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

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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.

[**Key words:** Polyhydroxyalkanoate; Acetate; Xylose; Recombinant *Escherichia coli*; Biomass; Hemicellulose]

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*-3-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

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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 pyruvate formate lyase (*pflA*) gene was confirmed using check primers: 5′-GCCACATCTGGAGAAACACC-3′ (forward primer) and 5′-AGAATGAAGCGCGGAATAAA-3′ (reverse primer). The plasmid pTVN118*pctpha*C1(ST/QK)AB (26) 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 $\cdot mL^{-1}$). To assess the effect of acetate on PHA production from xylose (Fisher Scientific, Hanover Park, IL, USA), 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*pctpha*C1(ST/QK)AB from xylose supplemented with acetate *E. coli* strains BW25113, JW0885, LS5218 and RSC10 were transformed with pTVN118*pctpha*C1(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*pctpha*C1(ST/QK)AB plasmid was measured by performing a control experiment using glucose (20 g$\cdot L^{-1}$) 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$\cdot L^{-1}$, samples 1–4) or xylose (20 g$\cdot L^{-1}$) and sodium acetate (25 mM, 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 ×*g* for 15 min. The cells were resuspended and washed twice in Nanopure water generated by a Barnstead Water Purification System (Fisher Scientific), 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*pctpha*C1(ST/QK)AB was grown for 15 h in LB media and 1 mL of this seed culture was used to inoculate 100 mL of LB supplemented with xylose (20 g$\cdot L^{-1}$). 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$\cdot L^{-1}$) at inoculation or at mid-exponential growth phase (OD_{600} = 0.6); acetate (25 mM) at inoculation or at OD_{600} = 0.6 (these four conditions were used as control; samples 4, 19, 17 and 18 respectively); xylose (20 g$\cdot L^{-1}$) and acetate (25 mM) added at inoculation (sample 8); xylose (20 g$\cdot L^{-1}$) and acetate (25 mM) added at OD_{600} = 0.6 (sample 14); xylose (20 g$\cdot L^{-1}$) added at inoculation and acetate (25 mM) added at OD_{600} = 0.6 (sample 15); and acetate (25 mM) added at inoculation and xylose (20 g$\cdot L^{-1}$) added at OD_{600} = 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 ×*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 analysis Gas chromatography (GC) 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$\cdot L^{-1}$) as an internal standard. The parameters for GC analysis were as follows: 1 μL injection volume, 30 m *Rtx-5* column (Restek, State College PA, USA), 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$\cdot min^{-1}$, ramp up to 200°C at 5°C$\cdot min^{-1}$, and increase to final temperature of 280°C at 30°C$\cdot min^{-1}$and hold for 2 min. Analysis was carried by split injection in a GC2010 gas chromatograph with a flame ionization detector (Shimadzu North America, Marlborough, MA, USA).

Nuclear magnetic resonance analysis Proton nuclear magnetic resonance (^{1}H 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$\cdot L^{-1}$), and xylose (20 g$\cdot L^{-1}$) 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, MA, 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 ^{1}H 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 (Thermofisher Acros Organics, Geel Belgium) 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 analysis Gel permeation chromatography (GPC) was used to determine weight-average molecular weight (M_w), number-average molecular weight (M_n) and polydispersity ($Đ_M = M_w/M_n$) 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) 8 × 300 mm with 5 μm porosity column (Polymer Standards Service, Mainz, Germany), 40°C column oven temperature, chloroform mobile phase at 1 mL$\cdot min^{-1}$ flow rate. The analysis was done with a LC-20 AD Liquid Chromatograph with a RID-10A refractive index detector (Shimadzu North America), using a polystyrene standard.

Differential scanning calorimetry analysis Glass transition (T_g), melting (T_m), and crystallization (T_c) temperatures were determined by differential scanning calorimetry (DSC) as previously described (21). Approximately 2 mg of polymer were loaded onto a Tzero aluminum pan (TA Instruments, New Castle, DE, USA) and heated to 200°C at 10°C$\cdot min^{-1}$, then cooled to −80°C at 5°C$\cdot min^{-1}$, followed by temperature increase to 200°C at 10°C$\cdot min^{-1}$. Analysis was carried out under a nitrogen atmosphere with a flow rate of 50 mL$\cdot min^{-1}$ 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 pTVN118*pctpha*C1(ST/QK)AB from xylose supplemented with acetate *E. coli* strains BW25113, LS5218 and their respective *pflA* mutants JW0885 and RSC10 harboring pTVN118*pctpha*C1(ST/QK)AB were grown in LB media supplemented with xylose or xylose and acetate to assess the effect of

TABLE 1. PHA accumulation in *E. coli* strains BW25113, LS5218, JW0885, and RSC10 harboring pTVN118*pctpha*C1(ST/QK)AB grown at 30°C and 250 rpm with xylose (20 g$\cdot L^{-1}$) as feedstock.

Sample	Strain	CDW (g L^{-1})	PHA (wt%)	PHA composition (mol%)[a]		Total PHA (g L^{-1})
				2HP	3HB	
1	BW25113	6.3 ± 0.0	60.4 ± 0.2	20.3 ± 0.1	79.7 ± 0.1	3.8 ± 0.0
2	JW0885	2.0 ± 0.0	51.2 ± 0.1	26.0 ± 0.1	74.0 ± 0.1	1.0 ± 0.0
3	LS5218	6.4 ± 0.4	49.0 ± 3.6	2.1 ± 0.1	97.9 ± 0.1	3.2 ± 0.2
4	RSC10	2.3 ± 0.2	47.9 ± 0.1	6.4 ± 0.8	93.2 ± 0.0	1.1 ± 0.2

All values are averages of duplicate experiments plus or minus the standard deviation about those averages.

[a] 2HP, 2-hydroxypropionate (lactate); 3HB, 3-hydroxybutyrate.