denoted X_t) was separated into PHA biomass (denoted X_{PHA}) and PHA-free residual cell mass (denoted X_r). Square braces are used to indicate titer or concentration. The specific sugar uptake rate ($mg_{sugar}/(g_{Xr}$ h)) was calculated as the sugar consumption within a particular time interval (mg/(L h)) divided by the average X_r titer determined during that interval. The overall PHA volumetric productivity (Q_v, mg_{PHA}/(L h)), specific PHA productivity (Q_s, $mg_{PHA}/(g_{Xr}$ h)), and yield coefficients of both PHA produced per sugars consumed ($Y_{PHA/S}$, g/g) and 3-HV from propionate ($Y_{3\text{-}HV/Pro}$, g/g) were determined as the slope of a plot of product titer (g/L) vs. substrate uptake (g/L).

Results and discussion

Growth characteristics/biomass production

Biomass synthesis over the 72-h cultivation period for the three strains is shown in Fig. 1. *P. sacchari* performed similarly regardless of whether the carbon source was a synthetic mixture of glucose and xylose or the TMP-Bio sugars, reaching early stationary phase within 24 h and [X_t] values of 4.85 (± 0.05) g/L and 5.57 (± 0.13) g/L cell dry mass (CDM) at 72 h for the synthetic sugar mixture and TMP-Bio sugars, respectively. The pH in the *P. sacchari* cultures rapidly dropped from the initial value of 6.85–4.30 by 24 h but then increased to 6.10 (± 0.02) by 72 h (Suppl. Fig. S1), indicative of the formation of a transient acid product.

The OD_{600} in *H. pseudoflava* cultures continued to increase past 24 h, reaching maximum values of 9.6 and 19.8 at 32 h when grown on the synthetic sugar mixture and TMP-Bio sugars, respectively, and declining slightly thereafter. The [X_t] was comparatively low when grown on synthetic sugars, reaching a maximum value of 2.82 (± 0.09) g/L at 48 h and declining thereafter. When grown on the TMP-Bio sugars, *H. pseudoflava* produced 6.50 (± 1.32) g/L of CDM by 72 h. The pH in *H. pseudoflava* cultures remained more constant than with *P. sacchari* (Suppl. Fig. S1). While the three strains generally produced more biomass from the TMP-Bio sugars with respect to the synthetic glucose-xylose mixture, the biggest increase was noted in *H. pseudoflava*. This could suggest that this strain shows a particular affinity for other compounds present in the TMP-Bio sugars such as alcohols, free fatty acids and soaps, organic acids and phenolic compounds. *B. megaterium* grew relatively well within the first 12 h, but biomass synthesis ceased between 12 h and 24 h and a sharp decline phase was observed afterward. The maximum total biomass values were observed at 24 h as 1.46 (± 0.07) and 3.53 (± 0.6) g/L of CDM from the synthetic sugar mixture and TMP-Bio sugars, respectively. The reasons for this sudden growth arrest are not clear and require further investigation. The pH dropped throughout the cultivation, reaching a final value of 4.41 (± 0.04) at 72 h when grown on synthetic sugars and 4.60 (± 0.13) at 72 h when grown on TMP-Bio sugars (Suppl. Fig. S1).

Sugar and ammonium uptake

The uptake of both sugars and NH_4-N are shown in Fig. 2. For *P. sacchari*, the cessation in biomass production by 24 h corresponded to NH_4 limitation, which was depleted to less than 1 mg/L NH_4-N within 24 h when grown on the synthetic sugar mixture. When grown on TMP-Bio sugars, NH_4-N was depleted below detectable limits within 12 h. In terms of sugar utilization, less than 0.5 g/L glucose remained by 24 h and less than 0.05 g/L remained by 72 h on the synthetic sugar mixture. Similar results were obtained when grown on TMP-Bio sugars; glucose was depleted to < 0.1 g/L within 24 h. In each case, glucose was co-consumed with xylose from the start of the fermentation, albeit the rate of glucose utilization was faster than xylose utilization (Table 1). Xylose was never depleted during the 72-h cultivation, remaining at or above 0.6 g/L. The depletion of glucose did not appear to affect the uptake rate of xylose. Amongst all strains, *P. sacchari* showed the highest specific uptake rates for both sugars when grown on the synthetic sugar mixture (0–24 h).

H. pseudoflava depleted NH_4 below detectable limits within 24 h when grown on both the synthetic sugar mixture and TMP-Bio sugars. The sugar uptake for *H. pseudoflava* corroborated the low biomass production as neither glucose nor xylose were depleted when grown on the synthetic sugar mixture. At 72 h, more than 1.6 g/L of both glucose and xylose remained, and there was little uptake of either sugar after 32 h. In contrast, when grown on the TMP-Bio sugars, *H. pseudoflava* depleted both glucose and xylose within 48 h. This was the only condition in which complete utilization of both sugars was observed. When grown on the TMP-Bio sugars, *H. pseudoflava* showed similar maximum specific

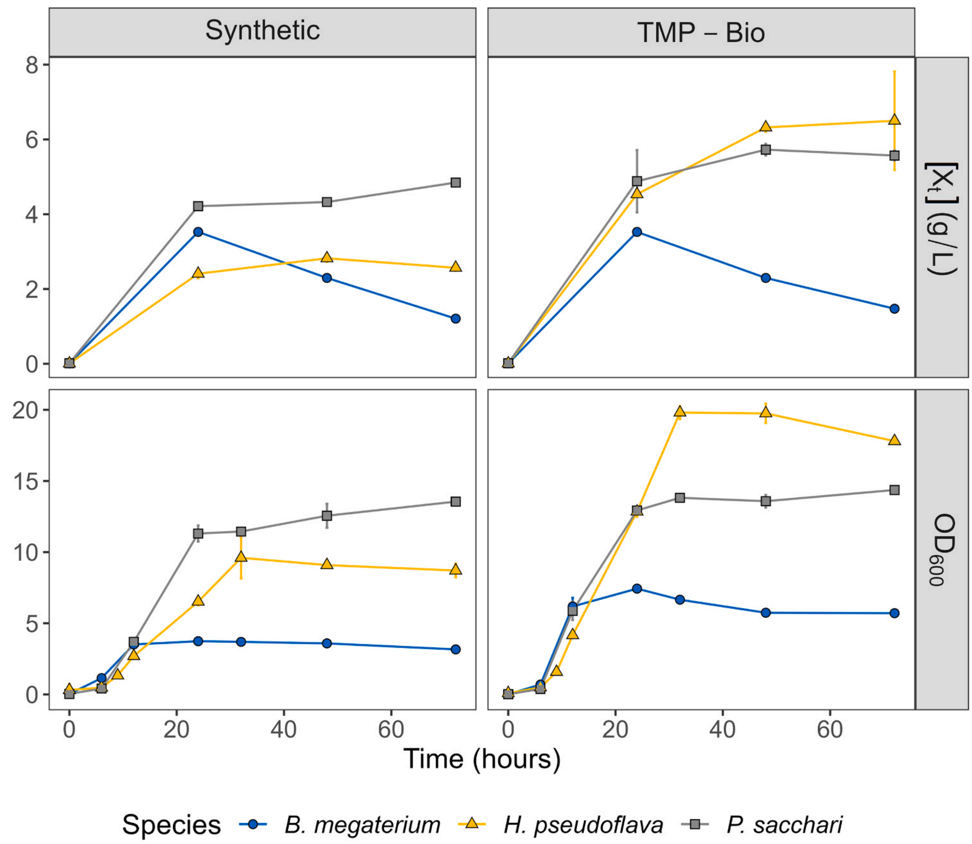

Fig. 1. Biomass production represented as OD_{600} (bottom panels) and CDM (top panels) for combined growth and PHA synthesis of three candidate strains grown on synthetic mixtures of glucose and xylose (left panels) and TMP-Bio sugars (right panels). Error bars represent standard deviations between biological triplicate flasks.

glucose uptake rates as *P. sacchari* (Table 1), and maintained the highest rate of xylose utilization after 24 h of any condition.

B. megaterium cultures did not reach NH_4-limiting conditions when grown on the synthetic sugar mixture as the concentration of residual NH_4-N remained stable around 70 mg/L after 24 h. As might be expected owing to the relatively poor growth observed in these cultures, neither sugar was completely utilized. Xylose did not appear to be consumed until after 12 h (corresponding to glucose concentrations of < 8 g/L) but then the two sugars were simultaneously consumed until 24 h, after which there was little uptake of either sugar. When *B. megaterium* was grown on the TMP-Bio sugars glucose was depleted to less than 1 g/L by 72 h, but xylose did not appear to be consumed to any significant extent.

PHA biosynthesis

Intracellular PHA content

Biomass samples were taken for PHA analysis at 24 h, 48 h, and 72 h, and a summary of the PHA content, $[X_{PHA}]$ and $[X_r]$ is shown in Fig. 3. The intracellular PHA content is shown in Fig. 3A. For each strain, the majority of the PHA was synthesized within 24 h. *B. megaterium* accumulated a maximum of 25.5 (± 2.8) % PHA within the first 24 h when grown on TMP-Bio sugars, and only 16.7 (± 0.2) % PHA at 24 h when the carbon source was the synthetic sugar mixture. The PHA content (along with $[X_t]$) steadily declined after 24 h, until a final value of 3 % was measured ($[X_t]$ = 1.47 (± 0.07) g/L) despite the presence of excess sugars. Using *B. megaterium*, the maximum $[X_{PHA}]$ was obtained at 24 h as 0.24 (± 0.11) g/L and 0.90 (± 0.11) g/L when grown on the synthetic mixture and TMP-Bio sugars, respectively.

When *H. pseudoflava* was grown on the synthetic sugar mixture, the biomass contained relatively high PHA content, reaching 51.2 (± 5.7) % at 24 h and 55.5 (± 2.0) % by 72 h. The maximum $[X_{PHA}]$ was 1.63 (± 0.22) g/L observed at 24 h. When grown on the TMP-Bio sugars, this strain accumulated up to 84.0 (± 3.1) % PHA at 72 h, resulting in a $[X_{PHA}]$ of 5.44 (± 0.91) g/L. These values are significantly higher than those obtained from pure sugars in a previous study [15]. Koller et al. [16] have suggested PHA synthesis is growth-associated in *H. pseudoflava*. The timing of NH_4 depletion between 12 and 24 h, compared to the relatively high intracellular PHA content observed at 24 h (51–65 %), corroborates this. However, it cannot be ruled out that in shake flask experiments with higher $[X_t]$, oxygen limitation may have driven reductive synthesis of PHA from glucose as a way to regenerate reduced electron carriers, which has been previously shown in *Azotobacter* spp. [17].

When grown on the synthetic glucose-xylose mixture, *P. sacchari* reached its maximum PHA content of 72.8 (± 1.1) % at 24 h, and this was followed by a slight reduction in PHA content thereafter under NH_4-limitation, but with excess xylose still present. When grown on TMP sugars, the PHA content reached 50 (± 2) % within the first 24 h, and then slowly increased to a final value of 56.9 (± 2.4) % by 72 h also under NH_4 limitation but with excess xylose, as well as other possible carbon sources contained within the TMP-Bio sugars. The corresponding maximum $[X_{PHA}]$ values were 3.07 (± 0.09) g/L at 24 h and 3.17 (± 0.21) g/L at 72 h when grown on the synthetic mixture and TMP-Bio sugars, respectively.

Productivities, rates, and yields

Table 2 shows the maximum volumetric and specific PHA productivities (represented by Q_v and Q_s, respectively) and also summarizes key performance data (intracellular PHA content, $[X_t]$, and $[X_{PHA}]$) in tabular format to complement Fig. 3. The Q_v was generally highest at 24 h for *P. sacchari* cultures (99–128 mg/(L h)), depending on the carbon source), although *H. pseudoflava* compared favorably when grown on TMP-Bio sugars (122 mg/(L h) at 72 h). Using either carbon source,

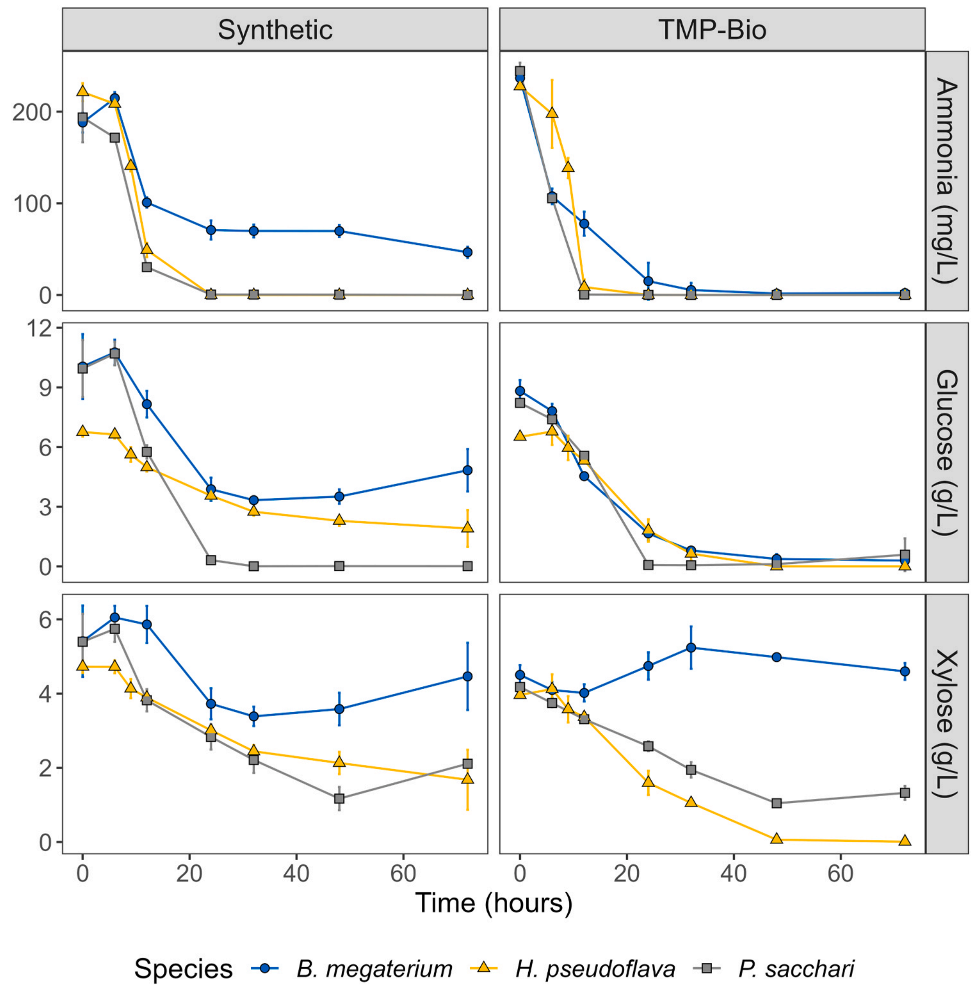

Fig. 2. Residual concentrations of ammonium (top panels), glucose (middle panels), and xylose (bottom panels) in flask cultures of three candidate strains grown on synthetic mixtures of glucose and xylose (left panels) and TMP-Bio sugars (right panels). Error bars represent standard deviations between biological triplicate flasks.

Table 1
Summary of glucose and xylose uptake rates over time (mg of sugar per g of X_r per hour) for three candidate strains grown on both a synthetic glucose-xylose mixture as well as TMP-Bio sugars. Errors represent standard deviations between biological triplicates.

	Glucose			Xylose		
	0–24 h	24–48 h	48–72 h	0–24 h	24–48 h	48–72 h
Synthetic sugar mixture						
B. megaterium	352	8	-	62	-	-
H. pseudoflava	203	69	14	108	38	16
P. sacchari	716	-	-	237	29	-
TMP Sugars						
B. megaterium	225	231 8	166	-	-	-
H. pseudoflava	233	161	92	114	46	2
P. sacchari	256	25	1	96	4	7

the Q_v for *B. megaterium* remained less than 40 mg/(L h), and the highest values were noticed in the first 24 h of growth. This suggests there may be limited return in prolonging the fermentation past 24 h, where generally only xylose remained.

The $Y_{PHA/S}$ values generally reached a maximum at 24 h and declined thereafter. Yields for *B. megaterium* reached a maximum of 0.13 g/g at 24 h on the TMP-Bio sugars. *P. sacchari* achieved yields of 0.23 (± 0.01) g/g and 0.27 (± 0.02) g/g at 24 h when grown on synthetic sugars and TMP-Bio sugars, respectively, which is similar to previous reports [18]. *H. pseudoflava* achieved a conversion yield of 0.28 (± 0.04) g/g when grown on synthetic sugar mixtures, but the yield achieved on TMP-Bio sugars was nearly double this, reaching a maximum value of 0.45 (± 0.03) g/g between 48 and 72 h. This is nearly the maximum theoretical yield of PHB from glucose of 0.48 g/g [19] and is further evidence that *H. pseudoflava* may have a particular affinity for other carbon sources present in the TMP-Bio sugar (but not measured in this work) for either growth, PHA synthesis, or both.

Copolymer production

The addition of 20 mM (1.48 g/L) propionic acid significantly slowed the growth of *P. sacchari*, and completely inhibited both *B. megaterium* and *H. pseudoflava*. During a 48-h cultivation, *P. sacchari* rapidly consumed propionate, with only 0.28 ± 0.13 g/L remaining at 24 h, and this was depleted below detectable limits by 48 h (Table 3;

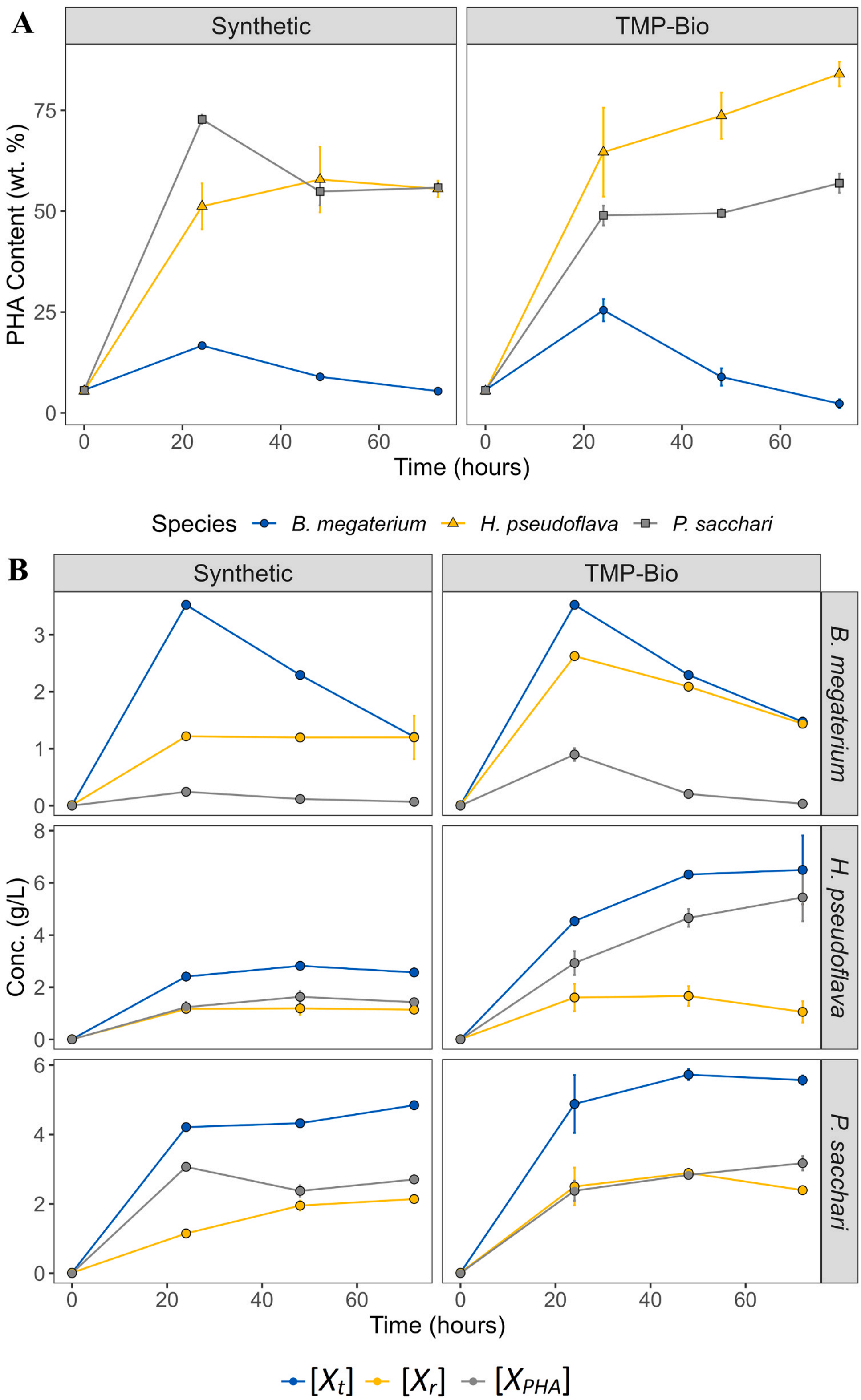

Fig. 3. Intracellular PHA content and titers of the different biomass components over time for each species grown on either a synthetic glucose-xylose mixture (left panels) or TMP-Bio sugars (right panels). **A)** $[X_{PHA}]$ and $[X_r]$ for each of the three candidate strains with $[X_t]$ overlaid for ease of interpretation. **B)** Intracellular PHA content over the time course of the fermentation process for each of the three candidate strains. Error bars represent standard deviations between biological triplicate flasks.

Suppl. Fig. S2). A copolymer was produced containing up to 5.8 mol% 3-HV, as determined by GC-FID. A previous study obtained copolymers with 28.4 and 65 mol% 3-HV subunits from 5 g/L glucose supplemented with either 2 g/L propionate or 1 g/L valerate, respectively [20]. In that work, the $Y_{3\text{-}HV/Pro}$ was only 0.06 (± 0.06) g/g, similarly to the $Y_{3\text{-}HV/Pro}$ values obtained in the present study (Table 3). This is not unexpected since *P. sacchari* is known to convert propionic acid to 3-HV with a yield of about 10% of the theoretical maximum (1.35 g/g) – similar to that of *Cupriavidus necator* [21]. This certainly could indicate propionyl-CoA was predominantly catabolized to build non-PHA cell mass, respiration, or maintenance [22]. Several studies have looked at genetic engineering to enhance the yield of 3-HV monomer subunits from propionate in *Burkholderia* spp. [23–25].

H. pseudoflava was severely inhibited by propionic acid at

Table 2
Comparing PHA biosynthesis characteristics of three candidate strains grown on both TMP-Bio sugars and a synthetic sugar mixture. Errors represent standard deviations between biological triplicates.

Strain	Carbon substrate	$[X_t]$ (g/L)	PHA content (%)	$[X_{PHA}]$ (g/L)	Q_v (mg_{PHA}/(L h))	Q_s (mg_{PHA}/(g_{Xr} h))	$Y_{PHA/S}$ (g/g)
B. megaterium	Synthetic mix	1.46 ± 0.07 (24 h)	16.7 ± 0.2 (24 h)	0.24 ± 0.01 (24 h)	10 ± 1 (24 h)	8 ± 0 (24 h)	0.04 ± 0.01 (24 h)
	TMP-Bio sugars	3.53 ± 0.05 (24 h)	25.5 ± 2.8 (24 h)	0.24 ± 0.01 (24 h)	37 ± 5 (24 h)	14 ± 2 (24 h)	0.13 ± 0.02 (24 h)
H. pseudoflava	Synthetic mix	2.82 ± 0.09 (48 h)	57.9 ± 8.1 (48 h)	1.63 ± 0.22 (24 h)	52 ± 8 (24 h)	44 ± 10 (24 h)	0.28 ± 0.04 (24 h)
	TMP-Bio sugars	6.32 ± 0.12 (48 h)	84.0 ± 3.1 (72 h)	5.44 ± 0.91 (72 h)	122 ± 19 (72 h)	82 ± 39 (24 h)	0.52 ± 0.09 (72 h)
P. sacchari	Synthetic mix	4.33 ± 0.04 (48 h)	72.8 ± 1.1 (24 h)	3.07 ± 0.09 (24 h)	128 ± 4 (24 h)	112 ± 7 (24 h)	0.23 ± 0.01 (24 h)
	TMP-Bio sugars	5.73 ± 0.16 (48 h)	56.9 ± 2.4 (72 h)	3.17 ± 0.21 (72 h)	99 ± 1 (24 h)	40 ± 4 (24 h)	0.27 ± 0.02 (24 h)

Table 3
Comparing PHBV biosynthesis characteristics of three candidate strains grown on both TMP-Bio sugars using different strategies for supplementing propionic acid, depending on strain tolerance. Errors represent standard deviations between biological triplicates.

Strain	Strategy	$[X_t]$ g/L	PHA content % CDM	3-HV content Avg mol% (max)	$Y_{3\text{-}HV/Pro}$ g/g	^{1}H NMR result mol%
P. sacchari	20 mM (batch)	3.80 ± 0.57	47.9 ± 4.3	4.9 ± 0.9 (5.8)	0.05 ± 0.01	7.5
	2 × 5 mM (pulse)	4.86 ± 0.44	62.1 ± 1.8	2.5 ± 0.0 (2.6)	0.09 ± 0.01	-
B. megaterium	5 mM (batch)	1.66 ± 0.19	16.7 ± 7.3	0.3 ± 0.1 (0.4)	< 0.01	-
	2 × 5 mM (pulse)	1.61 ± 0.12	15.5 ± 8.0	3.0 ± 0.2 (3.1)	0.03 ± 0.01	-
H. pseudoflava	5 mM (batch)	4.81 ± 0.18	63.1 ± 0.9	4.5 ± 0.3 (4.8)	0.32 ± 0.00	7.4
	2 × 5 mM (pulse)	4.57 ± 0.04	69.5 ± 6.0	7.4 ± 0.1 (7.5)	0.26 ± 0.00	11.5

concentrations of 10 mM (0.74 g/L) or higher, so the maximum concentration added to the medium was reduced to 5 mM (0.37 g/L), added either once at 0 h (batch strategy) or twice at 0 and 24 h (pulse strategy). Propionate was not detectable 24 h after its addition (Suppl. Fig. S3). The total biomass and intracellular PHA content were slightly lower than those obtained when TMP-Bio sugars were used as the carbon source without propionate addition (Table 3). The polymer resulting from the batch and pulse strategies contained a maximum of 4.8 and 7.5 mol% 3-HV subunits, respectively.

Thus, although easily inhibited by propionic acid, *H. pseudoflava* readily converted it to 3-HV monomer subunits with a higher yield ($Y_{3\text{-}HV/Pro}$ = 0.26 ± 0.00 g/g) than *P. sacchari* (Table 3). Others [15] suggested inhibition of both growth and polymer formation in *H. pseudoflava* by propionate at 1 g/L (13.5 mM) with complete inhibition at 3 g/L (about 40 mM). In that work, a copolymer with 45 mol% 3-HV subunits was produced from 10 g/L glucose in MSM supplemented with 2 g/L propionic acid. It was also reported [26] that *H. pseudoflava* could produce PHBV copolymers with 2.6–3.0 mol% 3-HV monomers, and even terpolymers containing up to 5.5 mol% 3-HV and 8.3 mol% 4-(hydroxybutyrate) subunits from structurally unrelated carbon sources – including sucrose and lactose. In the present study, the synthesis of polymer subunits other than 3-HB was not detected in the absence of propionate precursor, whether synthetic glucose-xylose mixture or the TMP-Bio sugars was used as carbon source.

Propionic acid was also severely inhibitory to *B. megaterium* at concentrations of 10 mM (0.74 g/L) or higher so the pulse feed strategy was also applied. The acid was poorly consumed by *B. megaterium*, as evidenced by the nearly 0.3 g/L (4 mM) remaining at 24 h, and the 0.52 g/L (7 mM) remaining at 48 h (Suppl. Fig. S4) following one or two additions of 0.37 g/L (5 mM) propionate, respectively. Biomass and PHA accumulation (Table 3) were similar to that observed when using TMP-Bio sugars as the C-source. The resultant polymer exhibited only small putative peaks for 3-HV that were frequently at or below the lower limit of quantification. This may be attributable to the class IV PHA synthase of *B. megaterium*, whereas both *H. pseudoflava* and *P. sacchari* have a class I PHA synthase [27,28]. Previous reports have shown that *B. megaterium* is capable of co-polymerizing 3-HV monomer subunits [29,30] and one study even reported production of PHBV from glucose as the sole carbon source in *B. megaterium* OU303A [31].

Previous work with either *P. sacchari* or *H. pseudoflava* used higher precursor-to-sugar ratios and obtained polymers with higher 3-HV contents [15,20]. Given that the propionic acid supplied to the cultures in this work was readily consumed, copolymers with higher 3-HV contents may have been produced earlier in the cultivation process. Moving forward, it will be important to carefully design a cultivation or feeding strategy that respects the tolerable limits of each strain, while also ensuring constant availability of the precursor molecules. This will in turn ensure that 3-HV subunits are randomly distributed in the co-polymer and not present as a less-desirable block copolymer, which could alter the polymer homogeneity [32].

Evaluation of polymer properties

Extraction and ^{1}H NMR analysis

During Soxhlet extraction of the dried biomass, it was noticed that the mass balance between the expected yield of PHA (determined from the intracellular PHA content measured by GC-FID) and the recovered mass of polymer post-extraction varied significantly depending on the strain. For example, dried biomass from *P. sacchari* consistently produced yields of 89.7 (± 3.3) % with respect to the polymer content detected in the GC-FID analysis. However, both *H. pseudoflava* and *B. megaterium* showed poor recoveries during chloroform extraction, with values of 29.0 ± 10.5 % and 35.4 ± 11.4 %, respectively. The latter value is similar to previous chloroform extraction results reported for the *Bacillus* genus [29]. Parameters including solvent or reagent, temperature, extraction duration, and physical pretreatment, amongst others, have been reported to affect the extractability of PHA from cells [33]. Less attention has been dedicated to the impact of the nature of the strains on the extraction yields. The different extraction yields obtained herein using the same conditions suggest a different accessibility of the solvent to the polymer granules in the three strains investigated. While further study is required to better understand the different accessibility to solvent, this may be due to: 1) strain-dependent differences in the characteristics of the non-PHA cell mass, specifically the thickness of the protein envelope surrounding the PHA granule, which is known to regulate the surface-area-to volume ratio of the granules themselves [34]; 2) different fragility associated with the cell envelope of different strains [33]; and 3) the amount and nature (molecular weight, crystallinity) of the polymer within the cells [35].

When sufficient quantities were obtained following extraction and purification, the isolated polymers were first examined by ^{1}H NMR. The resulting spectra showed high purity, comparable to that of the purchased standards (Suppl. Figs. S5–S8).

Polymer molecular weights

As shown in Table 4, the M_w of the polymers synthesized using *P. sacchari* ranged from 295.2 to 516.7 kDa, whereas polymers synthesized using *H. pseudoflava* ranged from 368.2 to 1270 kDa. These values are generally in the range of previous reports of PHA produced using sugar-based carbon sources summarized in Suppl. Table S1 [14,15,26,36,37], although others [38] reported higher molecular weights for *P. sacchari* grown on glucose or glucose-xylose mixtures. All polymers had PDI values in the range 1.19–1.45, indicative of fairly homogeneous molecular weights. Polymers synthesized using TMP-Bio sugars had higher M_w compared to the polymers synthesized from glucose-xylose mixtures. The addition of propionic acid increased the M_w of the PHA synthesized by *P. sacchari*. This was also true with *H. pseudoflava* for a single addition of 5 mM propionic acid, but further propionic acid addition (i.e., via the pulse strategy in *H. pseudoflava*) lowered the M_w and increased the polydispersity slightly. Previous reports have obtained M_w values ranging from 29 to 630 kDa for *B. megaterium* grown on sugar substrates [31,39–41], but in this work, the purified PHA from *B. megaterium* gave a broad refractive index peak distribution in the GPC analysis, such that it was difficult to assign an apex to the peak – causing unreliable interpretation of the chromatograms (Suppl. Figs. S9–S11). This could be the result of a very broad range of chain-lengths within the polymer, but may also be a function of the poor growth or extraction efficacy observed in this organism in the conditions tested.

Polymer thermal properties

The thermal properties obtained from DSC and TGA analyses for the various polymers are summarized in Table 5, and the corresponding thermograms are available as Supplementary information (Suppl. Figs. S12–S17). PHB homopolymers synthesized by either *P. sacchari* or *H. pseudoflava* showed similar melt behavior. The onset of the melt endotherm occurred at 164–167 °C, with the peak occurring between 171 and 174 °C. The PHB homopolymers synthesized by *B. megaterium* showed lower melting temperature, with the onset of the melt endotherm at 147–156 °C and the peak of the melt endotherm occurring at 162–163 °C. In all cases, PHB synthesized from TMP-Bio sugars had lower T_m than the corresponding polymers synthesized from synthetic glucose-xylose mixtures. The glass transition temperature values for PHB homopolymers varied from − 5.3 to 5.4 °C, and the effect of the carbon source on T_g was species-dependent. Similar X_c values were obtained on PHB synthesized from both *P. sacchari* and *H. pseudoflava* (57–60 %), but somewhat lower in *B. megaterium* (52–54 %). The X_c of the PHB homopolymers showed only slight differences in response to the carbon source. Overall, the thermal behavior and crystallinity for PHB homopolymers are within the range of values previously reported for these species when grown predominantly on sugar carbon sources (Suppl. Table S2) [14,15,31,36,38,39,41–44].

As shown in Suppl. Table S2, previous reports showed that increasing the 3-HV content of the polymer up to 94–95 mol% lowered the T_m from 177 °C to about 104 °C [38] and an even larger difference was noticed with the incorporation of 3-HHx monomers [37]. In this work, incorporation of 3-HV content up to 7.5 mol% did not have a significant impact on the melt endotherm, but did reduce its onset as well as ΔH_m, T_c, ΔH_c, T_g, and X_c, making the polymers more easily processable. During the study of PHBV copolymers synthesized using *Burkholderia cepacia* ATCC 17759, others [45] observed a maximum decrease in T_m at 20 mol% 3-HV (175.4–168.5 °C), with a subsequent increase at higher 3-HV contents. This study suggested that the relatively high T_d, low T_m, and more rapid crystallization of a copolymer containing 20 mol% 3-HV subunits may be ideal for injection molding.

Analysis by TGA showed that the decomposition temperatures of the PHB homopolymers and PHBV copolymers synthesized by either *P. sacchari* or *H. pseudoflava* were generally in the same range, at 293–299 °C, regardless of the presence of 3-HV monomers. In contrast, polymers synthesised by *B. megaterium* showed lower degradation temperatures (251–252 °C). The thermal stability of PHAs synthesized in the present study using either *P. sacchari* or *H. pseudoflava* compares favorably to previously reported data where T_d values ranging from 248 to 288 °C were measured for a variety of species [14,42,46].

In summary, *P. sacchari* and *H. pseudoflava* favored the production of thermally stable PHAs and the use of TMP-Bio sugars and propionate instead of synthetic sugars led to slightly lower melting temperatures. The obtained higher decomposition temperature and lower melting temperature are two desirable behaviors for plastic processing, which are expected to be even accentuated at higher incorporation of 3-HV.

Conclusions

This study has investigated the ability of three commercially available strains (*P. sacchari*, *B. megaterium* and *H. pseudoflava*) to valorize PHA biopolymers from hardwood hydrolysate produced by FPInnovations' TMP-Bio process. Although *P. sacchari* is well known for its ability to utilize sugar mixtures in such applications, this work has demonstrated that *H. pseudoflava* is highly competitive for the bioconversion of mixed sugars from hardwood hydrolysate into PHA. In addition to simultaneous uptake of C_5 and C_6 sugars and complete sugar utilization in batch fermentation conditions, *H. pseudoflava* actually achieved higher intracellular PHA content and PHA titer than did *P. sacchari*. Both strains showed the ability to incorporate supplemented propionic acid into a copolymer containing 3-HV subunits. Although propionic acid was inhibitory to *H. pseudoflava* at relatively low concentrations, the yield of 3-HV subunits from propionate was three-folds higher in

Table 4
Comparison of molecular weights determined by GPC for polymers synthesized from synthetic glucose-xylose mixtures or TMP-Bio sugars using each of the *P. sacchari* and *H. pseudoflava* strains, as well as selected PHBV copolymers synthesized from TMP-Bio sugars supplemented with propionic acid.

Strain	*P. sacchari*			*H. pseudoflava*			
Carbon source	Synthetic glc-xyl mix	TMP-Bio sugars	TMP-Bio + Propionate	Synthetic glc-xyl mix	TMP-Bio sugars	TMP-Bio + Propionate	TMP-Bio + Propionate (Pulse)
3-HV mol%	0	0	5.8 %	0	0	4.3 %	7.4 %
***Mn* (kDa)**	238.6	265.5	420.0	277.0	520.2	1070.0	372.5
***Mw* (kDa)**	295.2	361.2	516.7	368.2	704.3	1268.0	539.0
PDI	1.24	1.36	1.23	1.33	1.35	1.19	1.45

Table 5
Thermal properties determined using TGA and DSC for polymers synthesized from synthetic glucose-xylose mixtures TMP-Bio sugars using each of the three candidate strains, as well as selected PHBV copolymers synthesized from TMP-Bio sugars supplemented with propionic acid.

Strain	*P. sacchari*			*B. megaterium*		*H. pseudoflava*			
Carbon source	Synth. Glc-Xyl mix	TMP-Bio sugars	TMP-Bio + Propionate	Synth. Glc-Xyl mix	TMP-Bio sugars	Synth. Glc-Xyl mix	TMP-Bio sugars	TMP-Bio + Propionate	TMP-Bio + Propionate (Pulse)
3-HV mol %	0	0	5.8 %	0	0	0	0	4.3 %	7.4 %
T_d (°C)	298.1	297.4	294.4	251.8	251.3	293.3	298.6	295.6	299
T_m (peak,° C)	174.58	172.52	169.41	163.67	162.24	174.01	171.25	170.06	171.74
T_m (onset, °C)	167.48	164.83	161.19	156.06	147.03	165.91	164.11	162.98	154.79
ΔH_m (J/g)	84.10	83.56	74.73	79.28	77.13	87.22	83.18	74.97	67.41
T_c (peak,° C)	73.63	70.44	71.22	63.78	60.25	65.72	82.79	73.89	65.1
T_c (onset, °C)	93.60	85.15	82.49	77.57	82.65	79.87	94.10	90.71	84.23
ΔH_c (J/g)	57.2	52.57	51.15	50.87	26.58	42.59	66	50.4	20.7
X_c (%)[a]	57.6	57.2	51.2	54.3	52.8	59.7	57.0	51.4	46.2
T_g (°C)	4.78	1.57	6.26	-1.82	-5.29	1.62	5.41	4.36	-0.3

[a] Calculated from measured ΔH_m using a melt enthalpy value of 146 J/g for 100 % crystalline PHB, as previously done in [14].

H. pseudoflava than in *P. sacchari*, suggesting a more efficient propionate-to-3-HV metabolism. Finally, polymers synthesized by *H. pseudoflava* had higher molecular weight compared to those synthesized by *P. sacchari* under similar conditions, and high thermal stability. Future work will aim to optimize cultivation strategies toward copolymers synthesis from TMP-Bio sugars with consistent and reproducible 3-HV contents.

CRediT authorship contribution statement

Warren Blunt: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Project administration, Writing - original draft. **Purnank Shah:** Conceptualization, Data curation, Writing – review & editing. **Vinicio Vasquez:** Resources. **Mengwei Ye:** Resources. **Christopher Doyle:** Resources. **Yali Liu:** Resources. **Sajjad Saeidlou:** Resources. **Fanny Monteil-Rivera:** Project administration, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nbt.2023.06.005.

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