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Consolidated bioprocessing of poly(lactate-*co*-3-hydroxybutyrate) from xylan as a sole feedstock by genetically-engineered *Escherichia coli*

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Consolidated bioprocessing of lignocellulose is an attractive strategy for the sustainable production of petroleum-based alternatives. One of the underutilized sources of carbon in lignocellulose is the hemicellulosic fraction which largely consists of the polysaccharide xylan. In this study, *Escherichia coli* JW0885 (pyruvate formate lyase activator protein mutant, *pflA*$^{-}$) was engineered to express recombinant xylanases and polyhydroxyalkanoate (PHA)-producing enzymes for the biosynthesis of poly(lactate-*co*-3-hydroxybutyrate) [P(LA-*co*-3HB)] from xylan as a consolidated bioprocess. The results show that *E. coli* JW0885 was capable of producing P(LA-*co*-3HB) when xylan was the only feedstock and different feeding and growth parameters were examined in order to improve upon initial yields. The highest yields of P(LA-*co*-3HB) copolymer obtained in this study occurred when xylan was added during mid-exponential growth after cells had been grown at high shaking—speeds (290 rpm). The results showed an inverse relationship between total PHA production and LA-monomer incorporation into the copolymer. Proton nuclear magnetic resonance (^{1}H NMR), gel permeation chromatography (GPC), and differential scanning calorimetry (DSC) analyses corroborate that the polymers produced maintain physical properties characteristic of LA-incorporating PHB-based copolymers. The present study achieves the first ever engineering of a consolidated bioprocessing bacterial system for the production of a bioplastic from a hemicelluosic feedstock.

[**Keywords:** Polyhydroxyalkanoates; Xylanases; Recombinant *Escherichia coli*; Xylan; Biomass; Hemicellulose; Consolidated bioprocessing]

In recent years there has been increased interest in the use of woody or lignocellulosic biomass feedstocks to replace petroleum as a source for commodity products such as biofuels and bioplastics. Current commercial technologies to exploit lignocellulosics as a feedstock consist of three distinct steps: (i) pretreatment of biomass, (ii) enzymatic hydrolysis to release sugar monomers, and (iii) fermentation of biomass-derived sugars by microbes (1,2). This process may be made more efficient and environmentally friendly if the last two steps are combined. With this approach, enzymatic hydrolysis and fermentation are carried out in a single microbial event via a (1,3) Consolidated Bioprocessing (CBP) of the substrate to product. CBP consists of either (i) genetically engineering a microorganism to express and secrete biomass-hydrolyzing enzymes to generate single sugars that can be taken up by the same microbes and fermented into the desired bioproduct using a fermentative pathway native to the host microbe, or alternatively (ii) by engineering a desired fermentative pathway into microbes that natively degrade lignocellulosic biomass, or (iii) via a combination of both approaches (3,4).

Hardwood lignocellulose contains in its structure two polysaccharides: cellulose and xylan, a hemicellulose (5). Initial research efforts to develop CBP systems have mainly focused on cellulose utilization (4,6,7), but xylan is generally considered to be underutilized. Xylan has historically been used as a source for xylose in the production of xylitol and it has only recently emerged as potential feedstock for the production of petroleum-alternative products (8,9). Xylan is composed primarily of a main homopolymeric backbone of β-1,4 linked xylopyranose units and varying substituent branching groups the composition of which are species dependent. Acetylation in xylan is present in 7 out of 10 xylose monomers (5,8). The sugars present in xylan can be made accessible for microbial fermentation via hydrolysis of glycosidic bonds by xylanases (10,11). It has been proposed that efficient utilization of xylan as feedstock for the bio-production of petroleum-alternative products can be accomplished via a CBP strategy (7,11). In the CBP of xylan to value-added products, *Escherichia coli* has widely been the chosen microorganism due to its native ability to ferment xylose (12) and many strains have already been optimized for the biosynthesis of various commodity chemicals (13–17). In addition, xylanolytic strains of *E. coli* can be engineered by cloning xylanase-encoding genes into the microorganism (18–21). Quasi-CPB strategies with recombinant xylanolytic *E. coli* strains have been reported for the production of fatty-acid methyl-

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esters and increased production of succinate (18,19). In addition, using a binary microbial system, bioethanol production from xylan has also been achieved (21). However, these quasi-CBP systems required a single-sugar co-feedstock to supplement xylan and resulted in very low product yields. We previously achieved efficient utilization of commercial xylan as a co-feedstock in the production of bioplastics (i.e., polyhydroxyalkanoates, PHAs). PHA-producing *E. coli* LS5218 was genetically engineered to express two xylanase genes which were demonstrated to have extracellular activity when transformed into the microbe (20). PHA production was obtained when either xylose or arabinose was used as a co-feedstock in order to delay uptake of xylan-derived xylose via catabolite repression (20). With this approach production of up to 0.6 g/L of PHA bioplastic was obtained when xylan was supplemented with arabinose compared to 0.03 g/L when xylan was the only feedstock (20).

Despite these recent advances, a strictly consolidated bioprocessing system for xylan, (i.e., one that uses xylan as the sole feedstock for fermentation of bioproducts) has not been reported in the literature. Our previous results suggested that a strictly CBP system can be obtained by allowing the build-up of xylan-derived xylose in the media in order to divert excess substrate to anabolic pathways after energetic requirements for growth were met (20). Expanding upon our previous work, in the present study we tested our xylanolytic plasmid system in PHA-producing *E. coli* strain JW0885. *E. coli* JW0885 carries a mutation for the pyruvate formate lyase activator protein (*pflA*$^{-}$) and was selected for this study because it has been shown to have slower growth rates when xylose was used as feedstock (22,23). We relied on the slower growth rate of the strain in xylose to allow for the accumulation of xylan-derived xylose in the media and bypass the need of a co-feedstock to trigger catabolite repression.

E. coli JW0885 was genetically engineered to express an endoxylanase (XylB) from *Streptomyces coelicolor* and a β-xylosidase (XynB) from *Bacillus subtilis* to obtain xylanolytic *E. coli* strains. The *E. coli* strain JW0885 was additionally 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 PHA polymers. The co-expression of the xylanase system and the P(LA-*co*-3HB)-producing system in *E. coli* JW0885 show the strain to be capable of producing P(LA-*co*-3HB) when xylan was the only feedstock. Therefore, this study demonstrates first, the proof-of-concept of delaying xylose uptake for the efficient utilization of xylan as a fermentation feedstock, and second successful consolidated bioprocessing of polyhydroxyalkanoates by *E. coli* from xylan as a sole feedstock.

MATERIALS AND METHODS

Media and cultivation conditions All bacterial strains and plasmids are listed in Table 1. Removal of the kanamycin cassette from JW0885-1 to generate *E. coli* JW0885 was done according to the protocol developed by Datsenko and Wanner (24). Following successful removal of the kanamycin cassette via FLP recombination, removal of the *pflA* gene was confirmed using check primers: 5′-GCCACATCTG GAGAAACACC-3′ (forward primer) and 5′-AGAATGAAGCGCGGAATAAA-3′ (reverse primer). The plasmids pTVN118pctphaC1(ST/QK)AB and pBBRXBB2 were used for PHA production and xylan degradation, respectively. *E. coli* JW0885 were grown in Lennox Broth (LB, Difco) and for PHA production from xylan the media was supplemented with ampicillin (100 $\mu g \cdot mL^{-1}$) and kanamycin (50 $\mu g \cdot mL^{-1}$). Xylan supplementation was done using commercial beechwood xylan (>90% xylan, non-delignified, Sigma). All transformation, growth, and PHA production experiments were carried at 30 °C.

Assessment of growth rates of *E. coli* JW0885 strains with xylose as the only sugar feedstock Single colonies of *E. coli* JW0885 were inoculated separately into 50 mL of LB and grown at 30 °C and 250 rpm in a rotary shaker for 20 h. One milliliter aliquots of each culture were inoculated into 100 mL of LB media in 500 mL baffled flasks supplemented with xylose (20 $g \cdot L^{-1}$). In order to confirm that *E. coli* JW0885 has a lower growth rate when xylose is present in the media, a control experiment was carried by growing the strain in LB media without any sugar supplementation. All experiments were carried in triplicate and incubated at 30 °C for 48 h in a rotary shaker at 250 rpm. In order to compare growth rates between cells grown in media supplemented with xylose and media without sugar supplementation, the optical density (OD) at 600 nm every 1.5 h from the time of inoculation until the first 12 h and then every 12 h until the cells were growing for a total of 48 h.

Production of PHA from xylan by *E. coli* JW0885 harboring pTVN118pctSTQKAB and pBBRXBB2 *E. coli* JW0885 was transformed with pTVN118pctphaC1(ST/QK)AB (25) and pBBRXBB2 via electroporation (both plasmids were transformed simultaneously) and fresh transformants were used for all experiments. Successful transformants were selected by growth on LB plates supplemented with ampicillin and kanamycin. Maintenance of the pBBRXBB2 plasmid was confirmed by the appearance of halos around colonies on LB plates containing xylan and isopropyl β-D-1-thiogalactopyranoside (IPTG, 0.1 mM) (26). Maintenance of the pTVN118pctSTQKAB plasmid was done by carrying a control experiment using glucose (20 $g \cdot L^{-1}$) as 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, after which, 1 mL aliquots of the culture were inoculated into 100 mL of LB media in 500 mL baffled flasks supplemented with xylose (20 $g \cdot L^{-1}$), xylose (20 $g \cdot L^{-1}$) and xylan (10 $g \cdot L^{-1}$), or xylan (10 $g \cdot L^{-1}$). IPTG was added to a final concentration of 0.1 mM at an OD of 0.5–0.6 at 600 nm. All experiments were carried 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 (Barnstead), followed by lyophilization for a minimum of 24 h.

Effect of xylan concentration, shaking speed, and feeding times on PHA yields and composition One milliliter of *E. coli* JW0885 harboring pTVN118pctphaC1(ST/QK)AB and pBBRXBB2 overnight culture was inoculated into 100 mL of LB supplemented with xylan to a final concentration of 10 $g \cdot L^{-1}$ or 20 $g \cdot L^{-1}$. IPTG was added to a final concentration of 0.1 mM at an optical density (OD_{600}) of 0.6 and 0.4 for each concentration of xylan, respectively. Additionally, 50 mL of LB was inoculated with 1 mL of overnight culture and IPTG (0.1 mM) was added at an OD_{600} of 0.6. The cells were grown until the optical density doubled and the 50 mL culture was quickly transferred to 50 mL of pre-warmed LB media supplemented with xylan for a final volume of 100 mL at a final xylan concentration of 10 $g \cdot L^{-1}$ or 20 $g \cdot L^{-1}$. Each set of experiments was grown at 290 rpm or 180 rpm in a rotary shaker for 48 h at 30 °C in 500 mL baffled flasks. An additional set of experiments consisted of 50 mL of LB inoculated with 1 mL of overnight culture and incubated at 290 rpm or 180 rpm. The cells were IPTG (0.1 mM) induced at an OD600 of 0.6 and grown until the optical density was doubled. Each 50 mL cultured was transferred to 50 mL of pre-warmed LB media supplemented with xylan to obtain a final concentration of 20 $g \cdot L^{-1}$ in 100 mL of media. At this point the cells grown at 290 rpm were incubated at 180 rpm and cells initially grown at 180 rpm were incubated at 290 rpm for the remainder of the 48 h growth period. Growth was monitored over the course of the experimental period by measuring OD_{600} at time intervals of 12 h after inoculation with a Genesys 10UV spectrophotometer (Thermo Electron Corporation). At the end of the growth period the cells were harvested as outlined previously. All experiments were performed in triplicate.

TABLE 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Source or reference
E. coli JW0885-1	F–, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^{-}, *rph-1*, *ΔpflA726::kan*, *Δ(rhaD-rhaB) 568*, *hsdR514*	Coli Genetic Stock Center
E. coli JW0885	F–, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^{-}, *rph-1*, *ΔpflA726*, *Δ(rhaD-rhaB)568*, *hsdR514*	This study
pTV118NpctphaC1(ST/QK)AB	pTV118N derivative, *M. elsdenii pct*, *Pseudomonas* sp. *61-3 phaC1(ST/QK)*, *R. eutropha phaB*, *phaA*, Amp^{r}	25
pBBRXBB2	pBBR1-MCS2 derivative, *B. subtilis xynB*, *S. coelicolor xlnB*, Km^{r}	20
pCP20	FLP recombinase, Amp^{r}	24

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