Title: The effect of acetate as a co-feedstock on the production of poly(lactate-co-3-hydroxyalkanoate) by *pflA*-deficient *Escherichia coli* RSC10

Short title: Effect of acetate on P(HA-co-LA) biopolymer production

Authors: Lucia Salamanca-Cardona¹, Ryan A. Scheel¹, Kouhei Mizuno², N. Scott Bergey¹, Arthur J. Stipanovic¹, Ken'ichiro Matsumoto³, Seiichi Taguchi^{3,4} and Christopher T. Nomura^{1,5,6}

¹Department of Chemistry, State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210

²Department of Creative Engineering, National Institute of Technology, Kitakyushu College, 5-20-1, Shii, Kokuraminami-ku, Kitakyushu 802-0985, Japan

³Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-28, Kita-ku, Sapporo 060-8638, Japan

⁴CREST, JST, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan

⁵Center for Applied Microbiology, State University of New York, College of Environmental Science and Forestry, Syracuse, New York 13210

⁶Hubei Collaborative Innovation Center for Green Transformation of Bio-Resources, College of Life Sciences, Hubei University, Wuhan 430062, China

Corresponding Author:

Dr. Christopher T. Nomura

Department of Chemistry
318 Jahn Lab
1 Forestry Dr.
Syracuse, New York 13210

Phone: (315) 470-6854/470-6843

Email: ctnomura@esf.edu

Keywords: Polyhydroxyalkanoates; acetate; xylose; recombinant *Escherichia coli*; biomass; hemicellulose.

Abstract

Developing *Escherichia coli* strains that are tolerant to acetate toxicity is important in light of an increased interest in the efficient utilization of lignocellulosic biomass feedstocks for the biosynthesis of value-added products. In this study, four strains known to produce polyhydroxyalkanoates (PHAs) from the typical hemicellulosic sugar xylose were tested for their tolerance to acetate. *E. coli* RSC10 was found to be tolerant of acetate, both in growth and fermentation studies. In the presence of acetate the strain showed a >2-fold increase in overall yields compared to using xylose alone as the feedstock. More importantly, the strain was found to be able to utilize acetate as a feedstock for biosynthesis of PHAs, with complete depletion of acetate (25 mM) at 9 h when acetate was the sole feedstock. Higher concentrations of acetate showed greater inhibition of fermentation than growth with a reduction of 90% in PHA yields at 100 mM. Additionally, the present work provides data to support the potential of acetate as a modulator for the control of composition of PHAs that incorporate lactate (LA) monomers into the copolymer from hemicellulose derived sugars.

Introduction

Increased interest in utilization of lignocellulose as a feedstock for the biosynthesis of commodities (i.e. fuels, plastics) which are currently derived from petroleum requires focusing research efforts into engineering microbial factories that can not only synthesize the target product, but can also achieve high production yields. Pretreatment of lignocellulosic biomass can result in the accumulation of toxic byproducts that negatively affect yields either by slowing microbial growth or inhibiting fermentative processes (1-5). Acetate, furfural, and hydroxymethylfurfural are among the most common byproducts derived from the hemicellulose portion of lignocellulosic biomass subjected to thermochemical treatments (6-8). Acetate is a common byproduct released from pretreatment of xylan, a component of hardwood lignocellulose, which contains up to 29% of its weight as acetyl groups (9,10). Detoxification of lignocellulosic hydrolysates prior to fermentation is possible and many strategies have been developed (11-14); however, these processes significantly increase production costs (15,16). A potential strategy to bypass extensive detoxification processes is the development of bacterial strains that are more resistant to inhibitory effects of pretreatment byproducts.

In *Escherichia coli*, acetate has been shown to negatively affect growth and some biosynthetic pathways (17,18). Additionally, acetate produced from overflow metabolism of sugars is excreted into the media as growth progresses, becoming an obstacle for production of high yields of recombinant protein (19-21). Nevertheless, some *E. coli* strains have been found to be partially tolerant to acetate. For example, *E. coli* K011 showed only a slight reduction in ethanol titers over long production periods or by adjustment of culture pH,

albeit cellular growth was negatively affected (5). In the present study, the effect of acetate on the cell growth and fermentations of four *E. coli* strains (BW25113, JW0885, LS5218, and RSC10) known to produce polyhydroxyalkanoates (PHAs) from xylose (22-25) was examined using xylose and acetate as feedstocks. These strains were engineered to express the PHA synthase from *Pseudomonas* sp. 61-3 harboring a Ser325Th/Gln481Lys mutation [PhaC1Ps(ST/QK)], a propionyl- CoA transferase (PCT) from *Megasphaera elsdenii*, and a β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB) from *Ralstonia eutropha* under a *R. eutropha* constitutive promoter in order to produce the PHA copolymer poly(lactate-co-hydroxybutyrate) [P(LA-co-3HB)] (26).

E. coli strains BW25113, JW0885, and LS5218 showed reduced cell growth while utilizing xylose as a feedstock in the presence of acetate, leading to reduced overall yields of PHAs. Conversely, strain RSC10 showed tolerance to acetate both in terms of cellular growth and fermentation. Additionally, this strain was able to utilize acetate as feedstock leading to a >2-fold increase in overall yields of PHA compared to using xylose as sole feedstock. Yields were further increased by 33% when addition of substrates was delayed until mid-exponential growth with results suggesting this increase to be a result of improved fermentations with no adverse effect in cellular growth. These results expand and corroborate previous findings suggesting *E. coli* RSC10 as a strain with the capability to withstand acetate toxicity with no inhibitory effects in growth and PHA production (24). Furthermore, these results demonstrate that the relationship between *E. coli* RSC10 and acetate goes beyond tolerance, as the strain is also capable of utilizing it as a feedstock for the production of PHAs with increased incorporation of 2-hydroxypropionate (lactate [LA]) into the copolymer.

Materials and Methods

Media and cultivation conditions

All bacterial strains and plasmids are listed in Supplementary Table S1. Removal of the kanamycin cassette from JW0885-1 to generate *E. coli* JW0885 was done according to the protocol developed by Datsenko and Wanner (27). Following successful removal of the kanamycin cassette via FLP recombination, removal of the *pflA* gene was confirmed using check primers: 5'-GCCACATCTGGAGAAACACC-3' (forward primer) and 5'-AGAATGAAGCGCGGAATAAA-3' (reverse primer). The plasmid pTVN118*pctphaC1*(ST/QK)AB (Taguchi et al. 2008) was used for PHA production. The four *E. coli* strains were grown in Lennox Broth (LB, Difco™) and plasmids were maintained by supplementation with ampicillin (100 µg·mL⁻¹). To assess the effect of acetate on PHA production from xylose (Fisher Scientific), sodium acetate was added at varying concentrations as described. All transformation, growth, and PHA production experiments were carried at 30 °C and 250 rpm.

Production of PHA in *E. coli* strains BW25113, JW0885, LS5218 and RSC10 harboring pTVN118*pctSTQKAB* from xylose supplemented with acetate

E. coli strains BW25113, JW0885, LS5218 and RSC10 were transformed with pTVN118*pctphaC1*(ST/QK)AB via electroporation and fresh transformants were used for all experiments. Successful transformants were selected by growth on LB plates supplemented with ampicillin. The efficiency of the pTVN118*pctphaC1*(ST/QK)AB plasmid was measured by performing a control experiment using glucose (20 g·L⁻¹) as a feedstock for PHA production. A single colony was inoculated into 50 mL of LB and grown at 30 °C and 250 rpm in a rotary shaker for 20 h overnight. 1 mL of overnight culture was inoculated into 100 mL of LB media in 500 mL baffled flasks supplemented with xylose (20 g·L⁻¹, samples 1-4) or xylose (20 g·L⁻¹) and sodium acetate (25mM, samples 5-8) at the time

of inoculation. All experiments were carried out in triplicate and incubated at 30 °C for 48 h in a rotary shaker at 250 rpm. After incubation the cells were harvested by centrifugation at 4000 x g for 15 minutes. The cells were resuspended and washed twice in Nanopure water (Barnstead), followed by lyophilization for a minimum of 24 h.

Effect of acetate concentration and substrate-addition time in PHA production from xylose by *E. coli* RSC10

E. coli RSC10 harboring pTVN118-1). At the time of inoculation sodium acetate was added to the media to the following final concentrations: 10, 35, 50, 75, and 100 mM (samples 9-13). Each set of experiments was grown at 250 rpm in a rotary shaker for 48 h at 30 °C in 500 mL baffled flasks. To assess how the time of substrate addition affects growth and fermentation, an additional set of experiments was carried out consisting of 100 mL of LB inoculated with 1 mL of overnight culture with addition of xylose (20 g·L⁻¹) at inoculation or at mid-exponential growth phase (OD₆₀₀=0.6); acetate (25 mM) at inoculation or at OD₆₀₀=0.6 (these four conditions were used as control; samples 4, 19, 17 and 18 respectively); xylose (20 g·L⁻¹) and acetate (25 mM) added at inoculation (sample 8); xylose (20 g·L⁻¹) and acetate (25 mM) added at OD₆₀₀=0.6 (sample 14); xylose (20 g·L⁻¹) added at inoculation and acetate (25 mM) added at OD₆₀₀=0.6 (sample 15); and acetate (25 mM) added at inoculation and xylose (20 g·L⁻¹) added at OD₆₀₀=0.6 (sample 16). A control experiment was also performed by growing the cells on LB media only without any other substrates added (sample 20). The cells were grown at 250 rpm in a rotary shaker for 48 h at 30 °C in 500 mL baffled flasks. At the end of the growth period the cells were harvested as outlined previously. All experiments were performed in triplicate.

Extraction of PHA polymers from microbial cells

Lyophilized cells were resuspended in methanol, mixed by stirring at room temperature for 5 min, and centrifuged at 4000 x g for 15 min. The samples were resuspended and washed twice with Nanopure water and freeze dried for a minimum of 24 h. Polymer extraction of washed cells was carried by soxhlet extraction using 80 mL of refluxing chloroform at 95 °C for 5 h. Following chloroform extraction, the samples were cooled to room temperature and the chloroform was completely removed by evaporation in a rotary evaporator. The polymer was washed twice with ice-cold methanol, redissolved in 5-6 mL of chloroform and cast into a glass Petri dish allowing evaporation of chloroform at room temperature.

Gas Chromatography (GC) analysis

Gas chromatography was used as previously described (21) to determine PHA production yields and relative monomer composition. Approximately 10 mg of dried cells were treated with 2 mL of chloroform and 2 mL of methanol:sulfuric acid solution (85:15) and incubated for 140 min at 100 °C. Following incubation, the samples were cooled to room temperature and 1 mL of Nanopure water was added and mixed. Separation of the aqueous and organic layers was done by centrifuging the samples at 750 rpm for 2 min. The bottom organic layer was removed and filtered through a 0.45 µm polytetrafluoroethylene (PTFE) acrodisk syringe filter (Restek). A 0.5 mL portion of filtered sample was transferred to GC vials and mixed with 0.5 mL of caprylic acid (0.1 g·L⁻¹) as an internal standard. The parameters for GC analysis were as follows: 1 µL injection volume, 30 m *Rtx*®-5 column (Restek), 45 °C column temperature and a column oven heating profile of 45 °C initial temperature, hold for 7 min, ramp up to 100 °C at 20 °C·min⁻¹, ramp up to 200 °C at 5 °C·min⁻¹, and increase to final temperature of 280 °C at 30 °C·min⁻¹ and hold for 2 min.

Analysis was carried by split injection in a GC2010 gas chromatograph with a flame ionization detector (Shimadzu).

Nuclear Magnetic Resonance (NMR) analysis

¹H-NMR was used as previously described (21) to confirm the identity of the extracted polymers from samples 4, 8, 14, and 16 since the obtained yields of PHA were high, and for determination of xylose and acetate concentration over the first 12 h of growth for cells grown in acetate (25 mM), xylose (20 g·L⁻¹), and xylose (20 g·L⁻¹) and acetate (25 mM) with all substrates added at inoculation (samples 17, 4, and 8 respectively). For polymer analysis, approximately 12 mg of purified polymer were dissolved in 1 mL of deuterated chloroform (Cambridge Isotope Laboratories, Cambridge, USA) and passed through a glass-wool plug to remove insolubles. The sample was analyzed at 30 °C in a Bruker AVANCE III 600 spectrometer (600 MHz ¹H frequency) equipped with a 5 mm IXI z-gradient probe. Data and spectra were processed in TOPSIN v1.3 from Bruker BioSpin, Billerica, USA. The same parameters were used for determination of sugar concentrations in the media. The samples were prepared by collecting approximately 1.5 mL of media every 3 h separating the cells by centrifugation and filtering the supernatant through a 0.45 µm PES syringe filter. Glucosamine dissolved in deuterium oxide (Acros, Waltham, USA) was used as a reference compound and added to the samples in a 1:9 ratio to a final concentration of 10 g·L⁻¹ (28).

Gel Permeation Chromatography (GPC) analysis

Gel permeation chromatography was used to determine weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity ($\mathcal{D}_M = M_w/M_n$) of the extracted polymers, as previously described (21). Approximately 0.7 mg of polymer was dissolved in 1 mL of chloroform and filtered through a 0.45 µm PTFE acrodisk syringe filter into GPC vials. The GPC analysis parameters were as follows: 50 µL injection volume,

styrenedivinylbenzene (SDV) 8X300 mm with 5 μm porosity column (Polymer Standards Service), 40 $^{\circ}\text{C}$ column oven temperature, chloroform mobile phase at 1 $\text{mL}\cdot\text{min}^{-1}$ flow rate. The analysis was done with a LC-20 AD Liquid Chromatograph with a RID-10A refractive index detector (Shimadzu), using a polystyrene standard.

Differential Scanning Calorimetry (DSC) analysis

Glass transition (T_g), melting (T_m), and crystallization (T_c) temperatures were determined by DSC as previously described (21). Approximately 2 mg of polymer were loaded onto a TzeroTM aluminum pan (TA Instruments) and heated to 200 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}\cdot\text{min}^{-1}$, then cooled to -80 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}\cdot\text{min}^{-1}$, followed by temperature increase to 200 $^{\circ}\text{C}$ at 10 $^{\circ}\text{C}\cdot\text{min}^{-1}$. Analysis was carried out under a nitrogen atmosphere with a flow rate of 50 $\text{mL}\cdot\text{min}^{-1}$ in a DSC Q200 differential scanning calorimeter. Data were selected from the second heating cycle.

Results

Production of PHA in *E. coli* strains LS5218, RSC10, BW25113 and JW0885 harboring pTVN118pctSTQKAB from xylose supplemented with acetate

E. coli strains BW25113, LS5218 and their respective *pflA* mutants JW0885 and RSC10 harboring pTVN118pctphaC1(ST/QK)AB were grown in LB media supplemented with xylose or xylose and acetate to assess the effect of acetate in growth and PHA production. Table 1 shows results from the strains grown in xylose (20 $\text{g}\cdot\text{L}^{-1}$). Under these conditions strains BW25113 and LS5218 produced highest overall yields for the dried cell mass (CDW in $\text{g}\cdot\text{L}^{-1}$) reaching 6.4 and 6.3 $\text{g}\cdot\text{L}^{-1}$ respectively. Strain BW25113 had the highest PHA production as a fraction of total cell mass (wt%) with 60.4 and highest total PHA with 3.8 $\text{g}\cdot\text{L}^{-1}$. PHA production as wt% and total PHA reached 49.0 and 3.2 $\text{g}\cdot\text{L}^{-1}$ respectively.

The *pflA* mutants grown on xylose (20 g·L⁻¹) alone reached cell masses of 2.0 g·L⁻¹ for strain JW0885 and 2.3 g·L⁻¹ for strain RSC10. Total PHA production and PHA production as wt% of total cell mass was similar for both strains with 1.0 g·L⁻¹ and 51.2 for *E. coli* JW0885 and 1.1 g·L⁻¹ and 47.9 for *E. coli* RSC10. Incorporation of LA-monomers in copolymers, denoted as 2HP, produced by strains BW25113 and JW0885 were highest with 20.3 and 26.0 (mol%) respectively, and for strains LS5218 and RSC10 LA fraction reached only 2.1 and 6.4 (mol%) respectively.

When the four strains were grown on xylose (20 g·L⁻¹) and acetate (25 mM), total PHA yields were significantly reduced for *E. coli* BW25113 (1.3 g·L⁻¹), LS5218 (1.4 g·L⁻¹) and JW0885 (0.3 g·L⁻¹) as shown in Table 2. However, strain RSC10 showed an increase to 1.9 g·L⁻¹. This strain also showed an increase in final cell mass and LA fraction reaching 4.2 g·L⁻¹ and 13.1 (mol%) respectively, while PHA as a fraction of total cell mass was slightly reduced to 45.3. A slight increase in these two parameters was also obtained in strain JW0885 with 2.2 g·L⁻¹ of total cell mass and 28.0 (mol%) LA fraction, although this strain showed a markedly reduced PHA production as a fraction of cell mass reaching only 15.1 (wt%). Strain BW25113 also showed a significant decrease in cell mass and PHA (wt%) reaching 3.2 g·L⁻¹ and 40.7% respectively, and a similar pattern was obtained for strain LS5218 with 4.0 g·L⁻¹, and 36.0% respectively. However, the LA fraction was increased for *E. coli* LS5218 reaching 9.7 (mol%), while strain BW25113 showed a decrease in LA-monomer incorporation to 17.7 (mol%).

Effect of acetate concentration and substrate addition time in PHA production from xylose by *E. coli* RSC10

Based on the yields obtained from testing the effect of acetate on the four strains, *E. coli* RSC10 was selected to study the effect of acetate concentration in the media. The strain harboring pTVN118*pctphaC1*(ST/QK)AB was grown in LB supplemented with xylose and

246 varying concentrations of acetate ranging from 0 mM to 100 mM. All the cells grown on
247 media that contained any amount of acetate resulted in an increased LA fraction compared to
248 growth on xylose alone, with incorporation ranging between 10.3 and 13.1 (mol%) (Table
249 3). At 10 and 35 mM total cell mass yields were similar reaching $3.7 \text{ g}\cdot\text{L}^{-1}$. However total
250 PHA yields were significantly lower in cells grown with 35 mM acetate reaching only 0.7
251 $\text{g}\cdot\text{L}^{-1}$ compared to $1.8 \text{ g}\cdot\text{L}^{-1}$ for cells grown with 10 mM acetate, a result of lowered PHA
252 production as a fraction of cell mass in cells grown at 35 mM (Table 3). Yields for cell mass,
253 PHA (wt%), and total PHA production showed a pattern of decreasing as the acetate
254 concentration was increased from 25 mM, with PHA production as a fraction of cell mass
255 showing the steepest decline in values where at 100 mM it reached only 4.4 (wt%) while
256 total cell mass was lowered only to $2.9 \text{ g}\cdot\text{L}^{-1}$ (Table 3 and Figure 1).

257 To assess if yields obtained from growing the cells with xylose ($20 \text{ g}\cdot\text{L}^{-1}$) and acetate
258 (25 mM) could be improved, experiments were performed where substrates (separately or
259 together) were added either at inoculation or during mid-exponential growth phase
260 ($\text{OD}_{600}=0.6$). Results are shown in Table 4. Four control experiments were carried: two
261 using only acetate and two using only xylose as the substrate (samples 4, 8, 17, and 18). In
262 the case of the cells grown on acetate alone cell mass and PHA production yields showed no
263 difference when substrate was added at inoculation or at $\text{OD}_{600}=0.6$ (cell mass reaching 1.5
264 $\text{g}\cdot\text{L}^{-1}$ and PHA (wt%) reaching 15.2% and 15.5%). However, a significant difference was
265 observed in LA-incorporation where addition of acetate at $\text{OD}_{600}=0.6$ resulted in no LA
266 fraction detected while when acetate was added at inoculation LA fraction reached 4.7
267 (mol%).

268 Yields resulting from addition of xylose as the only substrate at inoculation have
269 been reported in the previous section (Table 1, sample 4) and yields obtained from addition
270 of xylose at $\text{OD}_{600}=0.6$ show only a small increase in yields with cell mass reaching $2.5 \text{ g}\cdot\text{L}^{-1}$

and 6.9 (mol%) of LA fraction (Table 2, sample 19). When both substrates were added at $OD_{600}=0.6$ (sample 14) or when xylose is added first followed by acetate at $OD_{600}=0.6$ (sample 15) there is no significant difference in overall cell mass yields ($5.0 \text{ g}\cdot\text{L}^{-1}$), PHA (wt%) (56.9% and 58.9%), or total PHA production ($2.9 \text{ g}\cdot\text{L}^{-1}$ and $3.0 \text{ g}\cdot\text{L}^{-1}$). The LA fraction was slightly increased when acetate is added later reaching 8.4 (mol%) compared to 7.1 (mol%) when both substrates are added at inoculation.

Addition of acetate at inoculation and xylose at mid-exponential growth phase results in slightly lower yields with cell mass reaching $4.5 \text{ g}\cdot\text{L}^{-1}$ and total PHA $2.7 \text{ g}\cdot\text{L}^{-1}$; however LA incorporation increased to 12.1 (mol%) (Table 4, sample 16). The yields obtained from these three feeding conditions are higher than those obtained when both acetate and xylose are added at inoculation, with the exception of LA fraction being highest (Table 1, sample 9) under these conditions.

Analysis of xylose and acetate concentration in the media during initial growth

In order to understand the metabolism of acetate and xylose during initial growth, aliquots from extracellular media from cells grown with acetate (25 mM), xylose ($20 \text{ g}\cdot\text{L}^{-1}$, or acetate (25 mM) and xylose ($20 \text{ g}\cdot\text{L}^{-1}$) (all added at inoculation) were collected every 3 h. The OD_{600} was recorded as a measure of growth rate, and the concentrations of acetate and xylose were determined by ^1H -NMR using the signals from protons attached to the α -carbon compared to glucosamine ($10 \text{ g}\cdot\text{L}^{-1}$) as a reference compound. With these conditions a standard curve was generated using xylose or acetate to obtain a response factor of 0.083 and 0.099 respectively. The growth rate of the cells under the three conditions show a constant increase up until 9 h when increase in optical density starts to plateau on the growth curve (Figure 2, S1-S3).

The cells grown on xylose alone appear to grow slightly faster than cells grown on acetate or xylose and acetate, which show identical growth for the first 6 h when growth on

acetate alone begins to slow (Figure 2a). When acetate is used as the sole feedstock, significant decrease in acetate concentration starts after 3 h with complete depletion of the substrate occurring by 12 h (Figure 2a, gray bars). Endogenous acetate is detected when cells are grown on xylose alone after the first 3 h with minimal increase over the next 9 h (Figure 2, white bars). Growth on acetate and xylose shows slower decrease of acetate in the media compared to samples grown on acetate alone, and even after 12 h acetate concentration remains above 65% of initial amounts (Figure 2a, black bars). The profile of xylose depletion in the first 12 h for samples grown on xylose or xylose and acetate shows a similar pattern, with xylose concentration in the samples growth on xylose alone decreasing faster (Figure 2b).

Analytical and thermal characterization of PHAs

Analytical and thermal characterization studies were done on polymer extracted from cells that resulted in significant yields of PHA: cells grown with xylose alone, xylose and acetate added at inoculation, xylose and acetate added at $OD_{600}=0.6$, and acetate at inoculation and xylose added at $OD_{600}=0.6$ (samples 4, 8, 14, and 16). 1H -NMR spectra for the four samples (Figure S4) showed characteristic peaks for polylactate (PLA) and poly(3-hydroxybutyrate) (PHB) confirming that all cells produced the same polymer poly(lactate-*co*-3-hydroxybutyrate). Physical properties for the polymers such as molecular weight (M_n , M_w), polydispersity (M_n/M_w), and glass transition (T_g), crystallization (T_c), and melting (T_m) temperatures are summarized in Table S2. Samples grown on xylose and xylose and acetate added at $OD_{600}=0.6$ show the highest M_n and M_w , as well as the lowest T_g with 6.8 °C and 7.2 °C. The T_m of all the samples show similar values for samples 8, 16, and 14. However a bimodal melting peak was detected at 145 °C and 155 °C for sample 4 (Table S2).

Discussion

Pretreatment of hardwood lignocellulosic biomass is a necessary step for its efficient utilization as a feedstock in fermentative processes for the sustainable production of value-added petroleum-alternative chemicals. During the pretreatment process sugars that make up the biomass are made more accessible; however, degradation products (e.g. aldehydes, carboxylic acids, organic acids, phenols) that can interfere with enzymatic hydrolysis and fermentation processes are generated during pretreatment, leading to lower sugar and end-product yields (1, 29-33). Among the most common byproducts are furfural and hydroxymethylfurfural which are dehydration products of xylose from xylan and glucose from cellulose, respectively. Acetate is released from deacetylation of xylan and can reach concentrations of up to $10 \text{ g}\cdot\text{L}^{-1}$ (34), thus significantly increasing toxic burden in *E. coli* fermentations that secrete acetate into the culture medium produced from overflow metabolism of sugars, further inhibiting growth and product biosynthesis.

Acetate metabolism in *E. coli* is carried out at the acetyl-CoA node where phosphotransacetylase (PTA) converts acetate into acetylphosphate which is in turn metabolized to acetate by acetate kinase (AckA) with the generation of ATP (Figure 3) (20). Both reactions are reversible allowing for the recycling of acetate after sugar feedstock depletion. PTA has been shown to be down regulated in the presence of exogenous acetate, with a wild-type strain grown in acetate showing similar transcriptional responses to a *pta* mutant grown without acetate (35,36). Perturbations in acetyl-CoA flux causing accumulation of pyruvate, lactate, and acetyl CoA have been observed in *pta* mutants, which may prevent generation of metabolites for energy production by down regulating the tricarboxylic acid cycle resulting in reduced growth (37,38). On the other hand, acetate has been shown to up-regulate expression of genes involved in the glyoxylate bypass (39). An additional pathway for acetate uptake involves the irreversible conversion of acetate to

acetyl-CoA by acetyl-CoA synthase (ACS) at the cost of 2 high energy phosphate bonds (Figure 3) (20). In this work, four *E. coli* strains capable of utilizing xylose for the biosynthesis of PHAs were tested in the presence of acetate.

E. coli BW25113 has been used as a parental strain for development of the Keio collection single-gene knockouts (27,40) and along with its *pflA*⁻ derivative strain JW0885 have been shown to produce high quantities of PHAs from xylose with JW0885 showing increased incorporation of LA-monomers into the copolymer (22). The *pflA* gene encodes for the pyruvate formate lyase activator protein (PflA), which is involved in the regulation of formate biosynthesis from pyruvate (Figure 3) via its action on pyruvate formate lyase protein (PflB) (41-43). A close relationship between acetate and formate metabolic pathways in *E. coli* has been established, indicating that elimination of formate biosynthesis via deletion of *pflA* in turn affects acetate metabolism (35,36,44).

Acetate had an adverse effect in both strains in terms of overall PHA yields, but the nature of decrease was different for each. Lower yields for *E. coli* JW0885 were a result of significant inhibition in fermentation where PHA as wt% cell mass decreased 60% from yields obtained when xylose was the sole feedstock. Despite lowered fermentation, cell mass and LA-fraction for strain JW0885 were slightly higher with acetate supplementation. On the other hand, strain BW25113 showed a 35% decrease in cell mass, with only a 33% decrease in PHA (wt%) production and a slight decrease in LA incorporation.

E. coli LS5218 carries two mutations (*fadR*⁻ and *atoC*_(con)) that have been suggested to increase acetate recycling from the extracellular media. (45,46). Furthermore, this strain has been shown to be resistant to inhibition by furfural-derived compounds during PHA production (22) and to be capable of utilizing xylose for PHA production, albeit with low incorporation of LA-monomers into the copolymer (24). *E. coli* LS5218 showed slightly higher tolerance to acetate than strain BW25113 with a decrease in cell mass of only 20%

and only 26% in PHA (wt%) production. However, the LA-fraction in the copolymer was increased by 450%. This inverse relationship between LA-fraction and PHA (wt%) production has been reported in previous studies focused on production of LA-incorporating PHB-based copolymers. (22,47). Nevertheless, this inverse relationship does not solely account for a reduction in total PHA yields of >50% obtained from *E. coli* LS5218 grown in the presence of acetate, confirming the inhibitory effect of this substrate in PHA production and growth. Regardless of the nature of the inhibition (i.e. preventing growth and/or affecting fermentation metabolism), the results presented here show that *E. coli* BW25113 and JW0885 have a low tolerance for acetate as feedstock, while the improved acetate recycling of LS5218 cushions the adverse effects of acetate in this strain.

E. coli RSC10 is a *pflA* deficient derivative of strain LS5218 and it showed capacity to utilize acetate as a feedstock for PHA production in addition to tolerance for exogenous acetate in growth and fermentation (Tables 1, 2). This is hypothesized to be a result of the synergistic effect of the three main mutations present in the strain: *pflA*⁻, *fadR*⁻, and *atoC*_(con). Formate biosynthesis is a competing pathway for lactate formation, and it occurs via conversion of pyruvate to formate by PflB with release of acetyl-CoA. Elimination of this pathway results in increased lactate biosynthesis as observed by an increase in LA fraction in *E. coli* JW0885 and RSC10 (Tables 1, 2) (44,48). However, with inactivation of PTA due to the presence of acetate, fluxes of acetyl-CoA and pyruvate are possibly perturbed resulting in accumulation of these metabolites. This accumulation in turn causes down-regulation of the TCA cycle, which combined with acetate-induced up-regulation of the glyoxylate bypass (GB) helps explain the significantly lower PHA yields obtained from *E. coli* JW0885 (Tables 1, 2) (37-39).

The *atoC*_(con) and *fadR*⁻ mutations are hypothesized to alleviate the disturbance in acetyl-CoA/pyruvate fluxes resulting in the increased yields (both in cell mass and PHA

production) obtained from *E. coli* RSC10. *atoC* encodes the gene for the transcriptional regulator (AtoC) of the *atoDAEB* operon which includes a transporter for short-chained organic acids (i.e. acetate, propionate, butyrate) allowing for the bypass of acetate transport at the PTA-AckA/ACS pathway level (49). FadR is a regulator protein of the GB, and *fadR* deficient *E. coli* constitutively expresses the enzymes involved in GB (50,51). Additionally, this mutation has been shown to significantly increase activity of PTA even in the presence of exogenous acetate (35,45). This demonstrates the importance of FadR in acetate utilization and regulation of acetyl-CoA fluxes, alleviating metabolic stresses that cause decreases in cellular growth due to down-regulation of the TCA cycle, which can now progress freely to metabolize any accumulated pyruvate due to the *pflA* mutation. The results obtained from *E. coli* LS5218 (Tables 1, 2) support this hypothesis since an active formate biosynthetic pathway would increase acetyl-CoA concentrations at the pyruvate/Acetyl-CoA node affecting cellular growth (Figure 3). Additionally, when strain RSC10 was grown on acetate alone (Figure 2a), a complete depletion of acetate by 12 h was observed, suggesting that the strain is capable of utilizing acetate as a carbon source.

When *E. coli* RSC10 was exposed to higher concentrations of acetate, fermentation was more severely affected than growth as concentration increased (Fig. 2), similar to the effect observed in strain JW0885 in the presence of acetate. The basis for the severe inhibition in fermentation possibly stems from the same metabolic perturbances caused by increased acetyl-CoA, which, in the case of strain RSC10 are caused by high acetate concentrations leading to higher conversion of acetate to acetyl-CoA by ACS and even by a functional PTA. Higher yields were obtained when the addition of the substrates (i.e. xylose and acetate) into the media was delayed until the cells reached an approximate $OD_{600}=0.6$. In all cases total PHA yields were improved between 42% and 58%, and in all cases the increased yields were a result of increased fermentation (Table 4).

The negative effects of acetate in production of recombinant proteins in *E. coli* have been well established (19,21). It is hypothesized that by allowing the cells to grow to mid-exponential phase without acetate stress, either due to exogenous or endogenous acetate produced from xylose catabolism (Figure 2a), expression of PTC, PhaA, PhaB, and PhaC was higher resulting in the observed increased fermentation levels. It is worth noting that the time of addition of acetate plays a significant role in overall yields for the LA-fraction. Addition of acetate at inoculation results in a higher LA-fraction when the cells were grown with or without xylose supplementation. For example, when acetate was the only feedstock, LA incorporation was detected only in cells where acetate had been added at inoculation (Table 4). These results suggest that acetate may be potentially used as a regulator in LA-monomer incorporation into the copolymer thus allowing for the control in the composition and, therefore, the physical properties of LA-incorporating PHB-based polymers (47,52).

¹H-NMR analysis carried on samples extracted from four selected experiments based on the observed yields and composition (Table S2) confirmed that the polymers were P(LA-co-3HB) copolymers (Figure S4), with samples containing a higher LA-fraction, as determined by GC, showing higher integration values for LA peaks (47). The molecular weight (M_w) shows a trend where polymers with higher LA incorporation have a lower M_w fitting earlier reports showing the inverse relationship between LA fraction and molecular weight (22,47,53,54). This trend is maintained in the thermal properties where samples with lower LA-fraction have higher T_g and T_c . All polymers had similar T_m values that fall within expected ranges for PHB-based copolymers. Two melting temperatures were detected for sample 4 which is in accordance with values obtained for copolymers that incorporate LA, where the lower temperature is suggested to be due to the presence of LA (21,47,52).

Development of strategies to alleviate acetate toxicity in *E. coli* is of particular interest in the utilization of lignocellulosic biomass as feedstock for the biosynthesis of value-added

products. Ideally a strain would be not only be tolerant of acetate in the media that might be present as a derivative of xylan, but would also be capable of using acetate as a carbon source to power growth or to provide metabolites for fermentation. In this work, four strains known to produce PHAs from xylose were tested for acetate tolerance. *E. coli* RSC10, a strain developed during preliminary work for the present study (24) was found to be tolerant of acetate both in growth and fermentation. More importantly, this strain was found to be able to utilize acetate as a feedstock for biosynthesis of PHAs. Additionally, the present work provides data to support the potential of acetate as modulator for the control of composition of PHAs that incorporate LA monomers into the copolymer.

Acknowledgments

The authors are grateful to Dr. B.R. Lundgren (SUNY-ESF) and Christian Felt for technical advice for this study and David Kiemle for assistance with NMR studies. This study was supported by NSF CBET-1263905 awarded to C.T. Nomura.

References

1. Klinker, H., Thomsen, A., and Ahring, B. Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Appl. Microbiol. Biotechnol.* **66**, 10-26 (2004).
2. Zaldivar, J., Ingram, L.O. Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. *Biotechnol. Bioeng.* **66**, 203-210 (1999).
3. Zaldivar, J., Martinez, A., Ingram, L.O. Effect of alcohol compounds found in hemicellulose hydrolysate on the growth and fermentation of ethanologenic *Escherichia coli*. *Biotechnol. Bioeng.*, **68**, 524-530 (2000).
4. Zaldivar, J., Martinez, A., Ingram, L.O. Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. *Biotechnol. Bioeng.*, **65**, 24-33 (1999).

5. Takahashi, C. M., Takahashi, D. F., Carvalhal, M. L., and Alterthum, F. Effects of acetate on the growth and fermentation performance of *Escherichia coli* KO11. Appl. Microbiol. Biotechnol. **81**, 193-203 (1999).
6. Cantarella, M., Cantarella, L., Gallifuoco, A., Spera, A., and Alfani, F. Comparison of different detoxification methods for steam-exploded poplar wood as a substrate for the bioproduction of ethanol in SHF and SSF. Process Biochem. **39**, 1533-1542 (2004).
7. Wyman, C. E., Dale, B. E., Elander, R. T., Holtzapple, M., Ladisch, M. R., and Lee, Y. Y. Coordinated development of leading biomass pretreatment technologies. Bioresource Technol. **96**, 1959-1966 (2005).
8. Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K., Ramakrishnan, S. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. Enzyme Res., **2011**, 1-17 (2011).
9. Teleman, A., Tenkanen, M., Jacobs, A., and Dahlman, O. Characterization of *O*-acetyl-(4-*O*-methylglucurono) xylan isolated from birch and beech. Carbohydr. Res. **337**, 373-377 (2002).
10. Sjöström, E. *The structure of wood. In Wood chemistry: fundamentals and applications* Gulf Professional Publishing, San Diego, CA. (1993). pp 1-12.
11. Jurado, M., Prieto, A., Martínez-Alcalá, Á., Martínez, Á. T., and Martínez, M. J. Laccase detoxification of steam-exploded wheat straw for second generation bioethanol. Bioresource Technol. **100**, 6378-6384 (2009).
12. Chandel, A. K., Kapoor, R. K., Singh, A., and Kuhad, R. C. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresource Technol. **98**, 1947-1950 (2007).
13. Schwartz, T. J., and Lawoko, M. Removal of acid-soluble lignin from biomass extracts using Amberlite XAD-4 resin. BioResources. **5**, 2337-2347 (2010).
14. López, M. J., Nichols, N. N., Dien, B. S., Moreno, J., and Bothast, R. J. Isolation of microorganisms for biological detoxification of lignocellulosic hydrolysates. Appl. Microbiol. Biotechnol. **64**, 125-131 (2004).
15. Palmqvist, E., and Hahn-Hägerdal, B. Fermentation of lignocellulosic hydrolysates. I: inhibition and detoxification. Bioresource Technol. **74**, 17-24 (2000).

16. Sivers, M. V., Zacchi, G., Olsson, L., and Hahn-Hügerdal, B. Cost analysis of ethanol production from willow using recombinant *Escherichia coli*. *Biotechnol. Progr.* **10**, 555-560 (1994).
17. Lawford, H. G., and Rousseau, J. D. Effect of acetic acid on xylose conversion to ethanol by genetically engineered *E. coli*. *Appl. Microbiol. Biotechnol.* **34**, 185-204 (1992).
18. Roe, A. J., O'Byrne, C., McLaggan, D., and Booth, I. R. Inhibition of *Escherichia coli* growth by acetic acid: a problem with methionine biosynthesis and homocysteine toxicity. *Microbiology.* **148**, 2215-2222 (2002).
19. Shiloach, J., and Fass, R. Growing *E. coli* to high cell density—A historical perspective on method development. *Biotechnol. Adv.* **23**, 345-357 (2005).
20. Wolfe, A. J. The acetate switch. *Microbiol. Mol. Biol. Rev.* **69**, 12-50 (2005).
21. Eiteman, M. A., and Altman, E. Overcoming acetate in *Escherichia coli* recombinant protein fermentations. *Trends Biotechnol.* **24**, 530-536 (2006).
22. Nduko, J.M., Matsumoto, K., Ooi, T., Taguchi, S. Effectiveness of xylose utilization for high yield production of lactate-enriched P lactate- *co*-3-hydroxybutyrate) using a lactate-overproducing strain of *Escherichia coli* and an evolved lactate-polymerizing enzyme. *Metab. Eng.*, **15**, 159-66 (2012).
23. Salamanca-Cardona L., Ashe C.S., Stipanovic A.J., Nomura C.T. Enhanced production of polyhydroxyalkanoates (PHAs) from beechwood xylan by recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.*, **98**, 831-842 (2014).
24. Salamanca-Cardona, L., Scheel, R. A., Lundgren, B. R., Matsumoto, K., Taguchi, S., Stipanovic, A. J., Nomura, C. T. Deletion of the *pflA* gene in *Escherichia coli* LS5218 and its effects in the production of polyhydroxyalkanoates using beechwood xylan as feedstock. *Bioengineered*, **5**, 284-287 (2014).
25. Salamanca-Cardona, L., Scheel, R.A., Bergey, N.S., Stipanovic, A.J., Matsumoto, K., Taguchi, S, Nomura, C.T. Consolidated bioprocessing of poly(lactate-*co*-3-hydroxybutyrate) from xylan as a sole feedstock by genetically-engineered *Escherichia coli*. *J. Biosci. Bioeng.* **122**(4). 406-414 (2016).
26. Taguchi, S., Yamada, M., Matsumoto, K., Tajima, K., Satoh, Y., Munekata, M., Ohno, K., Kohda, K., Shimamura, T., and Kambe, H. A microbial factory for lactate-

- 533 based polyesters using a lactate-polymerizing enzyme. Proc. Natl. Acad. Sci. **105**,
534 17323-17327 (2008).
- 535 27. Datsenko, K. A., Wanner, B. L. One-step inactivation of chromosomal genes in
536 *Escherichia coli* K-12 using PCR products. Proc. Natl. Aca. Sci., **97**, 6640-6645
537 (2000).
- 538 28. Kiemle, D.J., Stipanovic, A.J., Mayo, K.E. Proton NMR methods in the
539 compositional characterization of polysaccharides. ACS Symp. Ser., **864**, 122-139
540 (2004).
- 541 29. Duarte, G.C., Moreira, L.R.S., Jaramillo, P.M.D., and Filho, E.X. Biomass-derived
542 inhibitors of holocellulases. Bioenerg. Res. **5**, 768-777 (2012).
- 543 30. Ximenes, E., Kim, Y., Mosier, N., Dien, B., and Ladisch, M. Inhibition of cellulases
544 by phenols. Enzyme Microb. Technol. **46**, 170-176 (2010).
- 545 31. Ximenes, E., Kim, Y., Mosier, N., Dien, B., and Ladisch, M. Deactivation of
546 cellulases by phenols. Enzyme Microb. Technol. **48**, 54-60 (2011).
- 547 32. Nakagame, S., Chandra, R., and Saddler, J. The effect of isolated lignins, obtained
548 from a range of pretreated lignocellulosic substrates, on enzymatic hydrolysis.
549 Biotechnol. Bioeng. **5**, 871-879 (2010).
- 550 33. Leu, S., and Zhu, J. Substrate-related factors affecting enzymatic saccharification of
551 lignocelluloses: our recent understanding. Bioenerg. Res. 1-11 (2012).
- 552 34. Deutschmann, R., and Dekker, R. F. From plant biomass to bio-based chemicals:
553 latest developments in xylan research. Biotech Adv. **30**, 1627-1640 (2012).
- 554 35. Kirkpatrick, C., Maurer, L. M., Oyelakin, N. E., Yoncheva, Y. N., Maurer, R., and
555 Slonczewski, J. L. Acetate and formate stress: opposite responses in the proteome of
556 *Escherichia coli*. J. Bacteriol. **183**, 6466-6477 (2001).
- 557 36. Zhu, J., and Shimizu, K. Effect of a single-gene knockout on the metabolic regulation
558 in *Escherichia coli* for D-lactate production under microaerobic condition. Metab.
559 Eng. **7**, 104-115 (2005).
- 560 37. Chang, D. E., Shin, S., Rhee, J. S., and Pan, J. G. Acetate metabolism in a *pta* mutant
561 of *Escherichia coli* W3110: Importance of maintaining acetyl coenzyme A flux for
562 growth and survival. J. Bacteriol. **181**, 6656-6663 (1999).

38. Kakuda, H., Shiroishi, K., Hosono, K., and Ichihara, S. Construction of Pta-Ack pathway deletion mutants of *Escherichia coli* and characteristic growth profiles of the mutants in a rich medium. *Biosci. Biotech. Biochem.* **58**, 2232-2235 (1994).
39. Oh, M. K., Rohlin, L., Kao, K. C., and Liao, J. C. Global expression profiling of acetate-grown *Escherichia coli*. *J. Biol. Chem.* **277**, 13175-13183 (2002).
40. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Mori, H. Construction of *Escherichia coli* K12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.*, **2**, 2006:0008 (2006).
41. Sawers, G. A. R. Y., and Böck, A. Anaerobic regulation of pyruvate formate-lyase from *Escherichia coli* K-12. *J. Bacteriol.* **170**, 5330-5336 (1988).
42. Knappe, J., and Sawers, G. A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *FEMS Microbiol. Lett.* **75**, 383-398 (1990).
43. Crain, A. V., and Broderick, J. B. Pyruvate formate-lyase and its activation by pyruvate formate-lyase activating enzyme. *J. Biol. Chem.* **289**, 5723-5729 (2014).
44. Hasona, A., Kim, Y., Healy, F. G., Ingram, L. O., Shanmugam, K. T. Pyruvate formate lyase and acetate kinase are essential for anaerobic growth of *Escherichia coli* on xylose. *J. Bacteriol.*, **186**, 7593-7600 (2004).
45. Maloy S.R., Nunn W.D. Role of gene *fadR* in *Escherichia coli* acetate metabolism. *J. Bacteriol.* **148**, 83-90 (1981).
46. Spratt, S.K., Ginsburgh, C.L., Nunn, W.D. Isolation and genetic characterization of *Escherichia coli* mutants defective in propionate metabolism. *J. Bacteriol.*, **146**, 1166-1169 (1981).
47. Yamada, M., Matsumoto, K., Uramoto, S., Motohashi, R., Abe, H., Taguchi, S. Lactate fraction dependent mechanical properties of semitransparent poly lactate- co-3-hydroxybutyrate) s produced by control of lactyl-CoA monomer fluxes in recombinant *Escherichia coli*. *J. Biotechnol.*, **154**, 255-260 (2011).
48. Utrilla, J., Gosset, G., Martinez, A. ATP limitation in a pyruvate formate lyase mutant of *Escherichia coli* MG1655 increases glycolytic flux to D-lactate. *J. Ind. Microbiol. Biot.*, **36**, 1057-1062 (2009).
49. Jenkins, L. S., and Nunn, W. D. Regulation of the *ato* operon by the *atoC* gene in *Escherichia coli*. *J. Bacteriol.* **169**, 2096-2102 (1987).

- 595 50. Maloy, S. R., Bohlander, M., and Nunn, W. D. Elevated levels of glyoxylate shunt
596 enzymes in *Escherichia coli* strains constitutive for fatty acid degradation. J.
597 Bacteriol. **143**, 720-725 (1980).
- 598 51. Gui, L., Sunnarborg, A., and LaPorte, D. C. Regulated expression of a repressor
599 protein: FadR activates *iclR*. J. Bacteriol. **178**, 4704-4709 (1996).
- 600 52. Yamada, M., Matsumoto, K. I., Shimizu, K., Uramoto, S., Nakai, T., Shozui, F.,
601 Taguchi, S. Adjustable mutations in lactate LA)-polymerizing enzyme for the
602 microbial production of LA-based polyesters with tailor-made monomer
603 composition. Biomacromolecules, **11**, 815-819 (2010).
- 604 53. Song, Y., Matsumoto, K. I., Yamada, M., Gohda, A., Brigham, C. J., Sinskey, A. J.,
605 Taguchi, S. Engineered *Corynebacterium glutamicum* as an endotoxin-free platform
606 strain for lactate-based polyester production. Appl. Microbiol. Biotechnol., **93**, 1917-
607 1925 (2012).
- 608 54. Shozui, F., Matsumoto, K. I., Nakai, T., Yamada, M., Taguchi, S. Biosynthesis of
609 novel terpolymers poly (lactate-co-3-hydroxybutyrate-co-3-hydroxyvalerate) s in
610 lactate-overproducing mutant *Escherichia coli* JW0885 by feeding propionate as a
611 precursor of 3-hydroxyvalerate. Appl. Microbiol. Biotechnol., **85**, 949-954 (2010).

Figure Legends

Figure 1. Cell dry weight (CDW, gray bars) and PHA as a fraction of cell mass (wt%, black bars) yields from *E. coli* RSC10 harboring pTVN118*pctphaC1*(ST/QK)AB grown in LB media supplemented with xylose (20 g·L⁻¹) and varying concentrations of acetate (0-100 mM). As the concentration of acetate increases from 25 mM fermentation is more negatively affected than growth.

Figure 2. ¹HNMR analysis of acetate (a) and xylose (b) concentration in the media over the first 12 h of growth of *E. coli* RSC10 cells harboring pTVN118*pctphaC1*(ST/QK)AB grown in LB media supplemented with xylose (20 g·L⁻¹, white bars), acetate (25 mM, gray bars), or xylose and acetate (black bars). Growth of the cells at every 3h is shown for cells grown in xylose alone (squares), acetate alone (circles) or acetate and xylose (triangles)

648

649 **Figure 3.** Schematic representation of the metabolic pathways for PHA production from
650 xylose and acetate as feedstock by *E. coli* RSC10. The PHA-producing enzymes PCT, PhaA,
651 PhaB, and PhaC were engineered into *E. coli* for the synthesis of P(LA-co-3HB). PflA –
652 pyruvate formate lyase activator, PflB – pyruvate formate lyase, PDC – pyruvate
653 decarboxylase complex, LDH – lactate dehydrogenase, PTA – phosphotransacetylase, AckA
654 – acetate kinase, ACS – acetyl-CoA synthetase, PCT – propionyl-CoA transferase, PhaA - β -
655 ketothiolase, PhaB - NADPH-dependent acetoacetyl-CoA reductase, PhaC – PHA synthase.
656

PENTOSE PHOSPHATE PATHWAY

GLYCOLYSIS

FORMATE

PYRUVATE

ACETYL-CoA

ACETYLPHOSPHATE

ACETATE

LACTATE

LACTYL-CoA

R-3-HYDROXYBUTYRYL-CoA

POLY(LACTATE-co-3-HYDROXYBUTYRATE)