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ORIGINAL PAPER

The Use of *Azohydromonas lata* DSM 1122 to Produce 4-hydroxyvalerate-Containing Polyhydroxyalkanoate Terpolymers, and Unique Polymer Blends from Mixed-Cultures with *Burkholderia sacchari* DSM 17165

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Abstract
Azohydromonas lata DSM 1122 was utilized to synthesize short-chain-length (*scl*-) polyhydroxyalkanoate (PHA) terpolymers containing 3-hydroxybutyric (3HB) acid, 3-hydroxyvaleric (3HV) acid, and 4-hydroxyvaleric (4HV) acid from mixtures of glucose (GLC; 1 wt%) and levulinic acid (LevA; 0–0.4 wt%). LevA media concentrations greater than 0.4% completely inhibited cellular growth. At LevA concentrations ≤ 0.4%, the 3HV polymer content remained constant (3–5 mol%). The 4HV content was two-fold higher in the polymers derived from the 0.2% LevA-containing cultures reaching a maximum of 9 mol% (vs. 4 mol% in the 0.4% LevA-containing cultures). Polymer molecular weights (based on number-average molecular weight, M_n) were smallest (M_n = 240,000 g/mol) when synthesized in the presence of 0.2% LevA. At 0.4% LevA and at 1% GLC the average M_n values were 43% and 87% larger than the polymers synthesized in the presence of 0.2% LevA, respectively. Mixed-cultures containing *A. lata* and *Burkholderia sacchari* DSM 17165, a known poly-3-hydroxybutyrate-*block*-3-hydroxyvalerate (P3HB-*block*-3HV) producer, using LevA media concentrations ≤ 0.4% and staggered inoculations resulted in *scl*-PHA polymer mixtures with improved tensile properties. The results of this study show that LevA can be utilized in combination with simple sugars to produce unique *scl*-PHA terpolyesters and *scl*-PHA mixtures with enhanced properties.

Keywords *Azohydromonas lata* · Levulinic acid · Terpolyesters · 4-Hydroxyvalerate · Mixed cultures

Introduction

Plastics are quickly becoming a major concern around the world due to problems associated with their disposal. Recycling is one approach for plastic disposal but it is estimated that only about 9% of all plastics end up being effectively reprocessed [1]. That means that greater than 90% of the world's plastic is disposed of by some other means. The sad fact is that much of these potentially reusable plastics end up in landfills and in the world's waterways causing immense pollution problems. In fact, projections indicate that approximately 8 million metric tons of plastic waste enters the oceans annually [2]. One needs to look no further than the so-called 'Great Pacific Garbage Patch' that is currently swirling in the Pacific Ocean to see the ecological consequences of indiscriminant plastic disposal.

There are three potential approaches to reduce the amount of plastic waste in the biosphere. The first is to reduce or eliminate the use of recalcitrant plastics, particularly single-use plastics. In fact, many cities and countries around the world have initiated steps to control the use of single-use plastic by either instituting taxes or levies on them or by outright banning their use [3]. The second approach is through the scientific discovery of unique enzymes capable of efficiently breaking down commonly-used plastics, particularly poly(ethylene terephthalate) (PET) which is the most abundant petroleum-based polymer used in single-use plastics. In 2016 it was reported that the bacterium *Ideonella sakaiensis* 201-F6 produces an enzyme (PETase) that catalyzes the breakdown of PET [4]. A subsequent study characterized the 3-dimensional structure of the PETase, engineered the enzyme for improved PET degradation, and revealed that the

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enzyme could also degrade the PET replacement polyethylene-2,5-furandicarboxylate [5]. Unfortunately, the large-scale use of enzymes in plastic degradation (particularly in aqueous/marine environments) is hindered because of their low rates of reaction, salt-tolerance- and dilution- issues. A third approach is through the use of 'enviromentally-benign' plastic substitutes. Many of these materials can be produced through living systems either by whole-cell or enzymatic catalysis and are generally considered biocompatible, biodegradable, and biorenewable. The problem with this approach is that historically bio-based plastics have been prohibitively more expensive to produce than petroleum-based plastics, but faced with the choice of higher price vs. complete elimination of potential applications, bio-based plastic alternatives may be a favorable option provided their properties can be controlled to mimic those of petroleum-based plastics.

Polyhydroxyalkanoates (PHA) are one class of possible ecofriendly plastic substitutes. They are bacterial polyesters that are produced as carbon and energy reserve materials by a variety of bacterial species. These biopolymers are known to biodegrade to carbon dioxide and water in both terrestrial and aqueous/marine environments [6] and depending on the metabolic capabilities of the producing bacterial strain, they can be synthesized with either thermoplastic (short-chain-length PHA; *scl*-PHA) or elastomeric (medium-chain-length PHA; *mcl*-PHA) properties [7].

Typically, PHA synthesis occurs when surplus utilizable carbon is available but cellular metabolism is stunted by the lack of other vital nutrients. *Mcl*-PHA is typically synthesized by members of the genus *Pseudomonas* and is generally composed of 3-hydroxyalkanoic (3HA) acids ranging in length from 6 (3-hydroxyhexanoate; 3HHx) to 14 (3-hydroxytetradecanoate; 3HTD) carbons and whose side chains may be unsaturated and/or substituted with a host of chemical groups including but not limited to methyl, short-chain esters, phenoxy, cyanophenoxy, nitrophenoxy, epoxy among others [8]. Unfortunately, the elastomeric qualities in *mcl*-PHA come at a cost as the tensile strength and modulus are normally reduced in these polymers which can be problematic depending on the intended application. In contrast, *scl*-PHA is generally composed of 3HA acids with chain lengths ranging from 3 (3-hydroxypropionate; 3HP) to 5 (3-hydroxyvalerate; 3HV) carbons. Poly-3-hydroxybutyrate (P3HB) is the most well-known of the *scl*-PHA polymers but is stiff, brittle and challenging to process making it a less favorable option for widespread use. In order to improve the properties of *scl*-PHA, copolymers composed of 3-hydroxybutyric (3HB) acid, and 3-hydroxyvaleric (3HV) acid [9], 4-hydroxybutyric (4HB) acid [10] or 4-hydroxyvaleric (4HV) acid [11, 12] have been synthesized. These copolymers have markedly improved mechanical properties over P3HB but the precursor carbon substrates for these syntheses are comparatively more expensive and the yields lower than for petroleum-based polymers, thus hindering the cost differential between petroleum-based plastics and PHA plastics.

In order to help the economics of PHA biosynthesis, less expensive feedstocks have been the focus over the past few years. Carbon sources such as soy molasses [13], wheat hydrolysate [14–16] and crude glycerine [17–19] have all been tested as primary feedstocks for both *mcl*- and *scl*-PHA production, but in those instances where *scl*-PHA was the goal, a viable secondary carbon source was required to produce *scl*-PHA copolymers.

Levulinic acid (LevA; 4-oxovaleric acid) is an inexpensive molecule. In 2000, Bozell et al. predicted that the production costs of LevA could go as low as between $0.04 and $0.10/lb [20] depending on the scale of the operation. LevA can be produced by acid-catalysis from both 5- and 6-carbon sugars [21] and since it is a structural analogue to pentanoic acid and can be easily synthesized from lignocellulosic biomass, it has been assessed and successfully applied as a supplemental substrate in PHA biosynthesis. In 2011, Jaremko and Yu suggested a metabolic pathway for LevA in *Cupriavidus necator* [22]. This pathway demonstrated the possible enzymatic reactions responsible for the production of 3-hydroxybutyryl-CoA (3HB-CoA) and 3-hydroxyvaleryl-CoA (3HV-CoA), precursors for *scl*-PHA polymers containing 3HB and 3HV monomers, respectively. Evidence from previous studies has suggested that certain bacterial strains can, in fact, only synthesize 3HB and 3HV monomers from LevA. In one report, Keenan et al. reported that *Burkholderia cepacia* ATCC 17,759 could only synthesize P3HB-*co*-3HV when grown on xylose and LevA [23], while in a second study, Koller et al. reported that the same thing occurred when *Hydrogenophaga pseudoflava* DSM 1034 was grown on mixtures of whey permeate and LevA [24]. Interestingly, other bacterial strains have been reported to synthesize *scl*-PHA terpolyesters containing 3HB, 3HV, and 4HV. Valentin et al. reported the synthesis of *scl*-PHA terpolyesters in various strains of *Alcaligenes eutrophus, Alcaligenes xylosoxidans* ssp. *denitrificans*, and *Pseudomonas oxalaticus* when grown on 4-hydroxyvaleric acid or 4-valerolactone [11]. In that report, the 4HV content within the terpolyesters never exceeded 6.3 mol% which indicated that even when grown on closely related carbon substrates, these bacterial strains could only assimilate a small portion into the elongating polymer chains. The same phenomenon was reported by Gorenflo et al. for wildtype *Ralstonia eutropha* HF 39 (4HV content of 2.1 mol%) and *Pseudomonas putida* KT2440 (4HV content of 0.8 mol%). Recombinant strains of these same organisms gave increased 4HV polymer contents to as high as 30 mol% in recombinant *P. putida* [12]. Unfortunately, the metabolic pathway postulated by Jaremko and Ju [22] did not account for the synthesis of 4-hydroxyvaleryl-CoA (4HV-CoA), the precursor for 4HV production. A subsequent paper by Rand et al. did

account for the formation of 4HV-CoA from the metabolism of LevA. In that paper it was demonstrated that in *P. putida* KT2440 the *lvaRABCDEFG* operon (9,323 bp) was responsible for the formation of both 3HV-CoA and 4HV-CoA intermediates making possible the synthesis of 3HV- and 4HV-containing *scl*-PHA polymers [25].

In this paper we demonstrated that under suitable growth conditions *Azohydromonas lata* DSM 1122 can synthesize terpolymers of 3HB, 3HV, and 4HV from mixtures of glucose and LevA. *A. lata*, when used in mixed-cultures with *Burkholderia sacchari* DSM 17165 (a known producer of poly-3-hydroxybutyrate-*block*-3-hydroxyvalerate (P3HB-*block*-3HV) from mixtures of glucose, xylose, and LevA, [26]), could produce *scl*-PHA polymer mixtures with enhanced tensile properties. The use of inexpensive carbon feedstocks like LevA to produce unique *scl*-PHA copolymers with enhanced mechanical properties may make these environmentally friendly alternatives more appealing for use as substitutes for petroleum-based plastics and reduce the amount of plastic waste present in the environment.

Materials and Methods

Materials

Azohydromonas lata (*A. lata*; formerly classified as *Alcaligenes latus*) DSM 1122 *and Burkholderia sacchari* (*B. sacchari*) DSM 17165 were obtained from the Leibniz-Institut DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Stock cultures of both bacterial strains were prepared as glycerol stocks as described previously [27]. All simple salts were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Mallinckrodt Baker Inc. (Phillipsburg, NJ). D-(+)-glucose (≥ 99.5%), D-(+)-xylose (≥ 99%), and chloroform-*d* (99.8 atom %, containing 0.03% (*v*/*v*) tetramethylsilane; TMS) were purchased from Sigma–Aldrich Chemical Company. Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson (Sparks, MD). Levulinic acid (LevA; 98+%) was purchased from Acros Organics (Geel, Belgium). All organic solvents were HPLC grade and purchased from Honeywell Burdick and Jackson (Muskegon, MI) or Fisher Scientific (Fair Lawn, NJ).

Bacterial Cultivation Conditions

Azohydromonas lata Pure-Culture Experiments

All PHA-production experiments were conducted in triplicate in shake-flask cultures (1-L volumes contained in 2-L Erlenmeyer flasks) using simple salts media. The basal salts media was comprised of the following in g/L: Na_2HPO_4, 3.7; KH_2PO_4, 1.7; $(NH_4)_2SO_4$, 1.1; $MgSO_4 \times 7\ H_2O$, 0.2; ferric ammonium citrate, 0.07; $CaCl_2 \times 2\ H_2O$, 0.01 and 1.1 mL of microelement solution consisting of the following in 1 L of 0.1 N HCl: H_3BO_3, 0.3 g; $CoCl_2$, 0.1 g, $ZnSO_4 \times 7\ H_2O$, 0.2 g, $MnCl_2 \times 4\ H_2O$, 0.03 g; $Na_2MoO_4 \times 2\ H_2O$, 0.4 g; $NiCl_2 \times 6\ H_2O$, 0.02 g, $CuSO_4 \times 5\ H_2O$, 0.01 g. Simple salts basal media was initially prepared in 4-L volumes and 950 mL of this media was transferred into four separate 2-L Erlenmeyer flasks containing either 0, 2 g, 4 g, or 6 g of LevA. The pH of each production flask was adjusted to between 6.9 and 7.0 using sodium hydroxide pellets and each flask was autoclaved at 121 °C for 15 min to sterilize. Once each flask had cooled to room temperature, 50 mL of a 20% filter sterile stock solution of glucose (GLC) was aseptically added to obtain 1 L final media volumes with 0, 0.2, 0.4, or 0.6 wt% LevA contents.

The inoculum for each production flask (described above) was prepared by aseptically transferring 1.5 mL of *A. lata* DSM 1122 from a frozen glycerol stock culture into 50 mL of sterile Luria–Bertani (LB; 1% tryptone, 0.5% NaCl, 0.5% yeast extract) broth (in a 125-mL Erlenmeyer flask) and incubating the culture at 30 °C and 200 rpm in a shake incubator. After 24 h, the entire 50-mL volume was aseptically poured into a new sterile 200-mL LB broth culture (total 250 mL; in a 500-mL Erlenmeyer flask) and incubated as described above. After an additional 24 h, four separate 40-mL aliquots were removed from the LB culture and aseptically placed into separate sterile centrifuge tubes. The tubes were centrifuged (10,400 × *g*; 15 min; 4 °C) and the supernatants were discarded. Each cell pellet was aseptically suspended in 10 mL of sterile medium from one of the production flasks and each suspension was used as the inoculum for the respective 1-L production flasks. The production flasks were placed into the shake incubator at 30 °C and 200 rpm. 100-mL aliquots were aseptically harvested from each flask at 24 h intervals (up to 96 h) by centrifugation (conditions described above).

Azohydromonas lata/*Burkholderia sacchari* Mixed-Culture Experiments

The mixed-culture experiments were also conducted in triplicate using the same basal production media and culture conditions as described in the previous section with minor exceptions. It was previously reported that *B. sacchari* DSM 17165 was capable of utilizing xylose as a co-substrate for *scl*-PHA biosynthesis [26]. As such, the mixed-culture experiments were conducted utilizing a carbon source ratio of 1 wt% GLC:1 wt% xylose (XYL):0-0.4 wt% LevA. The basal simple salts medium was identically prepared in 2 separate 6-L volumes as described in the previous section and 900 mL of that bulk media was portioned into 12 separate

2-L Erlenmeyer flasks containing 0 (4 flasks), 2 g (4 flasks), or 4 g (4 flasks) of LevA (total 12 production flasks). The pH in each flask was adjusted to between 6.9 and 7.0 as previously described. Once sterilized and cooled, 50 mL of 20% filter-sterile stock solutions of GLC and XYL were aseptically added to each production flask to obtain 1-L final media volumes with either 0, 0.2, or 0.4 wt% LevA contents.

Staggered inoculation/growth patterns were used to induce variation in the recovered *scl*-PHA blends. All production cultures were grown for 72 h. *A. lata* was inoculated at the onset of each shake-flask culture and *B. sacchari* was inoculated at 24 h intervals resulting in *A. lata:B. sacchari* time/growth ratios of 72 h:72 h, 72 h:48 h, 72 h:24 h, and 72 h: 0 h (Table 1). The inocula for each production flask were prepared by initially transferring 1.5 mL of *A. lata* and 1.5 mL of *B. sacchari* from frozen cryovials into separate 125-mL Erlenmeyer flasks containing 50 mL of LB broth with incubation as described previously. At 24 h, all 50 mL from each overnight LB culture was aseptically poured into separate 1-L Erlenmeyer flasks containing 500 mL of fresh LB media (total volume = 550 mL) and the newly-inoculated flasks were incubated under the same conditions. After another 24 h, 12 separate 40 mL aliquots of overnight *A. lata* cells and 3 separate 40 mL aliquots of overnight *B. sacchari* cells were aseptically transferred to separate sterile centrifuge tubes and centrifuged (10,400 × *g*; 15 min; 4 °C). The supernatants were discarded and the pelleted cells were suspended in production media (*A. lata* cells were separately suspended in 10 mL of production media from all 12 production flasks while the *B. sacchari* cells (3 tubes) were suspended in 10 mL of production media from Set 1 (72 h:72 h time/growth ratio) of the production flasks including 0, 0.2, and 0.4 wt% LevA). The cells were then reintroduced back into the respective PHA production flasks and incubated as described previously. At the same time, 50 mL of overnight *B. sacchari* LB culture was aseptically added to a new 1-L Erlenmeyer flask containing 500 mL of fresh LB broth and incubated at 30 °C, 200 rpm. After an additional 24 h, the same procedure was carried out from the overnight culture and *B. sacchari* was inoculated into the second set (72 h:48 h time/growth ratio) of production flasks. This procedure was performed once more and the *B. sacchari* cells were inoculated into the third set (72 h:24 h time/growth ratio) of production flasks. The fourth set of production flasks (72 h:0 h time/growth ratio) were not inoculated with *B. sacchari*. At 72 h the entire 1-L volumes were harvested by centrifugation as described previously. At the conclusion of all the shake-flask experiments the cultures were processed to determine cell dry weight CDW), polymer concentration, carbon source utilization, and polymer characterization.

Table 1 Inoculation/growth patterns for the *Azohydromonas lata* DSM 1122/ *Burkholderia sacchari* DSM 17165 mixed-culture experiments

Set #	Time/growth ratio[a] (h)	Carbon source ratio (wt%) (GLC:XYL:LevA)
1	72 h:72 h	1:1:0
		1:1:0.2
		1:1:0.4
2	72 h:48 h	1:1:0
		1:1:0.2
		1:1:0.4
3	72 h:24 h	1:1:0
		1:1:0.2
		1:1:0.4
4	72 h:0 h	1:1:0
		1:1:0.2
		1:1:0.4

[a]First number corresponds to the duration in hours of *A. lata* in the cultures and the second number corresponds to the duration of *B. sacchari* in the cultures

Carbon Source Utilization, Cell Growth, and Polymer Yield

Carbon source (GLC, XYL, LevA) utilization from both the pure-culture and mixed-culture experiments was determined by High Performance Liquid Chromatography (HPLC) of culture supernatants while cell growth and PHA concentrations were measured gravimetrically. Upon harvesting, cells were centrifuged as previously described and 3-mL volumes of the supernatants were removed and sequentially filtered through separate 0.45-µm and 0.22-µm nylon filters into separate, clean auto-sampler vials. An Agilent 1200 series HPLC instrument equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, CA) and a refractive index detector was used for all analyses. The column was maintained at 60 °C. A 5-µL injection volume was used and eluted with 5 mM sulfuric acid (prepared in HPLC-grade water) at a flow rate of 0.6 mL/min. Data were processed using the Agilent ChemStation software. The instrument was calibrated with GLC, XYL, and LevA standards. Peaks from the unknowns were identified by comparative retention times. Each sample (n = 3) was injected twice (total injections per growth condition = 6) and the results were averaged to obtain the final residual carbon source concentrations in each flask.

Dry cell weights were determined from the cell pellets derived from each shake-flask culture after centrifugation. In all cases, cells were washed twice with deionized water, re-centrifuged and the water fractions discarded. The cell pellets were frozen, lyophilized to dryness and accurately weighed. Polymer concentrations were determined from the chloroform-extracted cell masses. The lyophilized cells were

extracted for at least 24 h in excess chloroform at 30 °C and shaking at 250 rpm. Cellular material was removed by vacuum filtration through Whatman #2 filter paper. The chloroform was evaporated under vacuum to give the crude polymers. Each crude polymer was dissolved in a small volume of chloroform and precipitated (3×) by dropwise addition into cold methanol. The polymers were placed into separate tared vials, dried in vacuo for at least 24 h and weighed. Polymer concentration was determined by weight difference. Volumetric productivities for both cell growth (Q_x) and PHA concentration (Q_p) were calculated as described in a previous publication using an initial dry cell mass of 0.30 ± 0.05 g/L [28].

Polymer Characterization

Repeat unit compositions were determined for each polymer isolate by Proton—Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR) as described in a previous publication [26]. In short, solution-state NMR spectra were recorded at 14.1 T on an Agilent DD2 NMR Spectrometer using a 5-mm OneNMR probe with Z-axis pulsed field gradients operating at 25 °C. All samples were dissolved in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) added as an internal reference. The proton spectra, at 600 MHz, had a sweep-width of 5387 Hz and were acquired with a 45-degree pulse angle and a 1 s relaxation delay. Compositions from replicate polymers derived from identical culture conditions (n = 3) were averaged and reported.

Thermal properties were determined for each polymer isolate (n = 3) using a Perkin Elmer Pyris 1 (Norwalk, CT) differential scanning calorimeter (DSC) that was calibrated using indium (T_m = 156 °C) and cyclohexane (transition temperatures at − 87 °C and 6 °C). Each PHA polymer sample was accurately weighed (~ 5 mg) and sealed in separate aluminum pans. The heating profile was as follows: (1) equilibration at 25 °C for 2 min, (2) heating to 200 °C at 10°C/min to eliminate thermal history, (3) isothermal step at 200 °C for 2 min, (4) cooling to − 50 °C at 100°C/min, (5) isothermal step at − 50 °C for 2 min, (6) reheat to 200 °C at 10°C/min. All thermal transitions were determined based on the second heat and the averages from replicate polymers were reported. The glass transition temperatures (T_g) were measured as midpoint temperatures, while the melting temperatures (T_m) and crystallization temperatures (T_c) were measured as peak temperatures from the melting endotherms and crystallization exotherms, respectively.

The molecular weights of the polymers were measured by gel permeation chromatography (GPC) using an Agilent Technologies (Santa Clara, CA) modular GPC system. A PLgel 10 µm MIXED-B (300 × 7.5 mm) column and a PLgel 5 µm MIXED-C (300 × 7.5 mm; Agilent Technologies) column were arranged in series and a calibration curve was generated using polystyrene standards (Polymer Laboratories, Amherst, MA) ranging in molecular weight (M_p) from 580 to 3.05×10^6 g/mol and narrow (≤ 1.13) polydispersities. Replicate PHA samples (n = 3) derived from identical culture conditions were dissolved in HPLC grade chloroform at a concentration of 0.5 mg/mL. The samples were filtered through a 0.45 µm nylon syringe filter and injected into the system with an injection volume of 100 µL. The column oven was kept at 40 °C and the flow rate was 1 mL/min. All data was processed using the Cirrus™ GPC/Multidetector Software (Agilent Technologies). Averages were determined from the replicate samples and reported.

Tensile properties were measured using solution cast films that were cast in aluminum dishes (dia. = 7.5 cm) from chloroform (250 mg PHA/10 mL $CHCl_3$) solutions. Five individual measurements were made from each polymer film cast from the polymers derived from each culture condition performed in triplicate (total = 15 measurements/polymer). Once cast, the films were allowed to air-dry for at least 24 h and were then carefully cut into strips (width = 7 mm). After incubation in an environmental chamber with relative humidity of 50 ± 5% for 24–48 h, an upgraded Instron mechanical property tester, model 1122 (Instron, Norwood, MA), and Testworks-4 data acquisition software (MTS Systems Corp., Minneapolis, MN) were used to measure tensile strength (MPa), elongation at break (%), Young's Modulus (MPa), and fracture energy (J/cm^3). All samples were measured with a sample length of 2 cm between the grips and a crosshead speed of 5 cm/min. All measurements were made in a conditioning room set at 23 ± 2 °C and 50 ± 5% relative humidity.

Results and Discussion

In an effort to reduce the amount of plastic pollution in the world, scientists have been actively engaged in the synthesis and characterization of carbon–neutral biopolymers that can be applied in various applications as petroleum-based plastic substitutes. Unfortunately, successes in this area of research have been thwarted to some extent by the high costs associated with bio-based syntheses. To overcome this obstacle, many inexpensive feedstocks have been tested for their applicability in biopolymer production. LevA is one such molecule. Relatively few past studies have documented the synthesis of *scl*-PHA polymers that contain 4HV [11, 12]. In this study we utilized LevA as a co-substrate for the synthesis of unique PHA biopolymers using a previously undocumented LevA-utilizing bacterial strain. *Azohydromonas lata*, when grown in pure-culture in the presence of 1% GLC and 0-0.4% LevA, was capable of cell growth and *scl*-PHA polymer synthesis (Table 2). In the absence of LevA, cell growth was initiated earlier in

Table 2 Cell growth, PHA concentration and polymer/cell content for the 48, 72, and 96 h pure-cultures of *Azohydromonas lata* DSM 1122 grown on 1% GLC:0-0.6% LevA (n = 3 for each growth condition)

LevA conc. (wt%)	Cell dry weight (CDW; g/L)			Q_x[a] (g/L/h)			PHA conc. (g/L)			Q_p[a] (g/L/h)			PHA/cell content (%CDW)		
	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h
0	4.6	5.0	4.4	0.090	0.065	0.043	1.8	1.7	1.1	0.038	0.024	0.011	39.1	34.0	25.0
0.2	3.2	4.5	4.8	0.060	0.058	0.047	1.1	1.8	2.0	0.023	0.025	0.021	34.4	40.0	41.7
0.4	1.0	0.7	0.9	0.015	0.006	0.006	0.2	0.1	0.1	0.004	0.001	0.001	20.0	14.3	11.1
0.6	0.4	0.5	0.4	0.002	0.003	0.001	0	0	0	0	0	0	0	0	0

[a]Q_x and Q_p represent volumetric cell and PHA polymer productivities, respectively. Q_x was calculated using an initial dry cell weight of 0.30 ± 0.05 g/L

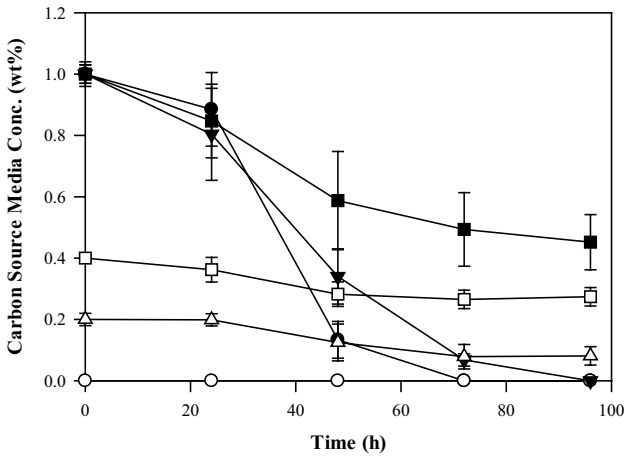

Fig. 1 Carbon source utilization by pure-cultures of *Azohydromonas lata* DSM 1122 (n = 3) grown on mixtures of 1% GLC:0 LevA (GLC, Black filled circle; LevA, open circle), 1% GLC:0.2% LevA (GLC, Black inverted triangle; LevA, open triangle), and 1% GLC:0.4% LevA (GLC, Black filled square; LevA, open square). Error bars represent ± standard deviation

the growth cycle resulting in CDWs of 4.6 and 5.0 g/L at 48 and 72 h, respectively. In fact, under these growth conditions, the GLC was entirely exhausted by 72 h (Fig. 1). At the same time, higher PHA concentrations (1.8 and 1.7 g/L) were achieved from those cell masses resulting in PHA/cell content values of 39.1 and 34.0%, respectively. In contrast, when LevA was introduced into the media, the rate of cell growth and PHA production was reduced but higher maximal PHA concentrations were achieved albeit, later in the growth cycle. Specifically, a 0.2% LevA media concentration lead to a maximum CDW of 4.8 g/L and PHA concentration of 2.0 g/L after 96 h signifying a maximal PHA/cell content of 41.7% which was the highest value achieved under the growth conditions tested. At LevA media concentrations of 0.4 and 0.6% very little-to-no cell growth or polymer synthesis occurred (Table 2). These results are supported by the carbon utilization patterns (Fig. 1). At a carbon source ratio of 1% GLC:0.2% LevA, the GLC was completely used up by 96 h while only 59% (1.2 g) of the available LevA was consumed. In comparison, at a carbon source ratio of 1% GLC:0.4% LevA, only 55% of the GLC and 31% (1.3 g) of the LevA was used after 96 h. Interestingly, equal amounts of LevA were utilized on a weight-basis from both the 0.2 and 0.4% LevA-containing cultures which indicated that GLC metabolism was the limiting factor between relatively large cell growth and smaller cell growth results.

The ^{1}H-NMR analyses of the PHA polymers produced from pure-cultures of *A. lata* are shown in Fig. 2. Integration of the resonances located between 4.9 and 5.3 ppm corresponding to the methine protons of 3HB (5.25 ppm), 3HV (5.16 ppm) and 4HV (4.91 ppm) showed that the polymers derived from GLC alone were composed solely of 3HB. As LevA was introduced into the cultures *scl*-PHA polymers were synthesized that contained low levels of both 3HV and 4HV components. Interestingly, the highest average content for non-3HB components (13 mol%) was realized when 0.2% LevA was present in the culture media as opposed to 0.4% LevA which resulted in polymers containing only 8 mol% non-3HB components (Table 3). These compositional deviations caused variation within the polymers with respect to their thermal properties. The polymers produced in the presence of 0.2% LevA exhibited T_m values of 150 °C regardless of the culture times, but the energy required to melt the samples (melting enthalpies; ΔH_m) decreased as the culture times prolonged. In addition, the crystallization temperatures (T_c), energies involved in crystallization (ΔH_c), and glass transition temperatures (T_g) all varied based on culture times. This indicated that the crystal dynamics within the polymers were different and dependent upon the length of the cultures, even when the compositions were relatively consistent. Molecular weight data revealed that the *scl*-PHA polymers synthesized in the presence of 0.2 and 0.4% LevA were on average 46% and 23% smaller (based on M_n data) respectively, than the P3HB homopolymers synthesized in the absence of LevA (Table 3).

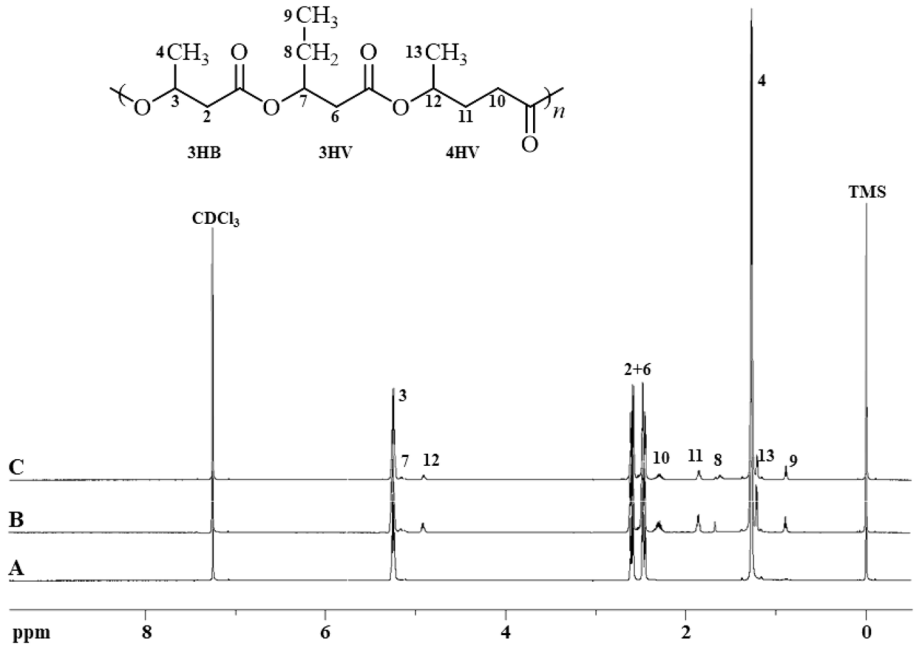

Fig. 2 Representative ^{1}H-NMR results for the *scl*-PHA polymers produced from pure-cultures of *Azohydromonas lata* DSM 1122 grown in the presence of 1% GLC:0 LevA (**a**), 1% GLC:0.2% LevA (**b**), and 1% GLC:0.4% LevA (**c**)

Table 3 PHA polymer characterization for the 48, 72, and 96 h polymers derived from pure-cultures of *Azohydromonas lata* DSM 1122 grown on 1% GLC:0–0.4% LevA (n = 3 for the polymers derived from each growth condition)

LevA conc. (wt%)	PHA composition (mol%)			Molecular weight ($\times 10^3$ g/mol)			Thermal properties				
	3HB	3HV	4HV	M_n	M_w	M_w/M_n	T_m (°C)	ΔH_m (J/g)	T_c (°C)	ΔH_c (J/g)	T_g (°C)
0 (48 h)	100	0	0	436	964	2.21	175	90.6	48	−42.1	3
0 (72 h)	100	0	0	385	996	2.59	175	86.0	48	−42.2	3
0 (96 h)	100	0	0	524	1179	2.25	175	87.4	50	−40.8	3
***x* =**	**100**	**0**	**0**	**448**	**1046**	**2.34**	**175**	**88.0**	**49**	**−41.7**	**3**
0.2 (48 h)	90	3	7	260	673	2.58	149	40.1	64	−39.1	1
0.2 (72 h)	86	5	9	250	657	2.63	150	32.3	68	−36.5	0
0.2 (96 h)	87	6	7	209	661	3.16	150	31.5	78	−31.7	0
***x* =**	**88**	**5**	**8**	**240**	**664**	**2.78**	**150**	**34.6**	**70**	**−35.8**	**0**
0.4 (48 h)	92	4	4	371	953	2.57	153	44.0	59	−38.7	2
0.4 (72 h)	92	4	4	414	978	2.36	153	46.8	54	−38.1	2
0.4 (96 h)	91	5	4	245	881	3.60	nd[a]	nd[a]	nd[a]	nd[a]	nd[a]
***x* =**	**92**	**4**	**4**	**343**	**937**	**2.73**	**153**	**45.4**	**57**	**−38.4**	**2**

'x =' refers to the average values derived from the reported results of each LevA concentration

[a]*nd* not determined due to a lack of sample

In a previous report we documented the ability of *Burkholderia sacchari* DSM 17165 to produce block copolymers of 3HB and 3HV from mixtures of GLC, XYL, and LevA [26]. In that study it was noted that the strain could grow and produce *scl*-PHA polymers at LevA concentrations as high as 0.8 wt% and that the LevA and carbohydrates present in the media were preferentially utilized (LevA > carbohydrate) resulting in controlled 3HB:3HV ratios and compositionally distinct block regions. Specifically, the 3HV content of the resulting copolymers was higher after 24 h than after 96 h, supporting the notion that LevA was preferred by the bacterial strain early in the culture. Once LevA was used, the strain could then metabolize the carbohydrate portion of the media. Since *B. sacchari* was proven to make these block copolymers from the same culture media and under the same growth conditions as *A. lata* DSM 1122, it was decided to utilize both of these bacterial strains in mixed-cultures to produce *scl*-PHA polymer blends from 1% GLC:1% XYL:0 to 0.4% LevA. Xylose was introduced into the media because *B. sacchari* was capable of metabolizing

XYL to produce *scl*-PHA and although *A. lata* cannot use XYL, it was thought that incorporating XYL into the media would aid in the production of specific polymer blends and establish *B. sacchari* growth compared to *A. lata* growth within the mixed-cultures. The LevA media concentrations (0–0.4 wt%) were chosen because both bacterial strains were capable of growth at these LevA concentrations (*B. sacchari* demonstrated in the previous study and *A. lata* determined from the pure-culture experiments described in this paper; Table 2). In order to introduce compositional variation into the system, a staggered inoculation pattern was used to allow bacterial growth of each strain for different lengths of time. *A. lata* was inoculated into all of the cultures at the onset of bacterial growth while *B. sacchari* was introduced into separate cultures at 24 h intervals (see "Materials and Methods" section and Table 1).

The CDWs, PHA polymer concentrations and PHA/cell content results for the mixed-cultures are shown in Table 4. When both bacterial strains were present in the media for 72 h (72 h:72 h samples), the lowest CDWs and PHA concentrations occurred in the absence of LevA. This was somewhat surprising. LevA has been reported to be toxic to some bacterial strains [29] while others seem to be able to withstand the toxic effects of LevA at media concentrations greater than 20 g/L [30]. In this study, the LevA in the media seemed to stimulate cell growth and polymer synthesis. In these mixed-cultures maximum CDWs and PHA polymer concentrations were obtained from the 72 h:72 h and 72 h:48 h cultures containing media with 0.2% LevA. At 0.4% LevA the CDWs and polymer concentrations decreased in all of the mixed-cultures (Table 4), which was expected based on the reduced cell growth and polymer production results from the pure-cultures of *A. lata* at 0.4% LevA (Table 2). These results were supported by the specific carbon source utilization results shown in Table 5. Interestingly, in all of the 72 h:72 h, 72 h:48 h, and the 72 h:24 h cultures some utilization of XYL was evident. *A. lata* does not possess the metabolic traits for XYL utilization (see Table 5, 72 h:0 h cultures) and therefore, any XYL utilization would signal *B. sacchari* growth within the mixed cultures. In the LevA-containing 72 h:72 h cultures almost all of the LevA was used up after 72 h. Logically, if both strains were growing and producing *scl*-PHA polymer equally well under these culture conditions, one would expect some 4HV present in the resulting polymers. ^{1}H-NMR analysis did not show any evidence of 4HV in these polymers (Table 6). In addition, the polymers produced from the 72 h:72 h cultures on 0.2 and 0.4% LevA both showed 2 T_g's, one at approximately − 11 to − 15 °C (close to poly-3-hydroxyvalerate; P3HV) and one at 3 °C (same as P3HB). These results, taken together, indicate that when both bacterial strains were inoculated into the production flasks containing LevA at the onset of the cultures (72 h:72 h), *B. sacchari* outcompetes *A. lata* to produce primarily P3HB-*block*-3HV copolymers. In contrast, the results

Table 4 Cell growth, PHA concentration and polymer/cell content for the mixed-cultures of *Azohydromonas lata* DSM 1122 and *Burkholderia sacchari* DSM 17165 grown on 1% GLC:1% XYL:0-0.4% LevA with staggered growth patterns (n = 3 for each growth condition)

Sample[a]	Cell dry weight (g/L)	Qx[b] (g/L/h)	PHA conc. (g/L)	Qp[b] (g/L/h)	PHA/cell content (%CDW)
72 h:72 h					
1:1:0	1.8 ± 0.1	0.021 ± 0.002	0.1 ± 0.0	0.001 ± 0.000	5.7 ± 0.2
1:1:0.2	4.6 ± 0.3	0.060 ± 0.004	2.3 ± 0.3	0.032 ± 0.004	50.0 ± 0.7
1:1:0.4	3.2 ± 0.3	0.040 ± 0.004	1.3 ± 0.3	0.018 ± 0.004	40.6 ± 1.3
72 h:48 h					
1:1:0	4.4 ± 0.9	0.057 ± 0.013	1.9 ± 0.9	0.026 ± 0.012	43.2 ± 6.3
1:1:0.2	5.3 ± 0.1	0.069 ± 0.001	2.8 ± 0.1	0.039 ± 0.001	52.8 ± 0.1
1:1:0.4	3.3 ± 0.6	0.042 ± 0.009	1.5 ± 0.4	0.021 ± 0.006	45.5 ± 3.8
72 h:24 h					
1:1:0	4.6 ± 0.1	0.060 ± 0.002	1.8 ± 0.0	0.025 ± 0.000	39.1 ± 0.1
1:1:0.2	4.4 ± 0.2	0.057 ± 0.003	2.2 ± 0.1	0.031 ± 0.002	50.0 ± 0.3
1:1:0.4	2.5 ± 0.3	0.031 ± 0.005	1.1 ± 0.1	0.015 ± 0.002	44.0 ± 1.1
72 h:0 h					
1:1:0	4.4 ± 0.1	0.057 ± 0.001	1.6 ± 0.0	0.022 ± 0.000	36.4 ± 0.1
1:1:0.2	3.7 ± 0.7	0.047 ± 0.009	1.7 ± 0.4	0.024 ± 0.005	45.9 ± 3.9
1:1:0.4	0.8 ± 0.2	0.007 ± 0.003	0.4 ± 0.1	0.006 ± 0.002	50.0 ± 6.7

[a]Sample designations (72 h:72 h, 72 h:48 h, 72 h:24 h, and 72 h:0 h) correspond to the growth times of *A. lata* and *B. sacchari* in each culture, respectively. The designations 1:1:0, 1:1:0.2, and 1:1:0.4 correspond to the carbon source ratios within the cultures. The first number = GLC concentration, the second number = XYL concentration, and the third number = LevA concentration, all in wt%

[b]Q_x and Q_p represent volumetric cell and PHA polymer productivities, respectively. Q_x was calculated using an initial dry cell weight for *A. lata* of 0.30 ± 0.05 g/L

Table 5 Selective carbon source utilization in the mixed-cultures of *Azohydromonas lata* DSM 1122 and *Burkholderia sacchari* DSM 17165 grown on 1% GLC:1% XYL:0–0.4% LevA with staggered growth patterns (n = 3 for each growth condition)

Sample[a]	GLC[b] (wt%)	XYL[b] (wt%)	LevA[b] (wt%)
72 h:72 h			
1:1:0	0.35 ± 0.04	0.87 ± 0.07	0
1:1:0.2	0.12 ± 0.02	0.70 ± 0.02	0.01 ± 0.01
1:1:0.4	0.77 ± 0.08	0.74 ± 0.02	0.01 ± 0.00
72 h:48 h			
1:1:0	0	0.73 ± 0.03	0
1:1:0.2	0	0.86 ± 0.08	0
1:1:0.4	0.36 ± 0.01	0.46 ± 0.10	0.01 ± 0.01
72 h:24 h			
1:1:0	0	0.83 ± 0.03	0
1:1:0.2	0.01 ± 0.01	0.82 ± 0.12	0.06 ± 0.02
1:1:0.4	0.71 ± 0.03	0.94 ± 0.03	0.14 ± 0.03
72 h:0			
1:1:0	0	0.99 ± 0.02	0
1:1:0.2	0.13 ± 0.05	0.98 ± 0.03	0.10 ± 0.01
1:1:0.4	0.45 ± 0.14	1.00 ± 0.00	0.28 ± 0.02

[a]For sample designations see the footnote for Table 4

[b]All values represent residual media carbon source concentrations after 72 h of cell growth from the growth patterns indicated

obtained from the 72 h:48 h and 72 h:24 h growth patterns showed relatively good cell growth and PHA concentrations but once again the maximum cell growth and polymer concentrations were realized at LevA media concentrations of 0.2% (Table 4). In these cases, the PHA/cell content values were calculated to between 39 and 53% indicating that under these culture conditions cell growth and polymer yields were near maximum. These results were once again supported by carbon source utilization data. In the 72 h:48 h cultures all of the GLC and LevA were consumed in the cultures containing 0 and 0.2% LevA. At 0.4% LevA, XYL utilization (0.54%) was more pronounced while some GLC was still present after 72 h. The 72 h:24 h cultures once again showed that the GLC media concentrations were essentially 0 upon harvesting the cultures containing 0 and 0.2% LevA. The polymers produced from the 72 h:48 h and 72 h:24 h cultures containing LevA exhibited polymer compositions containing small amounts of 4HV along with reduced T_m and ΔH_m values. In addition, the 0.4% LevA cultures showed multiple T_g values (Fig. 3). These data taken together indicate the presence of PHA terpolymer (composed of 3HB, 3HV, and 4HV):P3HB-*block*-3HV polymer mixtures with reduced crystallinities. The last series of cultures (72 h:0 h) contained no *B. sacchari*. The CDWs, polymer concentrations and PHA/cell content values resembled those obtained in the *A. lata* pure-cultures described previously (Table 2). As expected, these cultures showed no XYL utilization and reduced LevA utilization. The polymers synthesized under these conditions were composed of 88–90% 3HB, 4–5% 3HV, and 6–7% 4HV, similar to the pure-culture results reported earlier in this study, and exhibited a reduced T_m and only a single T_g value of 1–2 °C. The lack of a *B. sacchari* inoculum indicates that the *scl*-PHA polymers obtained from these cultures were not mixtures but were in fact synthesized solely by *A. lata*.

Table 6 PHA polymer characterization for the mixed-cultures of *Azohydromonas lata* DSM 1122 and *Burkholderia sacchari* DSM 17,165 grown on 1% GLC:1% XYL:0-0.4% LevA with staggered growth patterns (n = 3 for the polymers derived from each growth condition)

Sample[a]	PHA composition (mol%)			Thermal properties				
	3HB	3HV	4HV	T_m (°C)	ΔH_m (J/g)	T_c (°C)	ΔH_c (J/g)	T_g (°C)
72 h:72 h								
1:1:0	100	0	0	176	79	50	− 38	3
1:1:0.2	87	13	0	170	62	60	− 35	− 11; 3
1:1:0.4	61	39	0	165; 102	32; 3	64	− 24	− 15; 3
72 h:48 h								
1:1:0	100	0	0	175	82	51	− 38	3
1:1:0.2	84	11	5	152	24	78	− 24	1
1:1:0.4	56	42	2	156; 101	19; 1	78	− 16	− 13; 0
72 h:24 h								
1:1:0	100	0	0	175	84	50	− 41	3
1:1:0.2	83	10	7	153	18	81	− 16	1
1:1:0.4	52	44	4	152; 101	13	83	− 11	− 13; − 1
72 h:0 h								
1:1:0	100	0	0	175	82	49	− 41	3
1:1:0.2	88	5	7	154	29	73	− 32	1
1:1:0.4	90	4	6	152	45	64	− 38	2

[a]For sample designations see the footnote for Table 4

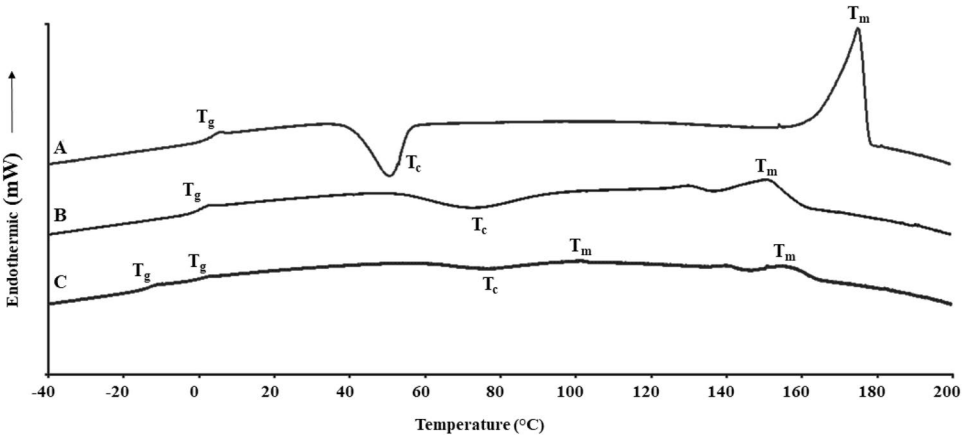

Fig. 3 Representative differential scanning calorimetry (DSC) results from the 72 h:24 h mixed-cultures grown in the presence of 1% GLC:1% XYL:0 LevA (a), 1% GLC:1% XYL:0.2% LevA (b), and 1% GLC:1% XYL:0.4% LevA (c)

One of the challenges in producing biopolymers with good tensile properties is maintaining tensile strength while reducing modulus and improving elongation. Typically, elastomeric polymers suffer from reduced tensile strength so finding polymers that can stretch yet maintain some physical strength can be a challenge. One way that these goals have been met is through the synthesis of co-polymeric PHA. The most well-known PHA copolymer is the P3HB-*co*-3HV copolymer which demonstrates isodimorphic behavior at various 3HV contents [31]. Another well-studied copolymer that has been particularly interesting based on its tensile properties is P3HB-*co*-4HB [32]. In both of these cases copolymeric PHAs were synthesized that improved the crystallization behavior and hence the tensile properties. In an effort to further improve the tensile properties among *scl*-PHA polymers, we synthesized natural polymer blends using mixed-culture strategies. The blends contained both terpolyesters composed of 3HB, 3HV, and 4HV as well as block copolymers composed of 3HB and 3HV. The results of those tensile property studies are shown in Fig. 4. The polymers produced using the 72 h:72 h and 72 h:0 h growth patterns originated primarily from *B. sacchari* and *A. lata*, respectively and therefore were not apparent mixtures. The 1:1:0 culture and the 1:1:0.4 culture from the 72 h:72 h and the 72 h:0 h growth patterns, respectively did not result in sufficient polymer to test. The P3HB homopolymers produced in the absence of LevA showed increasing tensile strength (between 23 and 30 MPa; Fig. 4a) and modulus (between 1041 and 1480 MPa; Fig. 4b). These results showed that the P3HB homopolymers were stronger and more brittle as *B. sacchari* spent less time in the mixed-cultures. The PHA polymer mixtures obtained from the 72 h:48 h, and the 72 h:24 h cultures at 0.2 and 0.4% LevA showed slightly decreased tensile strengths and moduli when compared to the polymers obtained from the individual bacterial strains in the 72 h:72 h and 72 h:0 h cultures. These results show that while tensile strength may be slightly reduced, they still maintain sufficient strength to merit further study. A reduced modulus is favorable in that these results indicate that the polymers from the mixed-cultures are more malleable than the stiff, brittle P3HB homopolymers.

Interestingly, the elongation and fracture energy from the polymers derived from all of the inoculation patterns showed comparable trends. Elongation values of the polymers derived from LevA-containing cultures were higher than the P3HB homopolymers but those copolymers/polymer mixtures derived from the 72 h:72 h, 72 h:48 h, and 72 h:0 h cultures showed maximum elongation values that were less than 50%. In contrast, the polymer mixtures derived from the 72 h:24 h cultures showed average elongations of 238% and 177% for the polymers produced in the presence of 0.2 and 0.4% LevA, respectively. These values were 349% and 378% higher than the polymer mixtures produced from the 72 h:48 h cultures containing 0.2 and 0.4% LevA (Fig. 4c). Fracture energy is defined as the energy required to open a unit area of crack surface and does not depend on the size of the structure (for a good review on fracture energy of elastomeric materials see reference [33]). In this study, the fracture energies of the polymers derived from LevA-containing cultures were greater than the P3HB homopolymers derived from the cultures in the absence of LevA. This was expected in that P3HB homopolymers are well-known to be relatively brittle materials. Once again, the polymers produced in the presence of LevA from the 72 h:72 h, 72 h:48 h, and 72 h:0 h cultures showed fracture energies that were between 3.3 and 9.9 J/cm^3. The fracture energies from the polymer films derived from the 72 h:24 h cultures containing LevA were 276% and 297% greater than the polymers produced under identical culture conditions from the 72 h 48 h cultures (Fig. 4d).

In conclusion, wildtype *A. lata* was demonstrated to be capable of utilizing LevA as a co-substrate for the synthesis of bacterial terpolyesters composed of 3HB, 3HV, and 4HV. These terpolymers were unique in that the 4HV content (9%) was the highest so far reported for a wildtype strain grown on LevA. By utilizing *A. lata* along with *B. sacchari*, a known

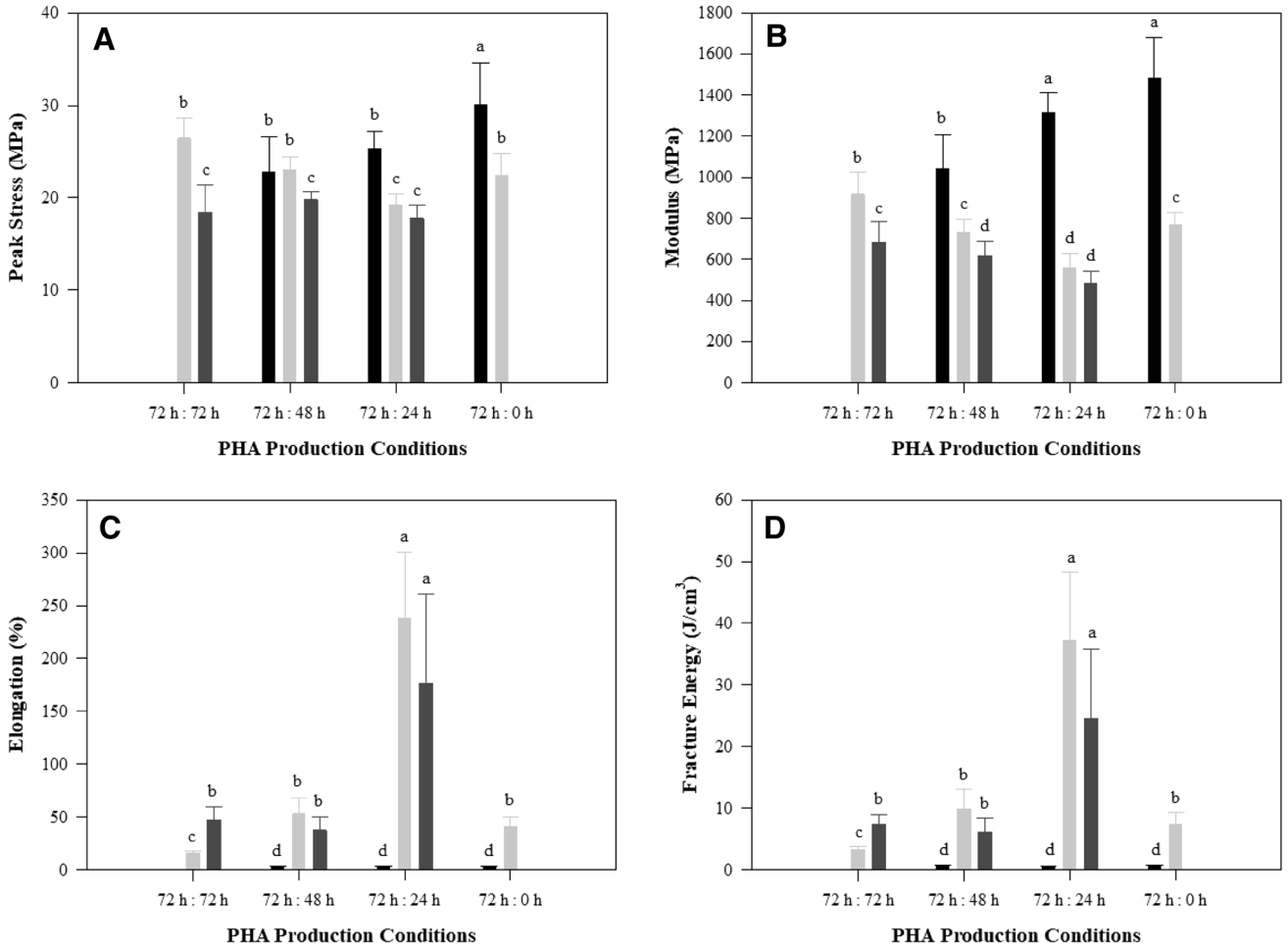

Fig. 4 Tensile property results (n = 5 measurements/polymer film derived from each culture condition using shake flask cultivation performed in triplicate; total = 15 measurements/polymer) for the PHA polymers derived from the 72 h:72 h, 72 h:48 h, 72 h:24 h, and 72 h 0 h mixed-cultures of *A. lata* and *B. sacchari*. Tensile strength (**a**), Modulus (**b**), Elongation (**c**), and Fracture energy (**d**) are shown for the polymers made in the presence of 1% GLC:1% XYL:0 LevA (Black filled square), 1% GLC:1% XYL:0.2% LevA (light grey filled square), 1% GLC:1% XYL:0.4% LevA (dark grey filled square). Error bars represent ± standard deviation. Different letters on bars within each graph indicate significant differences ($P < 0.05$)

3HB-*block*-3HV producer, in mixed-culture under staggered growth patterns, it was demonstrated that terpolyester/block copolymer mixtures could be obtained with enhanced tensile properties. As the scientific world explores new options for petroleum-based plastics, it is becoming imperative that the properties of the potential substitutes approach, if not mimic currently used materials and can be produced at competitive prices. Mixed-cultures are one means of controlling polymer properties. By using mixed-cultures polymer blends can be realized whose properties can be controlled based on the culture conditions and by using inexpensive carbon sources such as LevA, these mixtures can be produced more economically. The information presented in this paper demonstrates the feasibility of producing unique *scl*-PHA polymers and polymer blends and controlling their properties for targeted applications and reduced plastic waste.

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