Deletion of the \(\text{pflA}\) gene in *Escherichia coli* LS5218 and its effects on the production of polyhydroxyalkanoates using beechwood xylan as a feedstock

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Engineering of microorganisms to directly utilize plant biomass as a feedstock for the biosynthesis of value-added products such as bioplastics is the aim of consolidated bioprocessing. In previous research we successfully engineered *E. coli* LS5218 to produce polyhydroxyalkanoates (PHAs) from xylan. In this study we report further genetic modifications to *Escherichia coli* LS5218 in order to increase the lactic acid (LA) fraction in poly(lactic acid-co-3-hydroxyalkanoate) P(LA-co-HA) copolymers. Deletion of the \(\text{pflA}\) gene resulted in increased content of LA repeating units in the copolymers by over 3-fold compared with the wild type; however, this increase was offset by reduced yields in cell mass. Additionally, when acetate was used as a feedstock LA monomer incorporation reached 18.5 (mol%), which suggests that acetate can be used as a feedstock for the production of P(LA-co-HA) copolymers by *E. coli*.

Keywords: polyhydroxyalkanoates, xylanases, recombinant *Escherichia coli*, xylan, biomass, hemicellulose, acetate, pyruvate formate lyase

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**Introduction**

Consolidated bioprocessing (CBP) is a proposed approach to engineer microorganisms that are capable of utilizing hemicellulosic biomass for the biosynthesis of value-added products in a single microbial event.\(^1,2\) Previously we reported the first ever biosynthesis of polyhydroxyalkanoates (PHAs) by *E. coli* from hemicellulosic biomass (i.e., beechwood xylan) in an effort to obtain an *E. coli* CBP system for the production of bioplastics.\(^3\) *E. coli* was engineered to express and secrete xylanases to hydrolyze hemicellulosic xylan into xylose units to be metabolized by the same microorganism into poly(lactate-co-3-hydroxybutyrate) [P(LA-co-3HB)].\(^3\) Beechwood xylan is composed mainly of xylose units but it also contains up to 29% (w/w) acetate.\(^4\) Due the high content of xylan-derived acetate that can be hydrolyzed into the media, *E. coli* LS5218 was selected for development of the CBP system. This strain has two significant mutations, \(\text{atoC}_{\text{mut}}\) and a \(\text{fadR}\), that have been suggested to increase acetate recycling from the extracellular media.\(^5,6\) Our previously reported system relied on using either xylose or arabinose to delay uptake of xylan-derived xylose by the cells to increase yields of poly(lactate-co-3-hydroxybutyrate) compared with using xylose or arabinose alone as a feedstock.\(^3\)

Nduko et al.\(^7\) showed that using xylose as a feedstock for the production of P(LA-co-3HB) resulted in higher incorporation of LA into the copolymer compared with using glucose as a feedstock, making hemicelluloses an attractive substrate for the consolidated bioprocessing of PLA-based bioplastics. A separate study by Nduko et al. further showed that deletion of the \(\text{pflA}\) gene, a regulator of a competing pathway to lactic acid biosynthesis in *E. coli*, resulted in increased LA monomer incorporation when compared with the
wild type host strain. Our previous work showed that while the *E. coli* LS5218 system is capable of using xylan for the production of PHAs, incorporation of LA into the copolymer remained low. In this work, we report the deletion of the *pflA* gene in *E. coli* LS5218 and its effect on biosynthesis of P(LA-co-3HB) using the previously developed method for the utilization of beechwood xylan as a feedstock.

### Results and Discussion

Pyruvate is the end product of glycolysis in *E. coli* and can be further metabolized to acetyl-CoA for entry to other metabolic pathways including the tricarboxylic acid cycle, fatty acid biosynthesis, or acetate formation. However, pyruvate can also be metabolized to lactic acid directly by lactate dehydrogenase and to formate by pyruvate formate lyase (*PflB*). The production of formate is a competing reaction for the synthesis of lactic acid and acetyl-CoA and the enzyme responsible for this metabolic conversion (*PflB*) is regulated by pyruvate formate lyase activating protein (*PflA*). Deletion of the *pflA* gene should inactivate formate biosynthesis and increase flux of pyruvate into lactate and acetyl-CoA formation, effectively increasing the pool of LA precursors available for incorporation into P(LA-co-3HB) in the engineered *E. coli*. This suggests that deletion of *pflA* in *E. coli* LS5218 engineered for the biosynthesis of P(LA-co-3HB) from xylan is expected to result in higher incorporation of LA in the copolymer.

Experiments using *E. coli* RSC10 engineered to utilize xylan as a feedstock demonstrated that inactivation of *pflA* resulted in increased yields of LA monomer incorporation in the P(LA-co-3HB) copolymer when either xylene alone or xylene supplemented with xylan was used as feedstocks. Results from the P(LA-co-3HB) copolymer production experiments are summarized in Table 1. P(LA-co-3HB) copolymers synthesized in *E. coli* LS5218 incorporated 2.5 (mol%) and 2.1 (mol%) LA when grown in xylose alone and xylose supplemented with xylan, respectively. P(LA-co-3HB) copolymers produced by in *E. coli* RSC10 had a 3-fold increase in LA monomer incorporation, reaching 8.3 (mol%) and 7.8 (mol%) using xylene alone and xylene with xylan supplementation to the media. When xylene alone was used as feedstock, PHA biosynthesis was minimal for the wild type (*E. coli* LS5218) and the *pflA* mutant (*E. coli* RSC10) and there was no detectable LA incorporation in the polymers produced. Total cell mass yields from cells grown in xylene alone were slightly lower in the mutant (2.6 g∙L⁻¹ compared with 3.1 g∙L⁻¹ in the wild type). Cell mass yields were considerably lowered in *E. coli* RSC10 compared with the wild type when the cells were grown in xylene alone (1.9 g∙L⁻¹ and 7.0 g∙L⁻¹ respectively) or xylene supplemented with xylan (4.1 g∙L⁻¹ and 8.9 g∙L⁻¹ respectively). Further, as a control, cells were grown in glucose where cell mass yields reached 2.2 g∙L⁻¹ with total PHA accumulation of 45.1 (wt%) and LA incorporation of 0.9 (mol%). Despite the observed drop in cell mass yields in the mutant, PHA production as a percentage of total cell mass remained comparable to yields observed in the wild type *E. coli* LS5218 host strain. Because xylan can contain up to 29% (w/w) acetate (in the form of acetate esters that can be cleaved during pretreatment of biomass) exogenous acetate was used as a co-feedstock with xylene in order to mimic conditions resulting from addition of xylan to LB media. PHA production was low under these conditions reaching only 4.2 (wt%) but cell mass yields were improved (2.5 g∙L⁻¹) compared with using xylene alone. Surprisingly, LA incorporation from acetate supplementation reached 18.5 (mol%), which is >2-fold increase in LA incorporation compared with using xylene alone.

The results obtained from the growth experiments demonstrate that the inactivation of the pyruvate formate lyase pathway (Fig. 1) results in increased incorporation of LA into the copolymer when using xylene as a feedstock. However, after-fold increase in LA monomer incorporation in the P(LA-co-HB) copolymers produced by the *E. coli* RSC10 compared with *E. coli* LS5218, when used as a host strain, was offset by significantly lower yields in total cell mass. Deletion of the *pflA* gene in

### Table 1. PHA accumulation in *E. coli* LS5218 (LS) and RSC10 (RSC) harboring pTVN118pctphoC(ST/QK)AB and pBBRX8B2 grown at 30 °C grown on xylose, xylan, xylene, glucose, or xylene and acetate

| Sample               | CDW (g∙L⁻¹) | PHA (wt %) | PHA composition (mol%)* | Total PHA (g∙L⁻¹) |
|----------------------|-------------|------------|--------------------------|-------------------|
| LSXylose             | 7.0 ± 0.3   | 46.3 ± 1.5 | 2.5 ± 0.1                | 3.3 ± 0.1         |
| LSXylose/Xylan       | 8.9 ± 0.2   | 40.4 ± 1.2 | 2.1 ± 0.1                | 3.7 ± 0.1         |
| LSXylan              | 3.1 ± 0.0   | 1.1 ± 0.4  | 0                        | 0.03 ± 0.0        |
| RSCXylose            | 1.9 ± 0.1   | 41.1 ± 2.1 | 8.3 ± 0.5                | 0.8 ± 0.0         |
| RSCXylose/Xylan      | 4.1 ± 0.3   | 37.3 ± 7.0 | 7.8 ± 0.8                | 1.5 ± 0.2         |
| RSCXylan             | 2.6 ± 0.0   | 0.7 ± 0.0  | 0                        | 0.02 ± 0.0        |
| RSCGlucose           | 2.2 ± 0.1   | 45.1 ± 3.5 | 0.9 ± 0.1                | 1.0 ± 0.1         |
| RSCXylene/Acetate    | 2.5 ± 0.1   | 4.2 ± 0.3  | 18.5 ± 0.4               | 0.1 ± 0.0         |

All values are averages of triplicate experiments plus or minus the standard deviation about those averages. Cells were grown on LB supplemented with the respective feedstock to OD600 0.7 and IPTG induced (0.1 mM) and harvested at 48 h post inoculation. 2HP 2-hydroxypropionate (lactate); 3HB 3-hydroxybutyrate. *Data published previously.*

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E. coli LS5218 could potentially disturb the flux of pyruvate at the end of glycolysis (Fig. 1) leading to accumulation of pyruvate in the extracellular media thus lowering the amount of pyruvate available to enter the TCA cycle resulting in less energy accessible to power metabolism for cell proliferation. This is supported by the observed low yields obtained from the mutants grown in glucose alone and the final pH of the media for E. coli RSC10 cells grown on xylose or glucose alone, especially when compared with final pH values for the E. coli LS5218 (Table 2). Additionally, E. coli RSC10 cells grown in xylose supplemented with xylan produced higher cell mass yields and the final pH of the media remained closer to values observed for E. coli LS5218 under the same conditions suggesting less accumulation of acids in the extracellular medium and more energy available for cell growth. This could be a result of xylan-derived acetate present in the media that can be recycled into the cells as a result of the fadR and atoC (con) mutations in the strain.5,6 Further, xylose metabolism in E. coli has been shown to be affected by acetate metabolism along with disruptions in the pyruvate formate lyase pathways.12 The resulting yields obtained from growing the cells in xylose and acetate strongly suggest that acetate plays an important role in controlling LA monomer incorporation into the copolymers and that acetate could potentially be used by E. coli as a feedstock for the production of PHAs that incorporate lactate monomers.

Conclusions

Disruption of the pyruvate formate lyase pathway in E. coli LS5218 led to higher incorporation of LA monomers in the
P(LA-co-PHB) copolymer. Additionally, the studies performed using *E. coli* RSC10 for the production of PHAs from xylan as a feedstock have provided interesting data regarding the potential role that acetate can play in biosynthesis of PHAs by *E. coli*, particularly when hemicellulloses (which contain a considerable amount of acetate) are the desired substrate.

**Methods**

In order to increase LA monomer yields in our system the pflA gene was inactivated in *E. coli* LS5218 according to the protocol developed by Datsenko and Wanner.9 Gene-knockout primers used in the inactivation of the pflA gene: 5′-ATGTCATATA TGGTCCCGAT TACACTCTTT GAATCCTGTA GTGAGGGCTG GAGCTGTTCT-3′ (forward primer), 5′-TGAAGACATT ACCTATGGAC GGTACGGCTC AAGAATGCCG TTACCCCCGC TTTGGCCGAC-3′ (reverse primer). Homologous regions to the pflA gene sequence are underlined. Insertion of the kanamycin cassette at the pflA locus was confirmed with the following primers: 5′-CAGTCATAGC CGAATAGCCT-3′ and 5′-CGGTGCCCTG GCCACATCTG GAGAAACACC-3′ (forward primer) and 5′-AGAATGAAGC GCAGAATAAA-3′ (reverse primer).

The resulting mutant was designated *E. coli* RSC10 and was used in production of P(LA-co-3HB) from beechwood xylan with the same recombinant system and growth conditions previously reported.3 Briefly, *E. coli* RSC10 was co-transformed with the plasmids pBBRXBB2 and pTVN118cpctphaC(ST/OK)AB which harbor genes for xylan degradation and P(LA-co-3HB) biosynthesis respectively. Selection of successful co-transformants was done using ampicillin and kanamycin to a final concentration of 100 µg·mL−1 and 50 µg·mL−1 respectively. PHA production from xylan was carried by growing the co-transformants in Luria Bertani (LB) media supplemented with xylose, xylose and xylan, and xylan alone for 48 h at 30 °C and 250 rpm with orbital shaking. Control experiments were carried using glucose as a known feedstock for polymer biosynthesis or using acetate (33 mM) and xylose (20 g·L−1) as feedstocks to emulate carbon that would be derived from beechwood xylan.10 The expression of genes for polymer production was induced with IPTG to a final concentration of 0.1 mM at an approximate OD600 of 0.7. At the end of the growth period the cells were collected by centrifugation and freeze-dried. Composition of polymers produced by the co-transformants in each media was determined by gas chromatography (GC) as previously described.3

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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**Table 2.** Initial and final pH of the extracellular media for selected growth conditions of *E. coli* LS5218 (LS) and RSC10 (RSC) harboring pTVN118cpctphaC(ST/OK)AB and pBBRXBB2 grown at 30 °C grown on xylose, xylose and xylan, xylan, glucose, or xylose and acetate

| Sample                     | Initial pH | Final pH |
|----------------------------|------------|----------|
| LSXylose                   | 7.0 ± 0.0  | 8.5 ± 0.2|
| LSXylose/Xylan             | 6.5 ± 0.0  | 8.6 ± 0.3|
| RSCXylose                  | 7.0 ± 0.1  | 4.9 ± 0.1|
| RSCXylose/Xylan            | 6.5 ± 0.0  | 6.8 ± 0.2|
| RSCGlucose                 | 7.0 ± 0.1  | 4.9 ± 0.3|
| RSCXylose/Acetate          | 6.9 ± 0.1  | 8.1 ± 0.2|

All values are averages of triplicate experiments plus or minus the standard deviation about those averages. *Values recorded before inoculation with overnight seed culture. †Values recorded at 48 h post inoculation.*