

# Xylitol production by genetically modified industrial strain of *Saccharomyces cerevisiae* using glycerol as co-substrate

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Received: 27 October 2016 / Accepted: 29 January 2017 / Published online: 10 February 2017  
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**Abstract** Xylitol is commercially used in chewing gum and dental care products as a low calorie sweetener having medicinal properties. Industrial yeast strain of *S. cerevisiae* was genetically modified to overexpress an endogenous aldose reductase gene *GRE3* and a xylose transporter gene *SUT1* for the production of xylitol. The recombinant strain (XP-RTK) carried the expression cassettes of both the genes and the G418 resistance marker cassette KanMX integrated into the genome of *S. cerevisiae*. Short segments from the 5' and 3' delta regions of the Ty1 retrotransposons were used as homology regions for integration of the cassettes. Xylitol production by the industrial recombinant strain was evaluated using hemicellulosic hydrolysate of the corn cob with glucose as the cosubstrate. The recombinant strain XP-RTK showed significantly higher xylitol productivity ( $212 \text{ mg L}^{-1} \text{ h}^{-1}$ ) over the control strain XP ( $81 \text{ mg L}^{-1} \text{ h}^{-1}$ ). Glucose was successfully replaced by glycerol as a co-substrate for xylitol production by *S. cerevisiae*. Strain XP-RTK showed the highest xylitol productivity of  $318.6 \text{ mg L}^{-1} \text{ h}^{-1}$  and titre of  $47 \text{ g L}^{-1}$  of xylitol at

$12 \text{ g L}^{-1}$  initial DCW using glycerol as cosubstrate. The amount of glycerol consumed per amount of xylitol produced ( $0.47 \text{ mol mol}^{-1}$ ) was significantly lower than glucose ( $23.7 \text{ mol mol}^{-1}$ ). Fermentation strategies such as cell recycle and use of the industrial nitrogen sources were demonstrated using hemicellulosic hydrolysate for xylitol production.

**Keywords** *Saccharomyces cerevisiae* · Xylitol · Ty1 delta region · Hemicellulosic hydrolysate of corn cob · Glycerol

## Introduction

Xylitol is a polyol which has several applications as a low calorie sweetener and for prevention of dental cavities [33]. Microbial production of xylitol is desirable due to the high cost of the current industrial process based on chemical hydrolysis and hydrogenation [24]. Natural xylose metabolising microorganisms including yeasts such as *Candida* [32] have been studied for fermentation of xylose to xylitol. Microbial conversion of xylose to xylitol is carried out by a cofactor (NADPH or NADH) dependent enzyme xylose reductase (XR). Xylitol is further metabolized to xylulose by the enzyme xylitol dehydrogenase (XDH) present in the xylose metabolising microorganisms. Xylulose can then enter pentose phosphate pathway (PPP) where majority of the cofactor NADPH is regenerated [39]. However, application of the natural xylose metabolizing organisms for xylitol production is limited due to the poor efficiency and yield of xylitol on consumed xylose in pretreated hemicellulosic hydrolysates [7, 45].

For improvement in the xylitol production, synthetic biology approach has been used and genetically engineered bacteria [49] or yeasts such as *C. tropicalis* with deleted

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**Electronic supplementary material** The online version of this article (doi:10.1007/s10295-017-1914-3) contains supplementary material, which is available to authorized users.

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*XDH* gene and *S. cerevisiae* strains over-expressing heterologous XR have been developed. Synthetic biology is an excellent tool for design and construction of novel biological constructs to produce the desired products from various platform substrates or feed stocks [34]. Various approaches including DNA assembly tools, introduction of the DNA into the cells and identification of targets for pathway optimization have been employed to develop more efficient xylose fermenting strains [25]. Though considerable advancement has been made using the recombinant strains, development of industrial strains and using hemicellulosic hydrolysates is an important area for improvement in xylitol production [16, 30].

*Saccharomyces cerevisiae* is a Generally Regarded As Safe (GRAS) organism, and is a preferred host for the industrial fermentation processes. *Saccharomyces cerevisiae* demonstrates natural tolerance against the fermentation and metabolic inhibitors such as HMF and furfural by the virtue of elevated function of the PPP and oxidoreductase enzymes [14]. The early research efforts were focused on the use of laboratory strains of *S. cerevisiae* to express xylose reductase genes, due to availability of auxotrophic markers required for cloning gene cassettes [22]. The industrial strains are less amenable to genetic modifications due to varied ploidy levels and adaptations acquired making them robust to withstand the industrial process environments [40].

Although *S. cerevisiae* does not grow on xylose as the sole carbon source, it carries a gene *GRE3*, encoding a non-specific NADPH dependent aldose reductase, which converts xylose to xylitol [23]. It is expressed in the stress responses for hypoxia, osmotic or heat shock, etc. [10]. Overexpression of the endogenous *GRE3* gene in *S. cerevisiae* has been shown to facilitate xylose fermentation [18, 21, 22]. *Saccharomyces cerevisiae* is dependent on the HXT group of sugar transporters for xylose uptake. Expression of the *SUT1* gene encoding a xylose transporter from *P. stipitis* has been reported to improve xylose uptake in *S. cerevisiae* [12, 17, 46]. Though integration in high copy number is desirable for stable overexpression of the cloned gene, it requires iterative transformation of the cassette with different markers. The long terminal repeat sequences (LTR) of the retrotransposons have been previously reported for stable integration of multiple copies of the gene cassette in *S. cerevisiae* [20, 37].

In the present study, an industrial strain of *S. cerevisiae* was genetically engineered to carry stable integrated overexpression cassettes of the genes *GRE3* and *SUT1* in the genome. The gene cassettes were assembled and double crossover homologous recombination was facilitated by the flanking delta or long terminal repeat (LTR) regions of the Ty1 retrotransposon. It is desirable to use the industrial strains due to their high fermentation efficiency and

ability to withstand diverse stress conditions prevailing in the biorefinery processes [5, 47]. Xylitol production was evaluated using glucose as co-substrate in fed batch fermentation by the recombinant industrial strain. For the first time, glycerol has been demonstrated as cosubstrate to replace glucose in xylitol production by the recombinant *S. cerevisiae*. Xylitol production was evaluated with different cell densities and cell recycle fermentation using glycerol as cosubstrate. Finally, xylitol production has been evaluated with recombinant industrial strain using hemicellulosic hydrolysate of corn cob.

## Materials and methods

### Evaluation of glycerol utilization by *S. cerevisiae* strains

Cultures of the *S. cerevisiae* BY4741, *S. cerevisiae* Ethanol Red (ER) and *S. cerevisiae* XP strains were stored in the form of glycerol stocks at  $-80\text{ }^{\circ}\text{C}$ . The stocks were inoculated in 50 mL YPD medium in 250 mL flasks and cell growth was allowed to revive the culture. The overnight grown cells were transferred to the 50 mL medium containing  $10\text{ g L}^{-1}$  yeast extract,  $20\text{ g L}^{-1}$  of peptone and glycerol each (YPG) in 250 mL flask at an initial  $\text{OD}_{600\text{nm}}$  of about 0.4 and were incubated at  $30\text{ }^{\circ}\text{C}$  and shaken 150 rpm. The  $\text{OD}_{600\text{nm}}$  of the cultures was recorded at regular intervals to monitor the cell growth.

### Recombinant strain development

In our previous report, we reported cloning of the genes *GRE3* and *SUT1* in the vectors p426TEF and pD1211, respectively, under the strong and constitutive TEF1 promoter. The recombinant plasmids were maintained in the *E. coli* TOP10F' cells grown in LB medium with 80 ppm ampicillin [21]. The expression cassettes were PCR amplified from the respective purified plasmids using the primers 1 and 2 for the *GRE3* and 3, 4 for *SUT1* as enlisted in the Table S1. The KanMX cassette providing resistance to G418 was used as selection marker in the recombinant yeast strain. The marker cassette was obtained from the vector pJET1.2-B-KanMX. The overexpression cassettes were used as templates for PCR amplification of the segments G-*GRE3* (~1.6 kb) (primers 5, 6), G-*SUT1* (2.3 kb) (primers 7, 8) and G-KanMX (~1.6 kb) (primers 9, 10) using Q5 High-Fidelity DNA polymerase (0491S, NEB, UK). These segments were designed to carry overlapping regions for assembly of the recombinant vector pUC $\delta$ RTK $\delta$  which is shown in Fig. S1. The 5' (primer 5) and 3' (primer 10) delta regions were flanking the segments G-*GRE3* and G-KanMX, respectively. Amplified segments were purified by gel extraction as per kit protocol (28704,

Qiagen Netherlands). The vector pUC19 was used for cloning and maintenance of the construct in *E. coli*. Purified plasmid vector pUC19 was double digested using restriction enzymes *SacI* and *Sall* and linearized vector was then purified by gel extraction. The gene cassettes and the linearized vector were assembled to construct the circular plasmid pUC $\delta$ RTK $\delta$  as shown in Fig. S1, using the Gibson Assembly Cloning Kit (E5510S, NEB, UK) as per kit protocol. The construct was transformed into the *E. coli* competent cells (NEB 5-alpha, C2987, UK) using heat shock and transformants were selected on LB medium containing 80 ppm ampicillin. The transformants were verified for the presence of plasmid pUC $\delta$ RTK $\delta$  and selected recombinants were maintained in the selective LB medium as glycerol stocks at  $-80\text{ }^{\circ}\text{C}$ . The details of the primers, plasmids and the strains used in this study have been provided in Tables S1, S2 and S3, respectively.

The plasmid pUC $\delta$ RTK $\delta$  was purified from *E. coli* cells and digested using the *PsiI* enzyme. The 5.8 kb linear segment  $\delta$ RTK $\delta$  containing the flanking homology regions (40–45 bp each) and overexpression cassettes of *GRE3*, *SUT1* and KanMX, was purified by gel extraction for transformation in *S. cerevisiae*. The *S. cerevisiae* strain XP is a diploid industrial strain isolated from molasses-based distillery in India. *S. cerevisiae* cells were grown till mid exponential phase in the medium containing  $10\text{ g L}^{-1}$  yeast extract,  $20\text{ g L}^{-1}$  peptone with  $20\text{ g L}^{-1}$  glucose (YPD). Electroporation was performed in (BioRad Micropulser™) at 1.5 kV, 25  $\mu\text{F}$  capacitance and resistance of 200  $\Omega$ . The purified linear construct  $\delta$ RTK $\delta$  was transformed using 0.2 mm electroporation cuvette, maintained on ice. The transformants were selected on YPD plates containing 400 ppm of the antibiotic G418 and verified by PCR. Selected transformants of the recombinant industrial strain *S. cerevisiae* (XP-RTK) were grown and maintained in YPD medium with G418 as glycerol stocks at  $-80\text{ }^{\circ}\text{C}$ . Up to 150 recombinant colonies were re-patched on the YPD plates containing G418 at concentrations of 400–4000 ppm for screening for the degree of resistance to the antibiotic G418.

### Preparation of corn cob hemicellulosic hydrolysate

Corn cob having total solid content of 92% on the weight basis was subject to mechanical shear to obtain particle size of 20–40 mm, and soaked in water for nearly 30 min. The feed containing 30% solids was constantly supplied with steam, 2% sulphuric acid and 1% oxalic acid on the basis of the solid content for pre-treatment. The pre-treatment was performed in the continuous digester for 20 min at high pressure and temperature of 6 bars and  $160\text{ }^{\circ}\text{C}$ , respectively, maintained using the acids and steam. The pretreated slurry of the hemicellulosic hydrolysate of corn cob thus generated, contained about

18–20% solids [36]. Partial detoxification and concentration of the xylose stream was achieved by nanofiltration system having spiral wound membrane in the first stage. The membrane having a filtration area was  $0.37\text{ m}^2$  and molecular weight cutoff of 150–300 Da was selected based on the molecular weight of xylose ( $150\text{ g M}^{-1}$ ). The operating conditions were maintained at  $30\text{ }^{\circ}\text{C}$  and 30 bar pressure. In the second stage, the xylose stream was passed through anion exchange resin BF100 (Ion Exchange, India) column for detoxification [8]. The composition of the non-detoxified and detoxified hemicellulosic hydrolysate of corn cob used in fermentation trials has been provided in Table 1.

### Evaluation of glucose as cosubstrate in fed batch fermentation of xylose

The inoculums of the XP and XP-RTK cultures were prepared in YPD medium (pH 5.5) shaken at 150 rpm at  $30\text{ }^{\circ}\text{C}$ , for 24 h to achieve the  $\text{OD}_{600\text{nm}}$  of about 20. The fermentation medium containing  $10\text{ g L}^{-1}$  yeast extract and  $20\text{ g L}^{-1}$  peptone (referred as YP and prepared as  $10\times$  stock) and  $50\text{ g L}^{-1}$  of glucose solution were sterilized separately. The corn cob hemicellulosic hydrolysate was heated at  $80\text{ }^{\circ}\text{C}$  for 10 min before inoculation. The concentrated stocks along with the hemicellulosic hydrolysate were added just before inoculation to the bioreactor to prepare 400 mL fermentation medium (YPD) containing nearly  $40\text{ g L}^{-1}$  xylose. Fermentation was initiated by inoculating 15% v/v of seed culture. The pH of medium was maintained at 5.5 using 4 N NaOH or 4 N HCl. The fermentation was performed in 1 L fermenter (BioFlo/CelliGen 115, New Brunswick, USA) at temperature  $30\text{ }^{\circ}\text{C}$ , agitation of 150 rpm and at 0.2 vvm aeration. Process was operated in batch mode for first 24 h after inoculation. Once the initial glucose was completely consumed, glucose stock solution ( $300\text{ g L}^{-1}$ ) was continuously fed at a flow rate of  $2.5\text{ mL h}^{-1}$ . Antifoam was added as per requirement. All the chemicals were purchased from HiMedia (India).

**Table 1** Composition of non-detoxified and detoxified hemicellulosic hydrolysate of corn cob

Component	Concentration ( $\text{g L}^{-1}$ )	
	Non-detoxified	Detoxified
Xylose	65	54
Glucose	7	5.8
Acetic acid	7	0.44
HMF + furfural	0.7	0
Phenolics	3–4	1

### Mitotic stability estimation

The mitotic stability of the recombinant strain was assessed as the relative viability count of the recombinant cells in fermentation broth. Sample of broth at beginning and the end of the fermentation was spread onto plates of non-selective (YPD) and selective medium with 400 ppm G418 to estimate the fraction of recombinant cells that could retain the antibiotic resistance.

### Xylose fermentation with glycerol as co-substrate at different initial cell densities

For the batch fermentation study at different initial cell densities, the seed cultures of the strains XP and XP-RTK were grown in bulk in 2 L flasks each containing 400 mL of YPD medium. Cells were harvested at 24 h by centrifugation at 8000 rpm for 5 min and the pellets were resuspended to attain initial biomass concentration of 2, 4, 8, 12, 18, 24 g L<sup>-1</sup> DCW in the fermentation medium. Batch fermentation of xylose was performed in 50 mL medium containing 40 g L<sup>-1</sup> glycerol and xylose each (YPGX) 250 mL shaken flask at 150 rpm agitation and 30 °C.

### Cell recycle in batch fermentation of xylose with glycerol

Cell recycle studies were performed in batch fermentation of xylose using YPGX medium. Cells were harvested by centrifugation after 144 h of each batch of fermentation since glycerol was nearly completely consumed (Fig. 4c). The cells were inoculated to the fresh medium at 4 g L<sup>-1</sup> DCW. After each batch the biomass production and xylitol productivity were monitored.

### Xylitol production using alternative nitrogen sources

Xylitol production was studied while using alternative low cost sources of nitrogen with glycerol as co-substrate. The industrial nitrogen sources, corn steep liquor (CSL), urea and diammonium phosphate (DAP) were used in combinations, which contained the total nitrogen content equivalent to laboratory medium consisting of yeast extract and peptone (YP). Seed cultures of XP and XP-RTK grown in YPD medium were harvested by centrifugation and inoculated at 4 g L<sup>-1</sup> DCW to initiate fermentation. The fermentation media contained 40 g L<sup>-1</sup> of glycerol and xylose each. Batch fermentation of xylose was performed in 50 mL medium in 250 mL shaken flask at 150 rpm agitation and 30 °C.

### Xylitol production using detoxified hemicellulosic hydrolysate of corn cob

Preparation of the seed culture and the fermentation medium containing glycerol (40 g L<sup>-1</sup>) and CSL (4.2 g L<sup>-1</sup>) was similar as described earlier. Three different media combinations containing yeast extract and peptone or CSL as nitrogen source and pure xylose or detoxified hemicellulosic hydrolysate of corn cob were prepared. The synthetic xylose stock solution (400 g L<sup>-1</sup>) or detoxified corn cob hemicellulosic hydrolysate (dHHCC) were sterilized separately. Xylose or the hemicellulosic hydrolysate was then added to obtain the xylose concentration of nearly 40 g L<sup>-1</sup> in 50 mL volume of medium. The pH of the fermentation media was adjusted to 5.5 with 4 N HCl and then cells were inoculated at 4 g L<sup>-1</sup> DCW. Xylitol production using the hemicellulosic hydrolysate and pure xylose were compared in the batch fermentation by the recombinant *S. cerevisiae* XP-RTK strain.

### Specific xylose reductase activity measurement

Fermentation samples were centrifuged at 8000 rpm for 5 min to harvest 50 mg cells and were washed with distilled water. Crude cell-free extract was prepared by treating cells with 200 µL Y-PER™ Yeast Protein Extraction Reagent (Thermo Scientific, USA) and 4 µL Protease Inhibitor Cocktail (Sigma Aldrich, USA) and then centrifugation at 13,000 rpm for 10 min. The supernatant or crude cell free extract was immediately used for the enzyme assay and protein measurement or stored at -80 °C. The specific XR activity was estimated by the method reported earlier [44]. The assay reaction contained 150 µM NADPH, 500 mM Potassium phosphate buffer having pH 6, 200 µM xylose and diluted crude cell free extract in a total assay volume of 1 mL. Reaction mixture was allowed to stand on ice for 10 min and absorbance was read at 340 nm using the spectrophotometer (Schimadzu UV2450, Japan). One unit of XR activity is defined as the amount of enzyme required to oxidize 1 µM NADPH in 1 min. Protein content was determined by the standard method [26]. The specific XR activity was calculated by dividing the XR activity by the protein concentration in the samples.

### Mitotic stability measurement

The recombinant strains exhibited loss of vector during growth on non-selective media in fermentation studies. The stability of the recombinant strains was measured as the relative viability count. The fermentation broth was diluted appropriately and spread onto plates of selective (YPD with 400 ppm G418) and non-selective (YPD) media to estimate the fraction of recombinant cells that could retain the integrated segment.

## Analytical methods

The fermentation samples were analyzed for estimating concentrations of glucose, glycerol, xylose, xylitol, ethanol and other byproducts by HPLC (1100 system, Agilent Technologies, USA). Separation was done using the Aminex HPX-87H column (300 × 7.8 mm i.d.) (Bio-Rad, USA) at a column temperature of 55 °C. Sulfuric acid (5 mM) was used as mobile phase at a flow rate of 0.6 mL min<sup>-1</sup> and the compounds were detected using the refractive index detector. For estimation of the dry cell weight the fermentation samples were centrifuged at 8000 rpm for 5 min. The cell pellet was washed with distilled water. The cells were resuspended in 1 mL water and allowed to dry in a glass plate in the vacuum oven at 60 °C. The dry cell weight (DCW) was calculated in g L<sup>-1</sup> from the observed weight.

## Statistical analysis

The data was collected from at least three different and biologically independent experiments. The statistical analysis was done using student's *t* test null hypothesis and represented as mean ± standard error (SE).

## Results and discussion

### Evaluation of glycerol utilization by *S. cerevisiae* strains

Glycerol is a non-fermentative and non-repressive carbon source and it plays a significant role in osmoregulation in *S. cerevisiae* [4, 33]. Glycerol is an abundantly available, low cost carbon source hence it is favourable as a cosubstrate for growth, hence it has attained importance in development of industrially relevant bioprocesses by various microorganisms [27, 31]. Other co-substrates such as glucose, ethanol or cellobiose have been studied for NADPH regeneration during xylose metabolism by recombinant *S. cerevisiae*. Though sugars are abundantly available and

can be readily metabolized by yeasts, they exhibit certain demerits as co-substrates. Glucose exhibits catabolite repression and it is required in significantly higher amounts for xylose fermentation [28]. While utilization of ethanol resulted in lower rates of xylose fermentation than glucose [11, 29], cellobiose is a costly substrates [48]. Glycerol can be used to overcome the limitations of other co-substrates for production of various biochemicals at potentially higher yields [27, 31]. However, poor consumption and growth rate of *S. cerevisiae* on glycerol has been reported to restrict its use as a co-substrate [11, 45]. We compared the maximum specific growth rates ( $\mu_{\max}$ ) of different strains of *S. cerevisiae* on glycerol. The  $\mu_{\max}$  of different laboratory and industrial strains of *S. cerevisiae* on glycerol were measured and the highest  $\mu_{\max}$  of 0.26 h<sup>-1</sup> was obtained by the industrial strain XP (Table 2). Intraspecies diversity in growth characteristics on glycerol as sole carbon source has been reported earlier with *S. cerevisiae* isolates. The genetic diversity in the isolates was used as a tool to develop superior *S. cerevisiae* strain for improved glycerol metabolism [41, 42]. Based on maximum growth rate on glycerol, strain XP was selected for genetic modification to facilitate fermentation of xylose.

### Genome integration of gene cassettes

Earlier we have reported that overexpression of the genes *GRE3* and *SUT1* showed higher xylitol productivity in *S. cerevisiae* as compared to other xylose reductase genes [21]. In present study, we focused on genetically modifying the industrial diploid strain *S. cerevisiae* XP for the production of xylitol. The cassettes for overexpression of the genes *GRE3* (~1.6 kb), *SUT1* (2.3 kb) and the antibiotic marker *KanMX* (~1.6 kb) were synthesized by PCR from the respective plasmids. The 5' and 3' delta homology regions from Ty1 retrotransposon were incorporated using PCR flanking the cassettes of *GRE3* and *KanMX*, respectively. Recombinant circular plasmid pUC $\delta$ RTK $\delta$  was assembled and

**Table 2** Maximum specific growth rates of different strains of *S. cerevisiae* obtained on glycerol in batch mode

Strain	$\mu$ (h <sup>-1</sup> )	Media	References
<i>S. cerevisiae</i> BY4741	0.11 ± 0.08	Complex	This study
<i>S. cerevisiae</i> ER	0.18 ± 0.03	Complex	This study
<i>S. cerevisiae</i> XP	0.26 ± 0.01	Complex	This study
<i>S. cerevisiae</i> CBS 6412-13A	0.08	Synthetic medium	[41]
<i>S. cerevisiae</i> CBS 6412-13A	0.22	Adapted in synthetic medium	[41]
<i>S. cerevisiae</i> CEN. PK 113-7D	0.06	YNB <sup>a</sup>	[31]
<i>S. cerevisiae</i> MG16C	0.17	YNB	[31]
<i>S. cerevisiae</i> CBS 8066-FL20	0.19	Mineral medium	[35]
<i>S. cerevisiae</i> INVSc1	0.2	Complex with trace elements	[38]

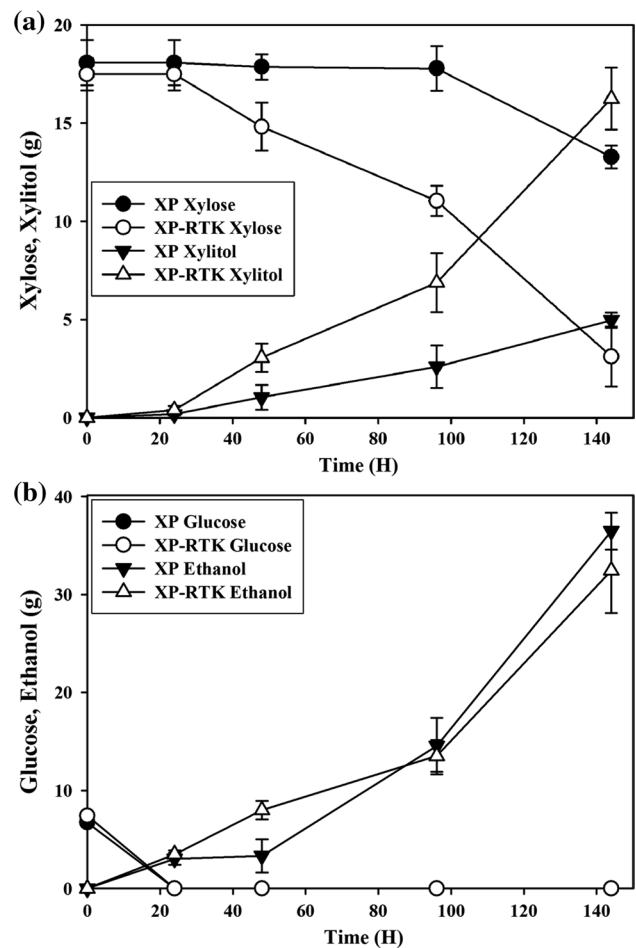
<sup>a</sup> Yeast nitrogen base medium

maintained in *E. coli* (Fig. S1). The plasmid DNA was purified and assembly was verified by PCR amplification and DNA sequencing of the construct (1st Base Laboratories, Malaysia). The linear construct  $\delta$ RTK $\delta$  consisting of the expression cassettes of the genes *GRE3*, *SUT1* and selectable antibiotic marker KanMX, was generated by digestion with *PsiI* and purified using gel extraction. The segment  $\delta$ RTK $\delta$  was then transformed into the electro-competent cells of the industrial strain *S. cerevisiae* XP and the transformants were selected on YPD medium containing antibiotic G418. The integration of the  $\delta$ RTK $\delta$  segment into the genome of recombinant *S. cerevisiae* XP was verified by PCR. Genome integration of the DNA assembly comprising multiple gene cassettes was achieved using the short (~45 bp) segments of the delta homology regions of the Ty1 retrotransposon of *S. cerevisiae*. The complete Ty1 elements or only delta segments of Ty1 exist in *S. cerevisiae* strains at the maximum frequency as compared to the other classes of retrotransposons. Also, the integration using Ty1 delta region has been reported to be more stable as compared to the homology regions from other members of Ty family [9, 19]. The plasmid based recombinant laboratory strains often show heterogeneous gene expression levels which is not desirable in industrial processes [34]. Plasmid based gene cloning results in segregational instability in the absence of selection pressure resulting in lower expression levels. The integration vectors using the auxotrophic markers for recombination allow a single or low copy number of the cloned gene. In our study, the *S. cerevisiae* XP transformants were selected on increasing concentrations of antibiotic G418 for selection of multicopy integrant. Selection of transformants with higher resistance to antibiotic has been reported to be correlated with high copy number integration using Ty delta homology regions [37]. After electroporation, 150 recombinant colonies were randomly selected among the transformants obtained on the plate containing 400 ppm G418 for further screening. The colonies were serially selected on the plates with 500, 600, 800, 1000, 1500, 2000, 3000 and 4000 ppm G418 concentration and the number of colonies that could grow were 138, 127, 79, 39, 13, 6, 2 and 2, respectively. The transformant showing resistance up to tenfold higher G418 concentration than the minimum inhibitory concentration (MIC) was referred to as strain XP-RTK and it was used for xylose fermentation studies.

#### Evaluation of glucose as cosubstrate in fed batch fermentation of xylose

Fed batch fermentation of xylose to xylitol by the recombinant industrial *S. cerevisiae* strain XP-RTK was studied

using glucose as cosubstrate. Xylose was obtained from the hemicellulosic hydrolysate of corn cob, which also contained sugars glucose in negligible amounts and the inhibitory compounds generated during pretreatment. Glucose was fed continuously for regeneration of the cofactor NADPH for the enzyme xylose reductase [13]. Glucose was fed at a growth limiting rate of  $2.2 \text{ g L}^{-1} \text{ h}^{-1}$ , to avoid catabolite repression of xylose metabolism. The recombinant strain produced xylitol with near theoretical yield, showing that xylitol could not be metabolized further. The overall xylose consumption by the recombinant strain XP-RTK (82%) was threefold higher than the control strain XP which showed 26% xylose consumption. Xylitol production was observed in the control strain due to expression of endogenous copy of the *GRE3* gene (Fig. 1a, b). The specific XR activity was compared in the recombinant strain, as well as the control strain using cells harvested from the fermentation broth. The specific activity in the cells of the recombinant strain XP-RTK ( $0.37 \text{ U mg}^{-1}$ ) was found to be



**Fig. 1** Xylitol production by *S. cerevisiae* strains XP and XP-RTK using non-detoxified hemicellulosic hydrolysate of corn cob with glucose fed as cosubstrate showing time profile of xylose, xylitol (a), and glucose, ethanol (b)

**Table 3** Xylitol production by XP and XP-RTK strains of *S. cerevisiae* using glucose (fed batch) or glycerol (batch) fermentation

Process	Fed batch		Batch	
	Co-substrate		Glycerol	
	XP	XP-RTK	XP	XP-RTK
$P_{\text{Xylitol}}$ (mg L <sup>-1</sup> h <sup>-1</sup> )	53.5 ± 18.0	212.0 ± 0.8**	146 ± 13.2	277 ± 17.4**
Specific XR activity (U mg <sup>-1</sup> )	0.20 ± 0.01	0.35 ± 0.02*	0.22 ± 0.05	0.37 ± 0.01*
Xylitol titer (g L <sup>-1</sup> )	5.0 ± 0.4	16.3 ± 1.6**	23.3 ± 0.75	47 ± 2.7**
$Y_{\text{xylitol/cosubstrate}}$ (mol mol <sup>-1</sup> )	–	2.12	–	0.042

Values are statistically significant over the control strain: \*\*  $p < 0.01$ , \*  $p < 0.05$

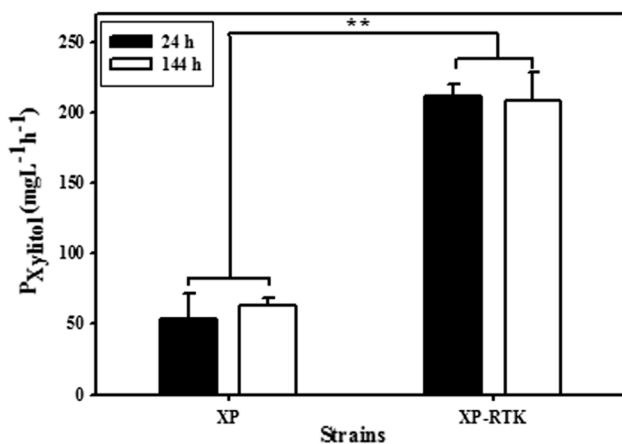
nearly twofold higher over the control strain (Table 3). The higher xylitol productivity by the strain XP-RTK could be attributed to the presence of multiple copies of the expression cassette integrated into the genome. During the batch phase, biomass growth occurred on glucose and the xylose consumption was not observed. In xylose fermentation phase, the specific xylitol productivity of 11 mg g<sup>-1</sup> h<sup>-1</sup> was shown by the recombinant XP-RTK strain. The specific productivity was maintained throughout fermentation, showing that the recombinant strain could stably retain  $\delta\text{RTK}\delta$  expression cassette over the xylose fermentation phase (Fig. 2). Stable genome integration could be facilitated using the short segments of the 5' and 3' delta regions.

**Xylose fermentation with glycerol as co-substrate at different initial cell densities**

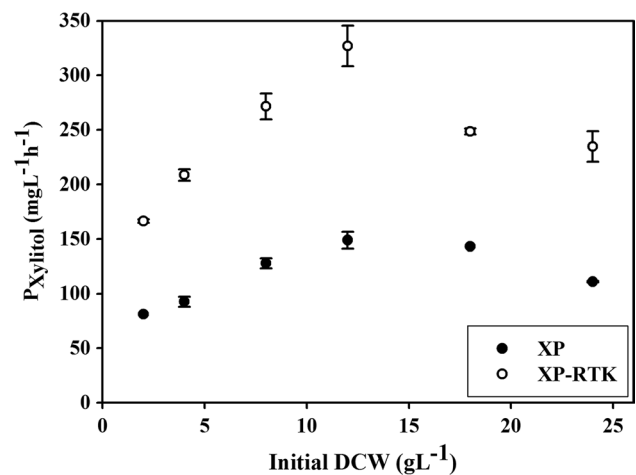
Xylose fermentation was performed using glycerol as co-substrate by the strains XP and XP-RTK at different initial cell densities (Fig. 3). Glycerol does not exhibit catabolite repression and could be co-metabolized with xylose. The aerobic metabolism of glycerol in *S.*

*cerevisiae* involves the gene *GCY1* encoding the enzyme glycerol dehydrogenase that converts glycerol to dihydroxyacetone while regenerating NADPH. The NADPH pool can be utilized for the activity of the enzyme xylose reductase for conversion of xylose to xylitol.

The strain XP-RTK showed a significant increase in xylitol productivity with increasing cell densities as shown in Fig. 3. After 144 h, xylitol productivity of 166, 208 and 271 mg L<sup>-1</sup> h<sup>-1</sup> were achieved at 2, 4 and 8 g L<sup>-1</sup> DCW, respectively, and the highest xylitol productivity of 318 mg L<sup>-1</sup> h<sup>-1</sup> was obtained at 12 g L<sup>-1</sup> DCW. Up to 12 g L<sup>-1</sup> biomass concentration, the yield of xylitol on the consumed glycerol ( $Y_{\text{xylitol/glycerol}}$ ) increased due to overall higher xylose reductase activity. The metabolism of glycerol led to increase in biomass concentration; which augmented the overall xylitol productivity. The control strain also showed highest xylitol productivity of 111 mg L<sup>-1</sup> h<sup>-1</sup> due to expression of endogenous copy of the *GRE3* gene at 12 g L<sup>-1</sup> DCW inoculum. However, the maximum xylitol produced (21 g L<sup>-1</sup>) by the control strain XP was significantly lower than the recombinant strain.



**Fig. 2** Specific productivity of xylitol by the strains XP and XP-RTK in fed batch fermentation with glucose as cosubstrate (\*\* $p < 0.001$ )



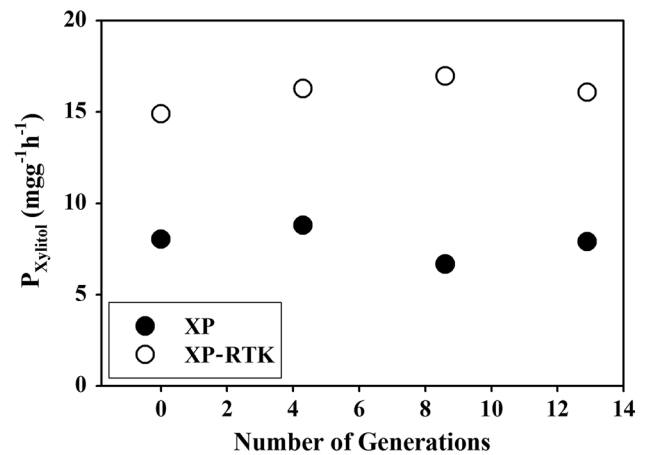
**Fig. 3** Effect of initial cell densities on xylitol productivity by the strains XP and XP-RTK

At cell densities beyond  $12 \text{ g L}^{-1}$ , decrease in xylitol productivity and the biomass formation were observed. This could be attributed to the limitation in aeration required for glycerol metabolism at increased cell densities in shake flask cultures. Higher cell densities lead to anaerobic conditions in the shake flask cultures shifting glycerol metabolism through the enzymes GUT1 and GUT2 which are independent of NADPH [33]. It was observed that equivalent biomass of nearly  $20 \text{ g L}^{-1}$  DCW was produced by the control and the recombinant strains on glycerol as a cosubstrate. Though the glycerol consumption was similar at different cell densities, xylitol productivity improved with the increasing initial cell densities.

The final xylitol titre of  $47 \pm 2.7 \text{ g L}^{-1}$  obtained in the batch fermentation was fourfold higher than the titre ( $12 \text{ g L}^{-1}$ ) achieved in fed batch process with glucose. One of the major advantages of glycerol as cosubstrate include xylose fermentation in batch mode as compared to the fed batch fermentation in glucose based process. Dilution of the broth during the fed batch process resulted in lower final titre of xylitol. The recombinant strain overexpressing *GRE3* gene required continuous regeneration of the cofactor NADPH which is provided by metabolism of the co-substrate. Assuming that the entire glucose is metabolized through PPP and completely oxidized to carbon dioxide, theoretically cells can regenerate 12 mol NADPH per 1 mol glucose consumed [3]. The amount of glucose fed to regenerate NADPH during fermentation of xylose was found to significantly exceed the theoretical requirement. In our studies, the molar yield of xylitol obtained on glucose was  $23.7 \text{ mol mol}^{-1}$ . The poor efficiency of cofactor regeneration with glucose as co-substrate can be explained by the consumption of glucose through the fermentation pathway apart from PPP, to produce ethanol and biomass [6]. Alternatively, glycerol is metabolized by the enzyme glycerol dehydrogenase under aerobic conditions to regenerate 1 mol of NADPH per mole of glycerol. The molar yield of xylitol on glycerol was found to be  $0.47 \text{ mol mol}^{-1}$ , which was closer to the theoretical requirement. This is the first report on xylitol production by industrial recombinant strain of *S. cerevisiae* using glycerol as co-substrate.

#### Cell recycle in batch fermentation of xylose with glycerol

The stability of the recombinant strain was studied during the cell recycle fermentation using non-selective media (YPGX) and xylitol productivity was evaluated. The control and the XP-RTK strain showed specific xylitol productivity of  $7.9$  and  $16 \text{ mg g}^{-1} \text{ h}^{-1}$ , respectively. The recombinant strain showed nearly twofold higher and stable specific



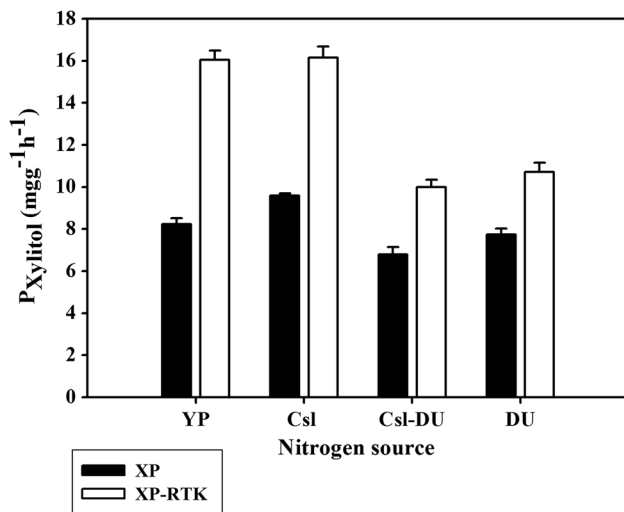
**Fig. 4** The specific xylitol productivity with cell recycle in batch fermentation of xylose with glycerol as co-substrate by the strains XP and XP-RTK

xylitol productivity over nearly 12 generations during four cells recycles (Fig. 4). The glycerol consumption and biomass produced by both the control and the recombinant strain were similar. However, during glycerol metabolism, the reducing power generated in the form of NADPH was efficiently utilized by the recombinant strain as a cofactor for xylitol production. The stable xylitol productivity was achieved by sustained expression of the *GRE3* gene. The stability of the strain during cell recycles also supported the selection of Ty1 retrotransposon delta segments for integration of the expression cassette.

#### Evaluation of industrial nitrogen sources for xylitol production

To improve the feasibility of the xylitol production by recombinant *S. cerevisiae*, industrial sources of nutrients were evaluated. The complex nitrogen sources such as yeast extract and peptone have been reported to enhance xylose metabolism in yeasts [2, 15, 43]. To replace the expensive components of the laboratory media such as yeast extract and peptone, alternative media components were evaluated for xylitol production by the recombinant strain to develop an industrially feasible process. Xylose fermentation was evaluated in media containing combinations of inorganic and organic nitrogen sources as shown in Fig. 5. The total nitrogen content in all the combinations was equivalent to the laboratory medium containing yeast extract and peptone. Among the different media combinations, CSL showed the highest productivity ( $232 \text{ mg L}^{-1} \text{ h}^{-1}$ ) of xylitol, which was comparable to that obtained using laboratory medium. The combination of DAP and urea as nitrogen source in the medium, led to significantly lower xylitol productivity of  $159 \text{ mg L}^{-1} \text{ h}^{-1}$  by the recombinant strain.





**Fig. 5** Specific xylitol productivity using alternative nitrogen sources, YP (10 g L<sup>-1</sup> yeast extract + 20 g L<sup>-1</sup> peptone), Csl (4.2 g L<sup>-1</sup> corn steep liquor), Csl-DU (2.5 g L<sup>-1</sup> corn steep liquor + 5.7 g L<sup>-1</sup> DAP + 1.3 g L<sup>-1</sup> urea), DU (8.5 g L<sup>-1</sup> DAP + 3.8 g L<sup>-1</sup> urea) by the strains XP and XP-RTK. All fermentations were conducted with 40 g L<sup>-1</sup> xylose and 40 g L<sup>-1</sup> glycerol and initial cell density of 4 g DCW per liter

Utilization of CSL as a complex nitrogen source led to significant improvement of about 1.6-fold in the specific productivity of xylitol by the XP-RTK strain (16.2 mg g<sup>-1</sup> h<sup>-1</sup>) over the control strain (9.6 mg g<sup>-1</sup> h<sup>-1</sup>) in spite of similar biomass formation. CSL is a complex nitrogen source consisting of several other macro and micro-nutrients that intensify growth and fermentation by the yeast cells [2, 15].

### Xylitol production using detoxified corn cob hemicellulosic hydrolysate

Xylitol production was compared in pure xylose and hemicellulosic hydrolysate of corn cob to evaluate the feasibility of using recombinant strain on industrial feed stocks. Fermentation using non-detoxified hemicellulosic hydrolysate showed negligible consumption of xylose using glycerol as cosubstrate (data not shown). The inhibitory compounds in hemicellulosic hydrolysate such as furfural, hydroxymethyl furfural (HMF) and acetic acid have been reported to exhibit redox stress in the microorganisms. Various oxidoreductase enzymes in PPP are the targets for inhibition affecting growth and metabolism of the microorganisms [1, 14]. During detoxification of the hemicellulosic hydrolysate, nanofiltration partially reduces the level of acetic acid and further removal of acetic acid along with other inhibitors like furfural, HMF and phenolics is achieved by treatment with anion exchange resin. The composition of the detoxified stream has been provided in Table 1, and it was used to study

xylitol production. The detoxified stream contained significantly lower or negligible amounts of the inhibitors as compared to the non-detoxified stream (Table 1). Xylitol productivity obtained by the strain XP-RTK using detoxified hemicellulosic hydrolysate (196 mg L<sup>-1</sup> h<sup>-1</sup>) or pure xylose (217 mg L<sup>-1</sup> h<sup>-1</sup>) were comparable using YP as nitrogen source. Use of CSL instead of YP for the fermentation of detoxified hemicellulosic hydrolysate also showed nearly similar xylitol productivity of 202 mg L<sup>-1</sup> h<sup>-1</sup> by the strain XP-RTK. The recombinant *S. cerevisiae* strain showed equivalent xylose fermentation rate with both, the industrial or laboratory sources of xylose and nitrogen used in the medium.

### Conclusion

In this study, we showed that recombinant industrial strains having different genetic backgrounds can be utilized for improvement in fermentation efficiency of the microorganism. The overexpression cassettes of the endogenous *GRE3* gene and *SUT1* were integrated into the genome using the short segment of the delta homology regions of the Ty1 retrotransposons to attain stable recombination. This is the first report on fermentation of xylose using glycerol as co-substrate by the recombinant industrial strain of *S. cerevisiae*. Cell recycle strategy and low cost industrial nutrients were utilized for xylitol production using the recombinant strain. The industrial strain showed ability to metabolise glycerol, which could be beneficial for the bio-refineries. Other advantages of using glycerol include, its low cost and formation of negligible byproducts, making glycerol a desirable co-substrate for the industrial fermentation and downstream processes. Our lab is currently working on developing the *S. cerevisiae* strain having higher rates of glycerol metabolism in the presence of inhibitors arising in the pre-treatment of biomass to avoid the detoxification process.

**Acknowledgements** This work was funded by Praj Industries Ltd. We would like to thank Prof. J. Thevelein (VIB, Belgium) for his kind gift of the vector pJET1.2KanMXP. We also thank the Analytical Sciences Team at Praj Matrix for the support in analysis of metabolites during the study.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was obtained from all individual participants included in the study.

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