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Conversion of levoglucosan and cellobiosan by *Pseudomonas putida* KT2440

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ABSTRACT

Pyrolysis offers a straightforward approach for the deconstruction of plant cell wall polymers into bio-oil. Recently, there has been substantial interest in bio-oil fractionation and subsequent use of biological approaches to selectively upgrade some of the resulting fractions. A fraction of particular interest for biological upgrading consists of polysaccharide-derived substrates including sugars and sugar dehydration products such as levoglucosan and cellobiosan, which are two of the most abundant pyrolysis products of cellulose. Levoglucosan can be converted to glucose-6-phosphate through the use of a levoglucosan kinase (LGK), but to date, the mechanism for cellobiosan utilization has not been demonstrated. Here, we engineer the microbe *Pseudomonas putida* KT2440 to use levoglucosan as a sole carbon and energy source through LGK integration. Moreover, we demonstrate that cellobiosan can be enzymatically converted to levoglucosan and glucose with β-glucosidase enzymes from both Glycoside Hydrolase Family 1 and Family 3. β-glucosidases are commonly used in both natural and industrial cellulase cocktails to convert cellobiose to glucose to relieve cellulase product inhibition and to facilitate microbial uptake of glucose. Using an exogenous β-glucosidase, we demonstrate that the engineered strain of *P. putida* can grow on levoglucosan up to 60 g/L and can also utilize cellobiosan. Overall, this study elucidates the biological pathway to co-utilize levoglucosan and cellobiosan, which will be a key transformation for the biological upgrading of pyrolysis-derived substrates.
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1. Introduction

Thermal processes for biomass deconstruction, such as pyrolysis and liquefaction, offer rapid, effective methods for the depolymerization of plant cell wall components (Czernik and Bridgwater, 2004; Mohan et al., 2006; Yang et al., 2007; Laird et al., 2009). These processes typically produce heterogeneous slates of compounds derived from polysaccharides and lignin that can potentially be upgraded simultaneously over chemical catalysts (Zhang et al., 2007; Mortensen et al., 2011; Xiu and Shahbazi, 2012; Wang et al., 2013), integrated into petroleum refinery streams (Talmadge et al., 2014), or fractionated through a wide variety of approaches and subsequently upgraded in a more selective manner to a broader slate of fuels and chemicals (Brown, 2005, 2007; Lian et al., 2010, 2012; Jarboe et al., 2011). Recently, there has been substantial emphasis placed on selective fractionation of pyrolysis-derived substrates (Lian et al., 2010; Pollard et al., 2012; Rover et al., 2014a, 2014b; Liang et al., 2013; Gooty et al., 2014a, 2014b) and the use of biological approaches to selectively upgrade at least some of the resulting fractions. Biological approaches are particularly attractive, as metabolic engineering enables both the broadening of substrate specificity as well as the targeted production of single products of interest (Brown, 2005, 2007; Jarboe et al., 2011). This general concept of combining thermal deconstruction with subsequent biological upgrading has been dubbed “hybrid processing” by Brown and coworkers (Brown, 2005, 2007; Jarboe et al., 2011).

One particular pyrolysis fraction of interest for biological upgrading consists of polysaccharide-derived substrates. In typical fast pyrolysis schemes, levoglucosan and cellobiosan are the most abundantly produced dehydration products of cellulose (Patwardhan et al., 2011, 2009; Bai and Brown, 2014; Bai et al., 2013). Multiple tandem catalytic-biological schemes have been developed to fractionate levoglucosan-rich streams from bio-oil, hydrolyze it to glucose, and upgrade it to ethanol, for example (Lian et al., 2010; Shafizadeh and Stevenson, 1982; Helle et al., 2007;

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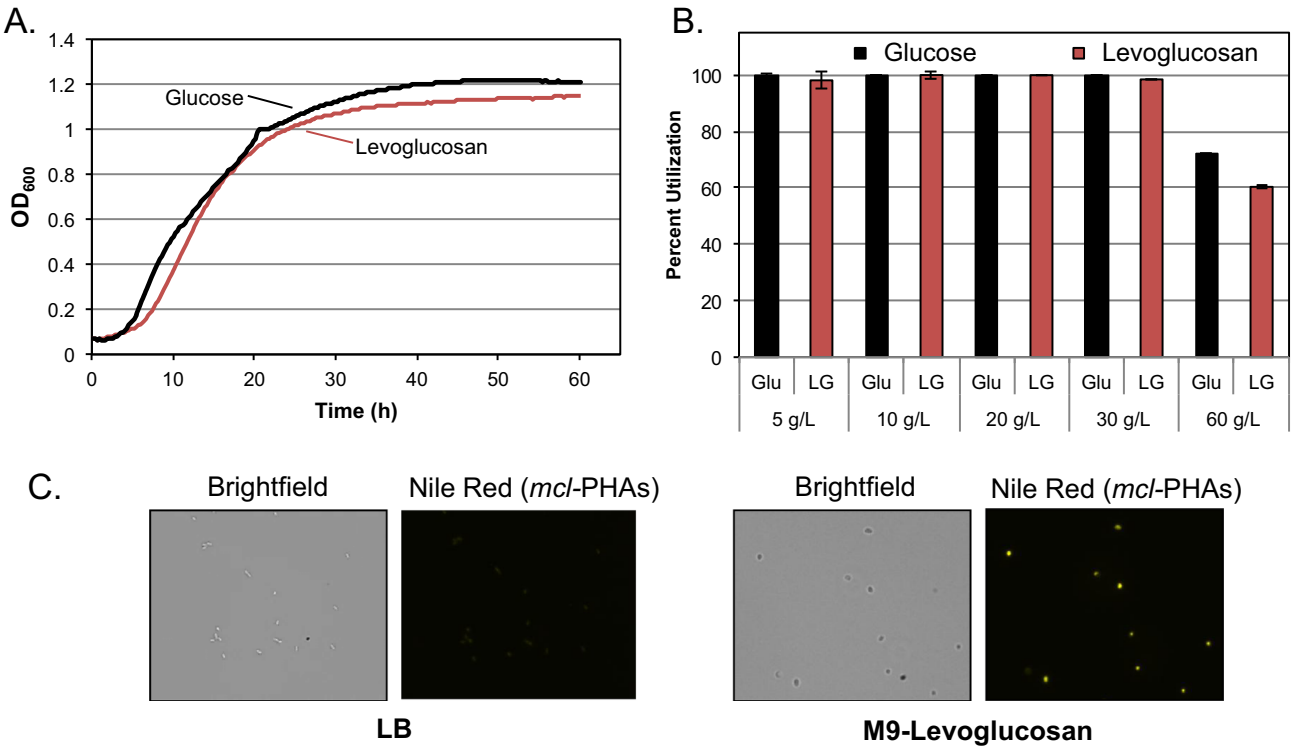

Fig. 1. The engineered *P. putida* strain is capable of growth and polyhydroxyalkanoate production using levoglucosan as the sole carbon source. (A) Growth curve analysis of levoglucosan in M9 minimal medium supplemented with either glucose or levoglucosan using a Bioscreen-C Automated Growth Curves Analysis System. (B) HPLC analysis shows the percent utilization of either glucose or levoglucosan from cultures of FJPO3 grown in shake-flasks. (C) Brightfield and fluorescence microscopy of strain FJPO3 grown on LB and M9-levoglucosan, with Nile Red staining prior to fluorescence microscopy to stain *mcl*-PHAs.

Bennett et al., 2009; Yu and Zhang, 2003a, 2003b; Chan and Duff, 2010). Lian et al. developed a process that used solvent fractionation to separate phenolics from pyrolytic sugars, hydrolyze the levoglucosan to glucose, and then use a biological step to either produce ethanol or fatty acids (Lian et al., 2010). Bennett et al. examined the optimal conditions to produce glucose from a levoglucosan-rich fraction of bio-oil, and noted a 216% yield of glucose, attributing the high yield to cellobiosan and other oligomeric forms of cellulose present in the bio-oil fraction (Bennett et al., 2009).

These types of processes require an intermediate catalytic step to produce glucose, but another approach has been developed that enables biological upgrading directly from levoglucosan (Prosen et al., 1993; Lian et al., 2013; Layton et al., 2011). Namely, levoglucosan can be converted to glucose-6-phosphate (G6P) through use of a levoglucosan kinase (LGK) (Layton et al., 2011; Zhuang and Zhang, 2002; Kitamura et al., 1991; Dai et al., 2009), the structure of which was recently reported (Bacik et al., 2015). Prosen et al. screened multiple fungi and yeasts on levoglucosan-enriched substrates and demonstrated substantial growth but only after the removal of lignin-derived aromatics, suggesting that tolerance to lignin-derived aromatic compounds is a major issue in these streams (Prosen et al., 1993). The observed growth was likely due to the presence of endogenous LGK enzymes (Kitamura et al., 1991). The LGK enzyme present in *Lipomyces starkeyi* has been shown to be quite active for conversion of levoglucosan to G6P (Dai et al., 2009). Jarboe et al. subsequently engineered the *L. starkeyi* LGK gene into an ethanol-producing strain of *Escherichia coli* (KO11), and were able to obtain near-complete conversion of levoglucosan and produce ethanol. Similar to observations from Prosen et al. (1993) and other studies (Chan and Duff, 2010), the authors note that substrate toxicity is likely to be a major factor for the feasibility of these biological approaches to upgrading pyrolytic sugar streams. Subsequently, Jarboe et al. conducted a detoxification study of pyrolytic sugars using an overliming method. The authors were able to demonstrate a nearly 10-fold improvement in ethanol production relative to no cleanup using the same engineered *E. coli* strain (Chi et al., 2013).

Along with levoglucosan, significant amounts of cellobiosan often form during fast pyrolysis (Rover et al., 2014b; Bai and Brown, 2014; Helle et al., 2007; Chi et al., 2013; Choi et al., 2014; Radlein et al., 1987; Scott et al., 1997; Johnston and Brown, 2014; Tessini et al., 2011). Radlein identified cellobiosan in 1987 as a major component of anhydrosugars after pyrolysis of Avicel (between 6% and 15% of the liquid product). To our knowledge, direct cellobiosan utilization has not been previously reported. However, to move towards a consolidated biological process for production of fuels or chemicals from pyrolytic sugar streams, it will be necessary to enable the biological utilization of these highly abundant molecules.

Here, we engineer a solvent-tolerant microbe, *Pseudomonas putida* KT2440, to utilize levoglucosan through the heterologous expression of the levoglucosan kinase from *L. starkeyi*. Furthermore, we demonstrate biological cellobiosan utilization using β-glucosidase-mediated hydrolysis of cellobiosan. Accordingly, our results provide a trajectory towards more complete biological utilization of pyrolytic sugar streams.

2. Materials and methods

2.1. Plasmid and strain construction

The *lgk* gene from *L. starkeyi* was codon optimized using Gene Designer software from DNA 2.0 and synthesized as a gBlock by Integrated DNA Technologies. The sequence of this codon optimized gene has been deposited at GenBank under the accession number KU377145. This fragment was cloned into plasmid pMFL76 which is

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