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Metabolic engineering of *Propionibacterium freudenreichii* subsp. *shermanii* for xylose fermentation

Peilian Wei [a,b,1], Meng Lin [b,c,1], Zhongqiang Wang [b,1], Hongxin Fu [b], Hopen Yang [b], Wenyan Jiang [b], Shang-Tian Yang [b,*]

[a] School of Biological and Chemical Engineering, Zhejiang University of Science & Technology, Hangzhou, Zhejiang 310023, China
[b] William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, 151 West Woodruff Ave., Columbus, OH 43210, USA
[c] Bioprocessing Innovative Company, 4734 Bridle Path Ct., Dublin, OH 43017, USA

HIGHLIGHTS

- *P. freudenreichii* cannot use xylose, the most abundant pentose in plant biomass.
- Xylose catabolic genes *xylA*, *xylT*, and *xylB* in *P. acidipropionici* were identified.
- They were overexpressed in *P. freudenreichii* subsp. *shermanii*.
- The engineered mutant can use xylose efficiently in the presence of glucose.
- The engineered *P. shermanii* can provide a novel cell factory for biorefinery.

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ABSTRACT

Propionibacterium freudenreichii cannot use xylose, the second most abundant sugar in lignocellulosic biomass. Although *Propionibacterium acidipropionici* can use xylose as a carbon source, it is difficult to genetically modify, impeding further improvement through metabolic engineering. This study identified three xylose catabolic pathway genes encoding for xylose isomerase (*xylA*), xylose transporter (*xylT*), and xylulokinase (*xylB*) in *P. acidipropionici* and overexpressed them in *P. freudenreichii* subsp. *shermanii* via an expression plasmid pKHEM01, enabling the mutant to utilize xylose efficiently even in the presence of glucose without glucose-induced carbon catabolite repression. The mutant showed similar fermentation kinetics with glucose, xylose, and the mixture of glucose and xylose, respectively, as carbon source, and with or without the addition of antibiotic for selection pressure. The engineered *P. shermanii* thus can provide a novel cell factory for industrial production of propionic acid and other value-added products from lignocellulosic biomass.

1. Introduction

Propionibacteria are Gram-positive, non-spore forming, facultative anaerobic bacteria. The dairy *Propionibacteria*, including *Propionibacterium acidipropionici*, *Propionibacterium shermanii*, and *Propionibacterium freudenreichii*, which are generally regarded as safe (GRAS), have long been used in manufacturing Swiss cheese (Thierry et al., 2011), vitamin B_{12} (Kosmider et al., 2012; Piao et al., 2004; Wang et al., 2012, 2014c, 2015a) and propionic acid (Wang and Yang, 2013; Wang et al., 2014a; Zhang et al., 2015). Propionic acid, mainly as calcium and ammonia salts, are widely used in food and feed industries to inhibit microbial growth. Because of consumer demand for naturally derived food ingredients and concerns over pollution caused by manufacturing processes using petroleum-based feedstocks, biobased propionic acid produced via fermentation is the desirable alternative to petroleum-derived propionic acid and other preservatives (Samel et al., 2014; Wang et al., 2013). In recent years, there has been increasing interest in biorefineries using renewable biomass, in particular, lignocellulosic biomass, the most abundant renewable feedstock available on earth, and bioprocesses for sustainable production of fuels, chemicals, and other value-added products (Buschke et al., 2013; ElMekawy et al., 2014; Singh et al., 2015; Wang et al., 2014d, 2016; Yang et al., 2015).

However, the use of lignocellulosic biomass in biorefineries is often limited by poor utilization of xylose, the second most abun-

* Corresponding author.
E-mail address: yang.15@osu.edu (S.-T. Yang).
[1] These three authors made equal contributions.

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dant sugar, after glucose, present in lignocellulosic biomass, in fermentation by many industrial microbes, including yeasts, *E. coli*, *Clostridium*, and some propionibacteria (Buschke et al., 2013; Yao and Shimizu, 2013; Yu et al., 2015). While *P. acidipropionici* can grow on and use both glucose and xylose efficiently for propionic acid production (Liu et al., 2012), it is difficult to metabolically engineer with available genetic engineering tools developed so far for propionibacteria (Faye et al., 2008; Jore et al., 2001; Kiatpapan and Murooka, 2001; Suwannakham et al., 2006). On the other hand, other propionibacteria such as *P. freudenreichii* (Ammar et al., 2013, 2014; Piao et al., 2004; Wang et al., 2014b, 2015b) and *Propionibacterium jensenii* (Liu et al., 2015) are more easily engineered, but they cannot utilize xylose as the carbon source (Loux et al., 2015). Metabolic engineering is a powerful tool to endow organisms with new metabolic pathways and has already been used to enable or improve xylose utilization in many microorganisms (Buschke et al., 2013), including yeast (Li et al., 2015), *E. coli* (Nichols et al., 2001), and *Clostridium* (Wu et al., 2015; Yu et al., 2015), but not *P. freudenreichii*. This study aimed to engineer *P. freudenreichii* subsp. *shermanii*, an important dairy microbe with many industrial applications (Thierry et al., 2011), for xylose fermentation.

As shown in Fig. 1A, xylose catabolism in bacteria usually involves a xylose transporter protein (XylT), which facilitates the cross-membrane transfer of xylose into the cell, xylose isomerase (XylA), which converts xylose to xylulose, and xylulokinase (XylB), which phosphorylates xylulose to xylulose-5-phosphate (Yu et al., 2015). Xylulose-5-phosphate is then further metabolized through the pentose phosphate pathway (PPP). The sequenced genome of *P. shermanii* (Falentin et al., 2010) contains only one gene, annotated as xylulokinase (Gene ID: 9283358), required for xylose metabolism. On the other hand, the sequenced genome of *P. acidipropionici* has a *xyl* operon containing genes annotated as xylose isomerase (*xylA*; PACID_RS01700), major facilitator transporter (*xylT*; PACID_RS01695), and xylulokinase (*xylB*; PACID_RS01690) (Parizzi et al., 2012). Although these genes have not been experimentally characterized for their function, we hypothesized that this *xyl* operon is responsible for xylose catabolism in *P. acidipropionici* and its expression in *P. shermanii* would enable it to use xylose for growth.

To prove the aforementioned hypothesis, we cloned *xylA*, *xylA-xylT*, and *xylA-xylT-xylB*, respectively, from *P. acidipropionici* ATCC 4875 and expressed them in *P. freudenreichii* subsp. *shermanii* DSM 4902. Mutants expressing these genes were then evaluated for their ability to use xylose in batch fermentation. The results showed that the *P. shermanii* mutant expressing all three *xyl* operon genes (*xylA*, *xylT*, and *xylB*) could utilize xylose efficiently without suffering from glucose-mediated carbon catabolite repression (CCR) (Görke and Stülke, 2008; Yao and Shimizu, 2013). This study is the first to experimentally verify the xylose utilization function of the *xyl* operon in *P. acidipropionici*. More importantly, the *P. shermanii* mutant capable of using xylose and glucose simultaneously can provide a novel and robust cell factory for industrial fermentation applications.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

Escherichia coli HST08 (Clontech, Mountain View, CA) used in gene cloning was cultivated aerobically in Luria-Bertani (LB) medium containing 100 μg/ml ampicillin at 37 °C with shaking in a rotary shaker. Stock cultures of *P. freudenreichii* subsp. *shermanii* DSM 4902 and *P. acidipropionici* ATCC 4875 were cultivated anaerobically at 32 °C in sodium lactate broth (NLB) containing 10 g/L yeast extract, 10 g/L trypticase soy broth and 10 g/L sodium lactate. Unless otherwise noted, the medium used in all batch fermentation studies contained (per liter): 25 g carbon source (glucose or xylose), 10 g yeast extract, 5 g trypticase soy broth, 0.25 g

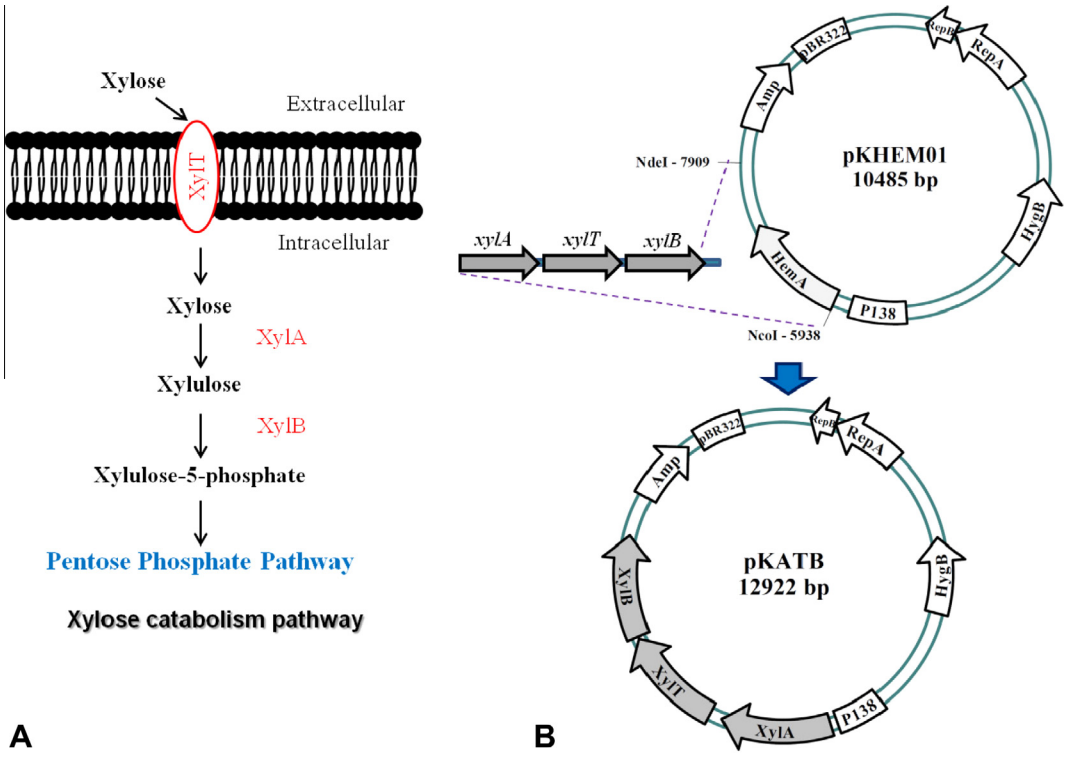

Fig. 1. Xylose utilization pathway of *Propionibacterium acidipropionici* (A) and construction of plasmid pKATB for expressing xylose utilization pathway genes (*xyl*A, *xyl*T, and *xyl*B) in *P. freudenreichii* (B). XylA is xylose isomerase, XylB is xylulose kinase, and XylT is a facilitator superfamily transporter protein. The xylose genes present on the same operon in the genome of *P. acidipropionici* were PCR-amplified and inserted into the plasmid pKHEM01 between the NdeI and NcoI restriction sites.