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Engineering of *Corynebacterium glutamicum* for xylitol production from lignocellulosic pentose sugars

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ABSTRACT

Xylitol is a non-fermentable sugar alcohol used as sweetener. *Corynebacterium glutamicum* ATCC13032 was metabolically engineered for xylitol production from the lignocellulosic pentose sugars xylose and arabinose. Direct conversion of xylose to xylitol was achieved through the heterologous expression of NAD(P)H-dependent xylose reductase (*xr*) gene from *Rhodotorula mucilaginosa*. Xylitol synthesis from arabinose was attained through polycistronic expression of L-arabinose isomerase (*araA*), D-psicose 3 epimerase (*dpe*) and L-xylulose reductase (*lxr*) genes from *Escherichia coli*, *Agrobacterium tumefaciens* and *Mycobacterium smegmatis*, respectively. Expression of *xr* and the synthetic *araA-dpe-lxr* operon under the control of IPTG-inducible P_{tac} promoter enabled production of xylitol from both xylose and arabinose in the mineral (CGXII) medium with glucose as carbon source. Additional expression of a pentose transporter ($araT_F$) gene enhanced xylitol production by about four-fold compared to the parent strain. The constructed strain *Cg-ax3* produced 6.7 ± 0.4 g/L of xylitol in batch fermentations and 31 ± 0.5 g/L of xylitol in fed-batch fermentations with a specific productivity of 0.28 ± 0.05 g/g cdw/h. The strain *Cg-ax3* was also validated for xylitol production from pentose rich, acid pre-treated liquor of sorghum stover (SAPL) and the results were comparable in both SAPL (27 ± 0.3 g/L) and mineral medium (31 ± 0.5 g/L).

1. Introduction

Xylitol is a pentahydroxy natural polyol renowned as a healthy sugar alternative in the food industry. Being a non-fermentable sugar alcohol with a cool and refreshing taste, it has become an excellent choice of sweetness without dental caries (Granstrom et al., 2007). Xylitol may find application as neutraceutical due to additional antidiabetic, antioxidant and anticarcinogenic properties (Mohammad et al., 2015). Consumption of xylitol, one of the top twelve economically significant value added chemicals from biomass refinery (Werpy et al., 2004), has reached an annual global value of $340 million and is expected to grow up to $540 million per year (Ravella et al., 2012). Industrial production of xylitol by chemical reduction of xylose, mainly derived from corn cob, soybean stalk, sugarcane bagasse, and light woods. Drawbacks of the chemical process are expensive downstream processing (Granstrom et al., 2007), a high energy requirement and environmental concerns due to toxic byproducts such as Ni^+ raised the interest in greener xylitol production processes (Su et al., 2013).

Microbial production of xylitol promises advantages over commercial chemical processes e.g. it has a much smaller energy demand (Canilha et al., 2013), and fewer requirements with respect to purity of the substrate xylose. Two biosynthetic pathways for conversion of xylose to xylitol are known in microorganisms: direct reduction of D-xylose to xylitol by NAD(P)H-dependent xylose reductase or isomerization of D-xylose to xylulose by xylose isomerase followed by reduction to xylitol by xylitol dehydrogenase (Akinterinwa et al., 2008). Several yeasts are known for their ability to produce xylitol (Granstrom et al., 2007; Li et al., 2015). However, their application in food industry has to be reduced since some of them are pathogenic (Fridkin and Jarvis, 1996). Only a few species of bacteria including *Corynebacterium* and *Mycobacterium* species produce very low mounts of xylitol naturally (Yoshitake et al., 1973; Izumori and Tuzaki, 1988). Heterologous expression of xylose reductase genes from various yeast sources improved direct conversion of xylose to xylitol, e.g. *E. coli* (Cirino et al., 2006; Hibi et al., 2007). The heterologous expression of a xylose transporter (*XylE)* gene in a *xylA* and *yhbC* deficient *E. coli* strain enabled xylose uptake and production of xylitol (Cirino et al., 2006; Hibi et al., 2007). Inactivation of cAMP-dependent-CRP system enhanced xylitol production in a strain expressing xylose transporter XylE or XylFGH genes (Khankal et al., 2008) and this

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Table 1
Microbial strains and plasmids used in this study.

	Microbial strains & plasmids	Characteristics	Reference
A	*E. coli*		
	DH5α	*DH5a Fthi-1 endA1 hsdr17(r-, m-) supE44 ΔlacU169 (f80lacZ_M15) recA1 gyrA96 relA1*	Hanahan, 1983
B	*C. glutamicum*		
	ATCC13032	Wild type (WT)	Abe et al., 1967
	Cg-xr1	Spec^R *C. glutamicum* with *pEKEx*$_3$ *–xr* for xylose conversion to xylitol	This study
	Cg-xr2	Spec^R *C. glutamicum* with *pEKEx*$_3$*-xrt* for pentose transport and xylitol synthesis from xylose	This study
	Cg-xr3	Spec^R *C. glutamicum* with *pEKEx*$_3$*-xrt*$_F$ for pentose transport and xylitol synthesis from xylose.	This study
	Cg-ab1	Kan^R *C. glutamicum* with *pVWEx*$_1$*-apx* vector for arabinose conversion to xylitol	This study
	Cg-ax1	Kan^R, Spec^R *C. glutamicum* with *pVWEx*$_1$*-apx* and *pEKEx*$_3$*-xr* xylitol synthesis from arabinose and xylose.	This study
	Cg-ax3	Kan^R, Spec^R *C. glutamicum* with *pVWEx*$_1$*-apx* and *pEKEx*$_3$*-xrt* $_F$for pentose transport xylitol synthesis from arabinose and xylose	This study
C	Vectors		
	pVWEx1	*E. coli-C. glutamicum* shuttle vector for regulated gene expression (*P*$_{tac}$, *lacIq, pCG1 oriVCg*)	Peters-Wendisch et al., 2001
	pEKEx3	*E. coli-C. glutamicum* shuttle vector for regulated gene expression (*P*$_{tac}$, *lacIq, pBL1 oriVCg*)	Stansen et al., 2005
	pEKEx3-xr	Derivative of *pEKEx*$_3$ for regulated expression of *xr* gene	This study
	pEKEx3-xrt	Derivative of *pEKEx*$_3$ for regulated expression of *xr* gene with *araT* gene	This study
	pEKEx3-xrt$_F$	Derivative of *pEKEx*$_3$ for regulated expression of *xr* gene with *araT*$_F$ gene	This study
	pVWEx1-apx	Derivative of *pVWEx*$_1$ for regulated expression of *araA, dpe, lxr* genes for xylitol synthesis from arabinose	This study

(Kan^R) Kanamycin resistance, (Spec^R) spectinomycin resistance.

recombinant *E. coli* strain co-utilized xylose and glucose simultaneously without catabolite repression. In addition, *Lactococcus lactis* equipped with xylose reductase from *Pichia stipitis* (Nyyssola et al., 2005) and *Bacillus subtilis* equipped with D-xylitol phosphate dehydrogenase from *Clostridium difficile* or *Lactobacillus rhamnosus* (Povelainen and Miasnikov, 2007) were shown to produce xylitol from xylose. Xylitol production from L-arabinose by recombinant *E. coli* strains was reported (Sakakibara et al., 2009).

An industrial process has to be environment-friendly and economically feasible. Lignocelluloses are considered as renewable and abundant sources of carbohydrates, mainly composed of cellulose, hemicelluloses and lignin. Dilute acid pretreatment (<2%, w/w) is the most accepted method for the release of cellulose from the lignocelluloses. The heterogeneous spent liquid obtained after acid hydrolysis is called acid pretreated liquor (APL). APL is a mixture of pentose sugars (xylose and arabinose), glucose, lignin and inhibitors (furfurals, acids, phenolic compounds, etc.) and its composition varies with the source of lignocellulose (Pienkos and Zhang, 2009). But the direct utilization of these sugars in APL is challenged by toxic derivatives formed during pretreatment process. However, most of the methods developed to detoxify pentose sugar rich APL are either expensive or inefficient (e.g. due to loss of sugars by non-specific adsorption). Direct conversion of pentose sugar rich APL by recalcitrant microbes will have enormous potential. Use of APL as carbon source for xylitol production has rarely been considered (Yoon et al., 2011).

The GRAS (Generally Recognized As Safe) actinomycete, *C. glutamicum* remains one of the best biocatalysts for the industrial production of amino acids and other fine chemicals for more than 50 years (Zahoor et al., 2012). The worldwide L-glutamate and L-lysine production by fermentation amounts to about 3 million and 2 million tons per year (Heider and Wendisch, 2015). A mycolic acid rich outer lipid layer of its cell wall enhances the structural integrity of the outer membrane and thereby provides osmotic stress tolerance towards high concentration of sugars and inhibitors (Bayan et al., 2003). C. glutamicum shows high tolerance to organic acid, furan, and phenolic inhibitors present in lignocellulose hydrolysates under oxygen deprivation conditions (Sakai et al., 2007). This enhanced tolerance helps to utilize blends of carbon sources and inhibitors present in biomass hydrolysates (Akinterinwa et al., 2008; Gopinath et al., 2011). A completely sequenced genome with well-established system metabolic engineering resources, which include CRISPR interference (Cleto et al., 2016) eases the scope of genetic manipulation of this bacterium (Kalinowski et al., 2003). Consequently, a variety of value-added chemicals including succinic acid (Okino et al., 2008), isobutanol (Smith et al., 2010), 1,2-propanediol (Niimi et al., 2011) organic acids (Wieschalka et al., 2013) monomeric precursors for polyamides (Hadiati et al., 2014) in addition to amino acids (Becker and Wittmann, 2012) using recombinant *C. glutamicum* enlightening its importance in various sectors of industrial biotechnology. *C. glutamicum* has been engineered for utilization of arabinose and xylose (Kawaguchi et al., 2008; Kawaguchi et al., 2006) and for production of amino acids, succinate and diamines from lignocellulosic hydrolysates (Meiswinkel et al., 2013; Gopinath et al., 2011; Buschke et al., 2011). *C. glutamicum* cannot utilize xylitol, but mannitol and arabitol (Laslo et al., 2012; Peng et al., 2011). Heterologous expression of the xylose reductase gene from *Pichia stipitis* in *C. glutamicum* led to xylitol production from pure xylose and glucose (Kim et al., 2010). Similarly, xylose reductase from *Candida tenuis* in recombinant *C. glutamicum* (*ΔldhA, ΔxylB, ΔptsF*) expressing the pentose transporter gene *araE* from *C. glutamicum ATCC31831* enabled xylitol production (Sasaki et al., 2010). However, arabitol-free xylitol production (Yoon et al., 2011) from complex substrate blends using recombinant *C. glutamicum* has not yet been reported. To the best of our knowledge, this is the first report on simultaneous conversion of both xylose and arabinose present in APL into xylitol using engineered *C. glutamicum*.

2. Materials and methods

2.1. Microbial strains and vectors

C. glutamicum ATCC 13032 (Abe et al., 1967) was used as a wild-type strain for our experiments. All microbial strains and vectors used in this study are listed in Table 1.