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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.

Table 2
List of custom oligo nucleotides used in this study.

Sl No	Name of Oligos	Sequences 5′ → 3′
01	*xr_PstI F*	AATATACTGCAGTAAAGGAGATATACATATGTCGCAGCAGATCCCC
02	*xr_Bam HI R*	ATATTAGGATCCTTACTGGATCTTGACCTGGTACTTGG
03	*araT BL F*	ATGGTTAATGAAAAGAAAATCTCAA
04	*ara T BL R*	TTACGCGCTTTTTCCGC
05	*araT_sig F*	ATGGCAGGGCACATCATCCGCTCAGACAGCCGGCCAATCGAAGGAGTAATGATGACAGA-GACTGTTCAACAAACCAAGAAAATCTCAAGTGGCTTCAT
06	*araT_ Bam HI F*	TATTATGGATCCCTATACTACTTTAAGAAGGAGATATACCATGGCAGGGCACATCATCCG
07	*araT_ EcoRI R*	TAGTAATGAATTCTTACGCGCTTTTTCCGCCTAC
08	*araA _xbaI F*	ATTATCTAGAAGGAGGACACGATAATGACG
09	*araA _EcoRI R*	ATATAGAATTCACGCGTCAGCGTCGCAT
10	*dpe_ EcoRI F*	TATACGAATTCAGGAGGGACACCCATGAAACAC
11	*dpe_ XhoI R*	TCATACTCGAGGCCGAACCGGGCGAC
12	*lxr_XhoI F*	GATATCTCGAGAGGAGGATATCTAATGACCACCACCGACGAC
13	*lxr_KpnI R*	TGTATGGTACCGGAGCTCTACCCG
14	*araA _gba F*	GCATGCCTGCAGGTCGACTCTAGAAGGAGGAACGATAATGACG
16	*araA_gba R*	GCCGTGTTTCATGGGTGTTCCTCCTGAATTCACGCGTCAGCGTCGCATC
17	*dpe_gba F*	GATGCGACGCTGACGCGTGAATTCAGGAGGAACACCCATGAAACACGGC
18	*dpe_gba R*	CGGTGGTGGTCATTAGATATCCTCCTCTCGAGGCCGAACCGGGCGACGGC
19	*lxr_gba F*	GCCGTCGCCCGGTTCGGCCTCGAGAGGAGGATATCTAATGACCACCACCG
20	*lxr_gba R*	GACGGCCAGTGAATTCGAGCTCGGTACCGGAGCTCTACCCGGCCAC

Underlined region represents restriction sites.

2.2. Chemicals

All the chemicals used were of analytical grade, procured from Sigma-Aldrich (Steinheim, Germany), Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Tryptone, Yeast extract, Luria Bertani (LB), Brain Heart Infusion (BHI) and Potato Dextrose Agar (PDA) was supplied by Merck, India. Enzymes and kits used for molecular biology were obtained from New England Biolabs (NEB, Ipswich, USA), Qiagen (GmbH, Hilden), Thermo scientific (Lithuania) and Sigma Aldrich (Steinem, Germany). Custom made oligonucleotides were procured from Genosys (Sigma-Aldrich).

Table 3
Composition of concentrated hydrolysate of dilute acid pretreated sorghum stover.

Components	Concentration (g/L)
Glucose	5.78 ± 0.2
Xylose	29.36 ± 0.1
Arabinose	5.64 ± 0.2
Mannose	0.82 ± 0.1
Galactose	0.56 ± 0.02
Acetic acid	0.06 ± 0.01
Fomic acid	0.03 ± 0.01
Furfural	0.58 ± 0.02
5-Hydroxymethylfurfural	0.25 ± 0.01

2.3. Media

The *E. coli* was maintained in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L sodium chloride). Yeast *Rhodotorula mucilaginosa* isolate NBT11 was cultured in nutrient medium (YXP medium) containing 40 g/L xylose, 10 g/L yeast extract, 5 g/L K_2HPO_4, 5 g/L KH_2PO_4 and 0.002 g/L $MgCl_2$ for RNA isolation. Recombinant *C. glutamicum* strains were grown at 30 °C in BHI medium containing 5 g/L Beef heart infusion, 12.5 g/L Calf brain infusion, 2.5 g/L Na_2HPO_4, 2 g/L glucose, 10 g/L peptone and 5 g/L NaCl. Fermentation studies were carried out using two sugar variants of medium containing CGXII salts (Keilhauer et al., 1993) with a combination of glucose, xylose and arabinose as carbon sources, assigned as CGXII-AXG and SAPL medium. SAPL medium contains concentrated APL of sorghum stover as sugar base. Based on the ratio of sugars present in the medium, they are further designated as CGXII-AXG1 medium and SAPL1 medium containing 10 g/L glucose, 15 g/L xylose and 15 g/L arabinose (1: 1.5: 1.5). Whereas CGXII-AXG2 and SAPL2 medium contains 10 g/L of each sugar (1: 1: 1). The fermentation media was sterilized by filtration (0.2 μM, Pall life Sciences, USA).The variations in the sugar composition of APL was adjusted with pure sugar for the preparation of SAPL medium.

2.4. Genetic modifications

All the standard molecular biology techniques were done as per the protocols described by Sambrook and Russell (2001). Genomic DNA isolations were done with Gen Elute genomic DNA isolation kit (Sigma, India). Plasmid isolations were done using GeneJet plasmid mini kit (Thermo Scientific, Lithuania) and the PCR product was purified by QIA quick PCR purification kit (Qiagen, Germany) as per the instructions provided by the manufacturers.

2.5. Construction of $pEKEx_3$-xrt_F plasmid

The xylose reductase (xr) gene was isolated from *R. mucilaginosa* isolate NBT11 (MTCC 12464) by cDNA synthesis (ProtoScript, NEB). PCR amplification of *xr* gene from cDNA was carried out using specific oligonucleotides 1 and 2 (Table 2) with ribosome binding site upstream (−7 bp) of the start codon with reference to the published sequences (GenBank accession number **HM038240**) (Xu et al., 2011). The 975 bp amplicon (GenBank accession number KT154803) cloned to $pEKEx_3$ under P_{tac} after digestion with PstI and BamHI restriction enzymes (NEB, USA).The resultant plasmid was designated as $pEKEx_3$-*xr* and it was further modified with arabinose/pentose transporter (AraT) to facilitate pentose transport across the membrane. *araT* gene from the genomic DNA of *B. licheniformis* (ATCC 14580) was amplified by PCR using specific oligonucleotides 3 and 4 (Table 2). The *araT* was customized by PCR (using oligonucleotides 4 and 5, Table 2) with an addition of 75 bp 5′ terminal gene segment adopted from *araE* gene of *C. glutamicum ATCC 31831* to obtain $araT_F$ fusion gene. The 1478 bp fusion gene ($araT_F$) was further amplified by PCR using oligonucleotides 6 and 7 (Table 2) with ribosome binding site upstream (−7 bp) of the start codon. The $araT_F$ gene was then cloned to $pEKEx_3$-*xr* under *xr* gene after digestion with Bam HI and EcoRI. The engineered plasmid so-called $pEKEx_3$-*xrt* was transformed into *E. coli* DH5α cells for maintenance. Transformants bearing $pEKEx_3$ derivatives were screened in LB medium supplemented with spectinomycin (0.1 g/L).

2.6. Construction of $pVWEx_1$-apx plasmid

The *araA* (**NC_000913**), *dpe* (**NC_003063**) and *lxr* (**NC_008596**) genes were amplified from genomic DNAs of *E. coli K12* (ATCC 47076), *A. tumefaciens strain C-58* (ATCC 33970) and *M. smegmatis* strain mc^2155 (ATCC 700084) respectively using oligonucleotides 8–13 followed by 14–20 listed in Table 2. The genes were tailored together in tandem with ribosome binding site in between by Gibson assembly (NEB, USA) as per manufacturer's instructions. The linear segment of genes was cloned after IPTG inducible (P_{tac}) promoter of $pVWEx_1$ to obtain a polycistronic transcript. The resultant plasmid $pVWEx_1$-*apx* was transformed into *E. coli* DH5α cells and the transformants were selected using LB medium containing kanamycin (0.05 g/L).

2.7. Transformation of C. glutamicum with engineered plasmids for xylitol synthesis

Freshly prepared electro competent cells of *C. glutamicum* were used for transformation (Van Der Rest et al., 1999). Transformants of $pVWEx_1$ derivatives were screened in BHI plate supplemented with kanamycin (0.05 g/L) and BHI with spectinomycin (0.1 g/L) for $pEKEx_3$ derivatives. Both antibiotics were supplemented for the recombinants bearing both plasmids.

2.8. Preparation of sorghum stover biomass hydrolysate by acid pretreatment

The Sorghum Stover hydrolysate was prepared by dilute acid hydrolysis using 2% sulphuric acid (w/v) at 121 °C for 70 min in an autoclave with 30% (w/v) solid loading. Resultant biomass was washed with deionized water and the washout liquid (APL) neutralized (pH ≥6.5) with calcium carbonate followed by NaOH. The diluted APL was concentrated in a vacuum concentrator to obtain the required pentose (mainly xylose) concentration for medium (SAPL medium) formulation and perceived the composition by HPLC analysis (Table 3). The APL concentrate was refined and sterilized by filtration through 0.2 μ filters.

2.9. Batch fermentation

50 mL of CGXII, CGXII-AXG1 and SAPL1 medium supplemented with respective antibiotic was dispensed in 250 mL Erlynmeyer conical flask and inoculated with 16 h old recombinant strains (2 g cdw/L). The immediate expression of recombinant genes was achieved by IPTG (1 mM) induction along with inoculation. The flasks were incubated at 200 rpm for 16 h at 30 °C. Samples were taken at every 2 h and analyzed for sugar utilization and xylitol production.

2.10. Fed batch fermentation

Fed batch fermentation was carried out in Infors HT Multifor parallel fermentors, equipped with calibrated and automated control systems for temperature, DO, pH, impeller speed and aeration rate. The total capacity of the fermentor was 750 mL, where the working volume was set to 200 mL with CGXII-AXG2 and SAPL2 medium. After sterilization, the medium was inoculated with16 h *Cg-ax3* cells (10 g cdw/L). The induction of genes (1 mM IPTG) was made immediately after inoculation. The fermentation was carried out under aeration of 0.1vvm at a variable stirring speed. Two rushton type six bladed impellors were used to ensure proper mixing of the fermentation media. The cascade system controlled dissolved oxygen (DO) at 10% by varying stirrer speed within a limit of 50–200 rpm. The temperature was maintained at 30 °C with a circulating water bath. The pH was maintained at 6.5 ± 0.2 using 1 N HCl and 1 N NaOH. The foaming was controlled by using 10 ppm antifoaming agent (Antifoam- O_{30}, Sigma). The DO and pH were measured through polarographic DO probe and platinum pH electrode (Mettler Toledo). Fermentation was carried out for 12 h with batch addition of 10 mL sugar mixture (for CGXII-AXG2) or concentrated APL (for SAPL2 medium) containing glucose, xylose and arabinose (10 g/L each) at every 4 h. The sampling was done at every 2 h interval for sugar and xylitol determination.

2.11. Spectrophotometric analysis

Cell density was determined spectrophotometrically at 610 nm using UV–vis spectrophotometer (UVA-6150, Shimadzu, Japan). Tecan Infinite 200 microplate reader was used to measure the NADPH concentration at 340 nm.

2.12. Enzyme analysis

Recombinant *C. glutamicum* cells grown in 50 mL CGXII-AXG1 medium were harvested after 6 h of IPTG induction (at 24 h after inoculation) by centrifugation at 6000*g* at 4 °C for 6 min. The cell pellets were washed twice with extraction buffer (50 mM Tris HCl pH 7.0) and dispensed in 2.0 mL of it. The cell suspension was sonicated using ultrasonic homogenizer (Vibra cell, Sonics and materials Inc, USA) in an ice bath for 10 min. The refined (0.2 μm filters, Pall life sciences) extracted protein was quantified by Bradford's method (Bradford, 1976) using Bovine Serum Albumin (BSA) as standard. The proteins were resolved in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide) and analyzed after stained with Coomassie-Brillian blue- R250 (Sambrook and Russell, 2001)

The xylose reductase activity was checked for crude protein isolated from *Cg-xr1*, where the oxidation of NADPH was monitored for enzyme activity (Sasaki et al., 2010). The xylose reductase activity was determined by monitoring the change in absorbance at 340 nm upon NADPH oxidation at 30 °C. The reactions were carried out in buffer containing 50 mM potassium phosphate (pH 6.0), 2 mM NADPH and 200 mM xylose. The decrease in absorbance after incubation at 30 °C was measured at 340 nm in a microplate reader (Infinite 200, Tecan). Enzyme activities are expressed as specific activity (units per mg of protein), where one unit corresponds to the conversion of 1 μmol NADPH per min under standard assay condition.

The intracellular protein from *Cg-ab1* was analyzed for L-arabinose isomerase (AraA), D-psicose 3 epimerase (Dpe) and L-xylulose reductase (Lxr) activity. The conversion of L-arabinose to L-ribulose was monitored for L-AraA activity, and synthesis of psicose from fructose was measured for Dpe activity. Both enzyme assays were carried out in a reaction mixture containing 100 mM Tris–HCl (pH 7.5), 100 mM L-arabinose for arabinose isomerase whereas 100 mM fructose and 1 mM $MnCl_2$ for D-psicose 3 epimerase at 30 °C for 5 min incubation (Sakakibara et al., 2009). The reaction was stopped by keeping it at 100 °C for 5 min. The arabinose depletion and psicose synthesis were quantified by HPLC analysis. The enzyme activity was determined in, enzyme unit (U), is defined as the amount of enzyme required for the conversion of 1 μmol substrate per min under standard assay condition and expressed in units per mg of protein.

The xylulose reductase activity was performed in buffer containing 100 mM Tris–HCl (pH 9.0), 0.5 mM $MgCl_2$, 2 mM $NADP^+$, and 100 mM of xylitol. Enzyme activity was determined based on the increase in absorbance at 340 nm caused by the reduction of $NADP^+$ to NADPH, up on the oxidation of xylitol to xylulose in standard assay conditions. One unit (U) is defined as the amount of

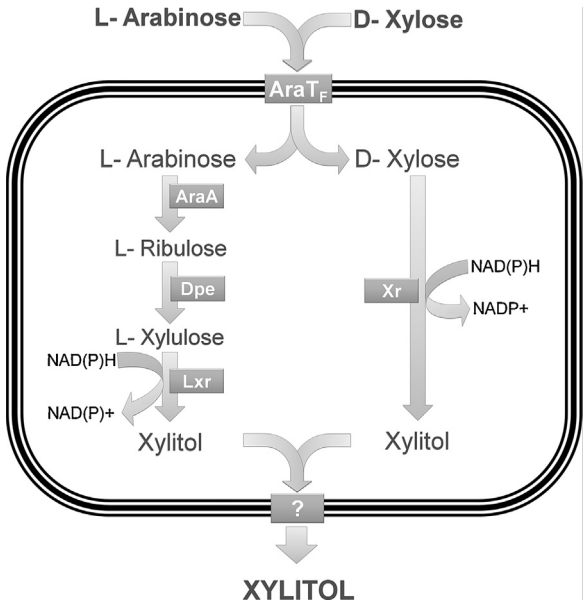

Fig. 1. Schematic representation of heterologus xylitol synthetic path way in recombinant *C. glutamicum* strain *Cg-ax3*. Shaded boxes showing recombinant enzymes involved in the pathway. Both L-arabinose and D-xylose were transported across the cell with the help of pentose transporter fusion protein ($AraT_F$). D-Xylose is converted to xylitol with the help of NAD(P)H dependant xylose reductase (Xr). L-Arabinose is converted to xylitol by the combined action of arabinose isomerase (AraA), D-psicose 3 epimerase (Dpe) and L-xylulose reductase (Lxr). The reducing equivalents (NAD(P)H) for the reactions generated by glucose (Pentose Phosphate Pathway). The xylitol produced inside the cell was secreted out through unknown (?) transporter.

enzyme required for the reduction of 1.0 μmol of $NADP^+$ to NADPH under the assay condition per minute (U/ mg of total protein).

2.13. Metabolite analysis

Sugars, xylitol and inhibitors were analyzed with automated High Performance Liquid Chromatography (HPLC) system (Prominence UFLC, Shimadzu, Japan) equipped with the solvent delivery pump (LC 20AD), auto sampler (SIL20AC-HT), and a column oven (CTO-20AC). The sugars and sugar alcohol were resolved with Phenomenex Razex RPM $Pb2^+$ cation exchange monosaccharide column (300 × 7.5 mm) with inline filters and guard column operated at 80 °C using deionized water (0.6 mL/min) as eluent. The sugars and xylitol was detected in RI detector (RID-10A Shimadzu, Japan). The inhibitors including organic acids were resolved in HPX-87H column (Bio-rad Aminex, USA) using 5 mM of H_2SO_4 as eluent at a flow rate of 0.5 mL/min at 50 °C was detected using PDA detector (SPDM-20A, Shimadzu, Japan). 20–50 μL of filtered sample (0.2 μM filters) used for analysis in both cases and quantification was made based on the calibration curves obtained from the chromatograms of the known standards.

3. Results

The objective of this work was to engineer *C. glutamicum* for the direct conversion of the lignocellulosic pentose sugars xylose and arabinose to xylitol (Fig. 1).

3.1. Expression and analysis of genes for xylitol conversion from xylose in C. glutamicum

The xylose reductase gene *xr* from *R. mucilaginosa* NBT11 was expressed in *C. glutamicum* ATCC13032 yielding strain *Cg-xr1*. Crude extracts of strain *Cg-xr1* showed NADPH-dependent xylose reductase activities of 6.4 ± 0.1 U/mg of protein. With NADH as reductant xylose reductase activities of 4.3 ± 0.1 U/mg were found. Crude extracts of the parental strains lacked NADPH- and NADH-dependent xylose activities. *C. glutamicum* ATCC 13032 is able to import arabinose and xylose, but consumption of these pentoses was enhanced when genes encoding a heterologous uptake system was expressed (Sasaki et al., 2009). The role of non specific sugar transporters for xylitol synthesis in *E. coli* and *C. glutamicum* has already been studied (Khankal et al., 2008; Sasaki et al., 2010). In this study, AraT from *B. licheniformis* was used. Native AraT was used in strain *Cg-xr2*. Alternatively, the signal sequence of AraE from *C. glutamicum* ATCC31831 was fused to the mature protein sequence of AraT from *B. licheniformis*. The recombinant strain *Cg-xr3* overproduced proteins $AraT_F$ and XR. SDS-PAGE analysis showed hints of expressions of $AraT_F$ (52.7 kDa) and Xr (35.4 kDa) by Coomassie-Brillian blue- R250 staining. Expression of $AraT_F$ improved xylitol titers from 1.2 ± 0.2 g/L to 6.2 ± 0.2 g/L (Table 4).

3.2. Expression and analysis of genes for xylitol synthesis from Arabinose

Arabinose can be converted to xylitol in 3 reactions catalyzed by enzymes of different origin (Sakakibara et al., 2009). After isomerization of L-arabinose to L-ribulose by arabinose isomerase (AraA) D-psicose 3-epimerase (Dpe) converts L-ribulose to L-xylulose. Finally, NADPH-dependent L-xylulose reductase (Lxr) reduces L-xylulose to xylitol. Expression of the genes *araA*, *dpe* and *lxr* as a synthetic operon and operated under IPTG inducible (P_{tac}) promoter of plasmid pVWEx1 in *C. glutamicum* yielded strain *Cg-ab1*. SDS-PAGE analysis revealed protein bands at 56 kDa, 31.5 kDa, and 26.9 kDa representing AraA, Dpe, and Lxr, respectively. Crude extracts of strain *Cg-ab1* showed specific activities for AraA of 4.3 ± 0.1 U/mg, Dpe of 5.6 ± 0.3 U/mg, and Lxr of 3.6 ± 0.2 U/mg. As expected, the Lxr activity was lower (0.8 U/mg) when NADH was used instead of NADPH for the enzyme assay.

3.3. Batch fermentation for xylitol production

The recombinant strains with xylose and arabinose transforming genetic makeup were studied for xylitol production from pentose sugars D-xylose and L-arabinose. The strains were analyzed for sugar utilization and xylitol production in CGXII media with individual carbon sources (Glucose, Xylose and Arabinose) (20 g/L each). The physical conditions for the fermentation were same as mentioned in materials and methods. The presence of glucose was found to be critical for xylitol production from xylose and arabinose, where the strain failed to produce xylitol in the absence of glucose even after 72 h of incubation. The supplementation of glucose (10 g/L) has initiated the xylitol synthesis from individual pentose sugars. The final recombinant strain *Cg-ax3* has produced 6.3 ± 0.4 g/L of xylitol from 6.5 ± 0.3 g/L of xylose and 4.4 ± 0.3 g/L of xylitol from 6.1 ± 0.3 g/L of arabinose utilized (Fig. 2A and B).Batch production of xylitol using recombinant strains were further validated in CGXII-AXG1 medium. This medium contained a blend of 10 g/L glucose, 15 g/L xylose and 15 g/L arabinose as carbon source. The physical conditions were same as described in materials and methods. The strain *Cg-ax3* has completely utilized glucose within 10 h of incubation and showed better xylitol production (Fig. 3A) from both pentose sugars (xylose and arabinose) present in the CGXII-AXG1 medium in comparison with other recombinant strains (Table 4). The xylitol producing competence of *Cg-ax3* was further verified by using in SAPL1 medium (based on acid pre-treated liquor of sorghum stover with a sugar concentration of 10 g/L glucose, 15 g/L xylose and 15 g/L arabinose) and showed comparable results obtained in CGXII-AXG1 medium. The xylitol production was 6.7 ± 0.4 g/L with a productivity of 0.28 ± 0.03 g/g

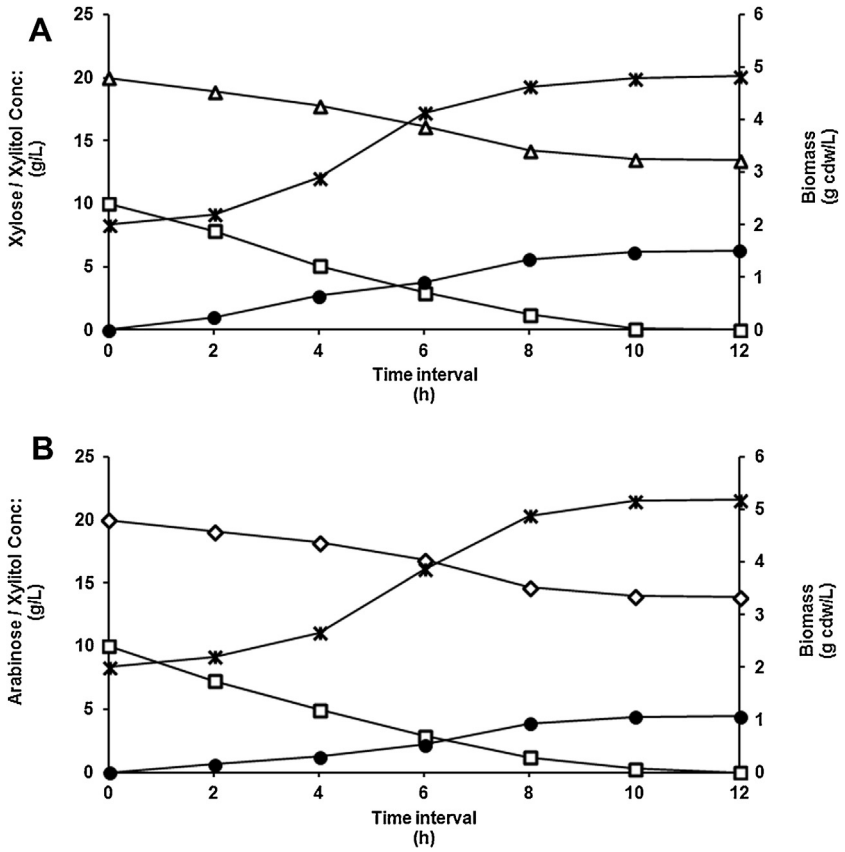

Fig. 2. Fermentation profiles of recombinant strain *Cg-ax3* (*pVWEx*$_1$*-apx* and *pEKEx*$_3$*-xrt*$_F$) (A) Batch fermentation profile in CGXII medium with xylose (20 g/L) and glucose (10 g/L) as carbon source. (B) Batch fermentation profile in CGXII medium with arabinose (20 g/L) and glucose (10 g/L) as carbon source. The graphs showing curves of biomass, sugar and xylitol concentrations during 12 h of incubation. Open squire (□) glucose; open triangle (Δ) xylose; open diamond (◊) arabinose; star (✱) biomass and filled circle (●) xylitol. The data shown are the average of triplicate experiment performed and the calculated standard deviation is less than 10%.

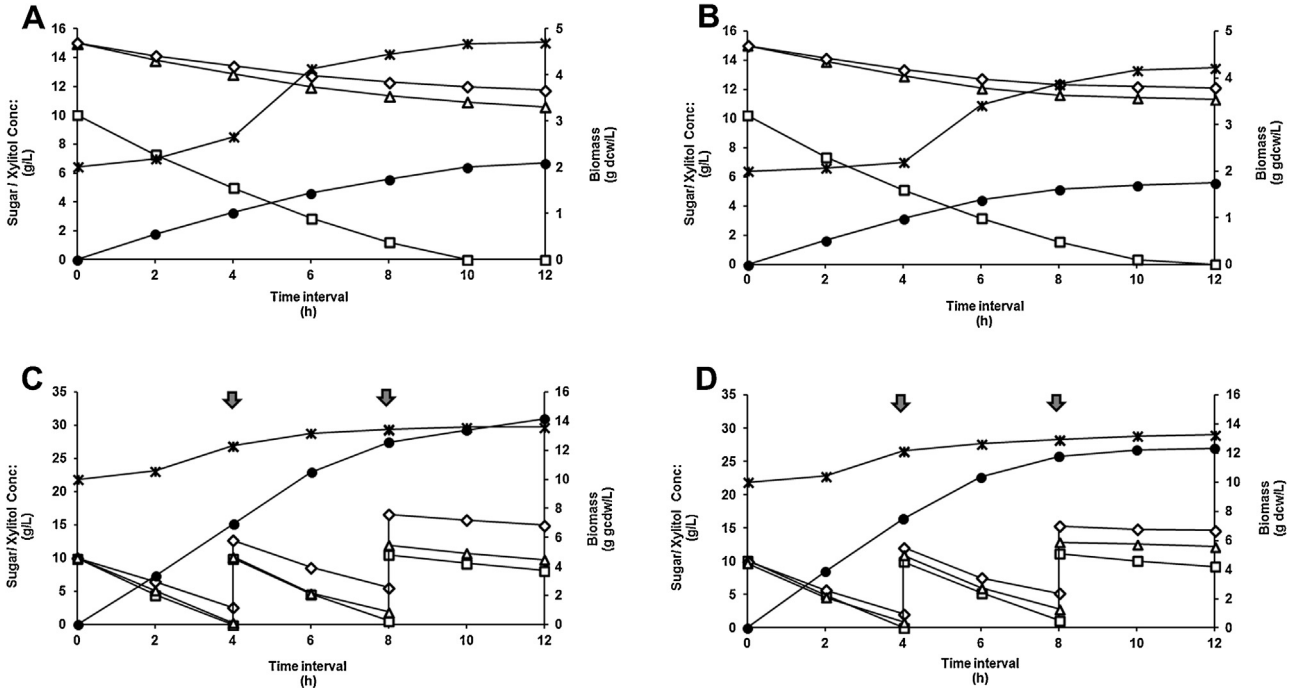

Fig. 3. Fermentation profiles of final recombinant strain *Cg-ax3* (*pVWEx*$_1$*-apx* and *pEKEx*$_3$*-xrt*$_F$) having xylose and arabinose bio conversion pathways for xylitol production. (A) Batch fermentation profile in CGXII-AXG1 medium. (B) Batch fermentation profile in SAPL1 medium. (C) Fed batch fermentation profile in CGXII-AXG2 medium (D) Fed batch fermentation profile in SAPL2 medium. The graphs showing curves of biomass, sugar and xylitol concentrations during 12 h of incubation. Open squire (□) glucose; open triangle (Δ) xylose; open diamond (◊) arabinose; star (✱) biomass and filled circle (●) xylitol. The downward arrows (↓) showing the time intervals of sugar additions. The data shown are the average of triplicate experiment performed and the calculated standard deviation is less than 10%.

Table 4
Sugar utilization and xylitol production by recombinant strains of *C. glutamicum* by batch fermentation using CGXII- AXG1 medium.

Sl No	Strains	Modification	Xylose utilization (g/L)	Arabinose utilization (g/L)	Xylitol production (g/L)	Productivity (g/g cdw/h)
1	*Cg-xr1*	*xr*	1.2 ± 0.2	–	1.1 ± 0.1	0.05 ± 0.01
2	*Cg-xr2*	*xr, araT*	1.7 ± 0.3	–	1.6 ± 0.2	0.07 ± 0.02
3	*Cg-xr3*	*xr, araT$_F$*	6.4 ± 0.3	–	6.2 ± 0.2	0.26 ± 0.02
3	*Cg-ab1*	*araA,dpe,lxr*	–	1.1 ± 0.2	0.8 ± 0.1	0.04 ± 0.01
4	*Cg-ax1*	*xr, araA,dpe,lxr*	1.1 ± 0.1	0.9 ± 0.1	1.8 ± 0.2	0.08 ± 0.01
6	*Cg-ax3*	*xr, araT$_F$, araA,dpe,lxr*	4.4 ± 0.1	3.4 ± 0.2	6.7 ± 0.4	0.28 ± 0.03

Table 5
Comparison of xylitol production and productivities by engineered *C. glutamicum* under fed batch conditions.

Recombinant *C. glutamicum* genotype	Sugars used for fermentation	Biomass used (g dcw/L)	Xylitol concentration (g/L)	volumetric productivity of xylitol (g/L/h)	Specific productivity of xylitol[d] (g/g cdw/h)	Reference
xyl 1	Glucose + Xylose	9.2	34[a]	0.9	0.09	Kim et al., 2010
araE, Δldh, CtXR, ΔptsF, ΔxylB	Glucose + Xylose	40	166[b]	7.9	0.20	Sasaki et al., 2010
xr, araT$_F$, araA, dpe, lxr	Glucose + Xylose + Arabinose	10	31[c]	2.6	0.26	This work

[a] after 40 h of incubation.
[b] after 21 h of incubation.
[c] after 12 h of incubation.
[d] calculated based on initial biomass used for fermentation.

cdw/h in CGXII-AXG1at 12 h of fermentation. Similarly, the xylitol production in SAPL1 medium was 5.6 ± 0.2 g/L with a productivity 0.23 ± 0.03 g/g cdw/h under same fermentation conditions (Fig. 3B)

3.4. Fed batch fermentation using recombinant strain Cg-ax3

Fed batch fermentation was performed in both CGXII-AXG2 (minimal medium with 10 g/L glucose, 10 g/L xylose and 10 g/L arabinose) and SAPL2 (medium based on acid pre-treated liquor of sorghum stover with a sugar concentration of 10 g/L glucose, 10 g/L xylose and 10 g/L arabinose) medium using recombinant strain *Cg-ax3*. Based on the batch fermentation studies, an inoculum concentration of 10 g cdw/L was used to reduce the lag phase. At regular intervals of 4 h, sugar mixtures or APL sugar concentrate was fed. The xylitol production in CGXII-AXG2 reached 31.0 ± 0.5 g/L (Fig. 3C) and a specific productivity of 0.26 ± 0.05 g/g cdw/h at 12 h of fed batch fermentation. Similarly, fed batch fermentation based on SAPL2 medium led to xylitol titers of 27.0 ± 0.3 g/L (Fig. 3D) and a specific productivity of 0.22 ± 0.1 g/g cdw/h.

4. Discussion

The efficient utilization of sugar mixtures e.g. from lignocellulosic hydrolysates is a critical component in biorefinery concepts. Here, we describe how xylose and arabinose present in pentose-rich hydrolysates can be transformed to xylitol in a single bioprocess using genetically modified *C. glutamicum*. To the best of our knowledge, this is the first report demonstrating efficient bio conversion of lignocellulosic pentose sugars (both xylose and arabinose) from sorghum stover biomass acid pretreated liquor based mineral medium (SAPL medium) to xylitol.

In the present study, *R. mucilaginosa NBT11* was the source of xylose reductase gene for xylitol production in *C. glutamicum*. *Rhodotorula* was isolated from APL as a contaminant. Its natural ability to produce xylitol from APL containing xylose with 50% efficiency was recognized subsequently (data not shown). The amino acid sequence showed 97% similarity to the reported sequence of xylose reductase from *R. mucilaginosa* up on BLASTP analysis. Most of the xylose reductases from yeasts also act on arabinose to produce arabitol. Recombinant strains with these xylose reductases are likely to produce arabitol along with xylitol when hemi cellulosic hydrolysates are used as the substrate. This poses a problem for xylitol production since arabitol cannot be separated easily from xylitol during downstream processing (Yoon et al., 2011). Notably, the xylose reductase from *R. mucilaginosa NBT11* employed here did not show detectable arabitol synthesis from arabinose (data not shown). Thus, the process developed here ensures arabitol-free xylitol production in both CGXII-AXG and SAPL media.

Heterologous expression of arabinose/pentose transporter genes in *C. glutamicum* to improve arabinose and xylose uptake was studied earlier (Sasaki et al., 2010). AraT and AraE are as low affinity proton-symport systems driven by proton motive force which is usually inefficient to transport sugars at lower concentration at alkaline pH (at low H^+ concentration) (Lam et al., 1980). The strain *Cg-xr2* overproducing AraT from *B. licheniformis* did not show improved xylitol production compared to *Cg-xr1*. AraT possesses 74% of amino acid similarity to AraE of *C. glutamicum* ATCC 31831 except at its N-terminal region. Thus, it was likely that its signal peptide was not functional in *C. glutamicum*. Indeed, fusion of the signal peptide of AraE from the related *C. glutamicum* strain ATCC 31831 to AraT increased xylitol production 5.3 fold increase. The specific productivity of *Cg-xr1* and *Cg-xr3* (Table 4) were higher than the previously reported strain for xylitol production (Table 5) even though volumetric productivity and final yield were comparatively low (Sasaki et al., 2010). The strain *Cg-xr3* is a superior strain for xylitol productivity than the reported modified strains such as *Lactococus lactis* (0.05 g/g cdw/h) and *Saccharomyces cervisiae* (0.19 g/g cdw/h) (Kim et al., 2010).

Although xylose is the major sugar present in APL, the amount of arabinose present in APL is non negligible for a variety of lignocellulosic biomass. This arabinose has to be utilized either as a carbon source to fuel the cell growth or can be used as a substrate for xylitol synthesis for better economic viability of the process. Xylitol synthesis from arabinose was first described by synthetic path way in *E. coli* lacking arabinose operon (Sakakibara et al., 2009). This pathway is more suitable for *C. glutamicum* compared to *E. coli* while wild type *C. glutamicum* lacks natural arabinose catabolism and allosteric sugar competition. The recombinant strain *Cg-ax3* showed a 3 fold increase in xylitol production from arabinose when compared to other strains (*Cg-ab1*and *Cg-ax1)* (Table 4). The conversion efficiency was lower (73%) than the engineered *E. coli* (92%) (Sakakibara et al., 2009). The presence of functional xylulokinase XylB in *C. glutamicum* may be the probable reason for the reduced conversion efficiency of arabinose to xylitol. XylB may catalyze the phosphorylation of L-xylulose, an intermediate of the arabinose conversion pathway to xylitol. The xylulose 5 phosphate formed

might be utilized by the bacterium though pentose phosphate pathway.

Waste free bio conversion of multiple sugars (xylose and arabinose) present in the APL to xylitol can improve the economy of the process to a great extend. The genetically modified *C. glutamicum* (*Cg-ax3*) with xylose and arabinose metabolism for xylitol synthesis have successfully validated in fermentation medium containing these sugars. The xylitol production efficiency of *Cg-ax3* with respect to the concentration of sugar utilized, were 97% and 73% for xylose and arabinose respectively (Fig. 2). Interestingly, a small amount (2.2 g/L) of xylitol was produced by *Cg-ax3* at 48 h using glucose as sole carbon source (20 g/L) by *Cg-ax3*. This may be due to the xylulose intermediate formed due to the action of xylB during glucose metabolism which was driven towards xylitol synthesis by xylulose reductase present in the strain. The molar yield of xylitol was 1 mol per 10.8 mol of glucose utilized. Additionally, the recombinant strain *Cg-ax3* was not detected for xylitol utilization in CGXII medium supplemented with xylitol (2%) as sole carbon source (data not shown). Although few sugar alcohol uptake systems for inositol (IolT1 and IolT2) (Krings et al., 2006), arabitol (RbtT) and mannitol (Laslo et al., 2012; Peng et al., 2011) were characterized earlier., there are no reports available about dedicated xylitol uptake or transport system in *C. glutamicum*.

The CGXII-AXG medium contained a combination of sugars similar to that present in SAPL to rule out contributions to xylitol formation from other components of SAPL. The xylitol productivity of *Cg-ax3* strain in batch fermentation with SAPL1 media (0.23 ± 0.03 g/gdcw/h) and CGXII-AXG1medium (0.28 ± 0.03 g/gdcw/h) were comparable. The hydrolysate-based medium containing various sugars and inhibitors was also suitable for fed batch fermentation with *Cg-ax3* (Fig. 3d) media. The productivity was obtained maximum at 6 h of fed batch fermentation, in both CGXII-AXG2 and SAPL2 media. There was also a similarity in productivity observed in CGXII-AXG1 (Fig. 3a) and SAPL1 (Fig. 3b) medium at 6 h under batch fermentation. This is the highest specific productivity reported for xylitol producers (Table 5). Theoretically, one molecule of glucose can yield two molecules of NADPH by pentose phosphate pathway. Single molecules of NADPH and xylose or arabinose can together yield one molecule of xylitol (ie.,) the maximum theoretical yield of xylitol with respect to the NADPH generated by single molecule of glucose is two. From the results obtained by fed batch fermentations the average molar yield of xylitol was about 1.6 mol per mole of glucose used when the cell growth was at stationary phase. These results showed that the developed strain required only about 3/4 concentrations of glucose required in comparison to xylose or arabinose concentration for xylitol synthesis. The xylitol productivity of strain *Cg-ax3* in xylose and arabinose containing medium was independent on pentose sugar proportions. This indicates its direct application for transformation of crude APL to xylitol without adjusting the sugar concentrations under fed batch conditions. Recent advances in process strategies comprise substrate preparation, process engineering, and metabolic engineering improved the range of xylitol production. The volumetric productivity of xylitol may be further increased by increasing the inoculum load as has been shown for xylitol production by yeasts species including *Candida tropicalis* (3.86 g/L/h) (Choi et al., 2000), *Debaryomyces hansenii* DBX 12 (2.24 g/L/h) (Pal et al., 2013), *S. cerevisiae* D-10-BT (1.5 g/L/h) (Oh et al., 2013) and *Kluyveromyces marxianus* YZJ015 (4.43 g/L/h) (Zhang et al., 2014). The xylitol conversion efficiency of *K. marxianus* YZJ015 was limited to 89%. As far as specific productivity is concerned, modified *E. coli* (0.29 g/g cdw/h) (Cirino et al., 2006) and yeast *Saccharomyces cerevisiae* (0.34 g/g cdw/h) (Oh et al., 2007) showed the best reported values. But the exploitation of *S. cerevisiae* for direct transformation of APL was ineffective due to inhibitors present in the hydrolysate (Iwaki et al., 2013). Similarly, application of genetically modified *L. lactis* (Nyyssola et al., 2005) and *E. coli* (Khankal et al., 2008) were also restricted to pure substrates (xylose and glucose). Moreover, there is no data available on xylitol formation when mixed sugars (hydrolysate) are used as substrate. In comparison to the previous reports, the *C. glutamicum* strain *Cg-ax3* showed superior specific productivity with xylitol conversion efficiencies based on the concentrations of pentose sugars utilized. Inactivation of xylitol import, elucidation and enhancement of the unknown xylitol secretary system and pentose transporter system are the future possibilities of further improvement of the strain.

5. Conclusions

The recombinant *C. glutamicum* strain constructed in this study allowed for xylitol production from xylose, arabinose and blends of these pentoses without concomitant formation of arabitol as by-product. Morover, arabitol-free xylitol production was achieved using the pentose-rich hemicellulosic hydrolysate acid pre-treated liquor of sorghum stover by direct fermentation.

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