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Metabolic engineering for sustainable xylitol production from diverse carbon sources in *Pichia pastoris*

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Abstract

Xylitol, known for its health benefits, is a valuable compound in the food and pharmaceutical industries. However, conventional chemical production methods are often unsustainable for large-scale applications, prompting the need for alternative approaches. This study demonstrates a significant enhancement in xylitol production using microbial cell factories, optimized through metabolic engineering. Two synthetic pathways were combined, and the introduction of a novel NADPH-dependent xylitol dehydrogenase further boosted xylitol yields, achieving 0.14 g xylitol/g glucose—a record-high yield for microbial systems. Additionally, the use of sustainable feedstocks, such as glycerol and methanol, led to the production of 7000 mg/L xylitol with a yield of 0.35 g xylitol/g glycerol, and 250 mg/L xylitol from methanol. These results underscore the potential for eco-friendly, cost-effective xylitol production, providing a robust foundation for future industrial-scale biotechnological applications.

Keywords Metabolic engineering, Xylitol biosynthesis, Sustainable carbon sources, *Pichia pastoris*

Background

Xylitol, a naturally occurring five-carbon polyol, is widely used in the food and pharmaceutical industries due to its favorable properties, including its low glycemic index, function as a sweetener, and ability to reduce decay-causing bacteria in the mouth [1]. The United States Department of Energy (USDOE) has identified xylitol as one of the 12 key value-added chemicals [2, 3]. Currently, the xylitol market generates approximately $1.01 billion in annual sales and is expected to grow to $1.37 billion by 2029 [4]. The increasing demand for xylitol underscores the need for more efficient production methods.

The conventional industrial production of xylitol involves multiple steps, beginning with the selection and pretreatment of hemicellulosic biomass, followed by xylose extraction, and culminating in the chemical conversion of xylose to xylitol [5]. However, this process is hindered by high costs, harsh reaction conditions, safety

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concerns, and environmental issues [6]. As an alternative, microbial production of xylitol from xylose reduction—achieved through the expression of xylose reductase—has garnered significant interest for its reliability and sustainability. Microorganisms such as *Pichia pastoris* [7], *Saccharomyces cerevisiae* [8, 9], and *Escherichia coli* [10] have been employed to produce xylitol from D-xylose or hemicellulose hydrolysates. However, xylose is approximately eight times more expensive than glucose, making it a cost-prohibitive raw material [11]. Identifying more cost-effective carbon sources, such as glucose, methanol, and glycerol, is crucial to improving the economic and environmental sustainability of xylitol production.

Glucose, as a more affordable alternative to xylose, has attracted considerable attention for xylitol production. Previous study has demonstrated a microbial process for converting glucose to xylitol using a three-step fermentation with three bacterial strains [12]. This method was later optimized to a two-step process by engineering *Gluconobacter oxydans* to overexpress its native NAD-dependent xylitol dehydrogenase (*XDH*) gene, doubling xylitol productivity [13]. Despite these improvements, the multi-step process remained impractical for industrial applications. Advances in genetic engineering, however, have made it increasingly feasible to construct microbial cell factories capable of directly converting glucose into a range of valuable products, including xylitol.

Xylitol can be produced from xylulose, a common intermediate in many organisms, via xylitol dehydrogenase catalysis. Two primary pathways have been applied to xylulose synthesis for xylitol production. The first is the Xu5P-dependent pathway, where ribulose-5-phosphate (Ru5P), an intermediate of the pentose phosphate pathway (PPP), is converted into xylulose-5-phosphate (Xu5P) by D-ribulose-5-phosphate-3-epimerase, followed by its conversion to xylulose by xylulokinase (*Xks1*). In one study, the xylitol dehydrogenase gene *Xyl2* was expressed in transketolase-deficient *S. cerevisiae*, leading to xylitol production from glucose via the Xu5P-dependent pathway, though yields remained low, with only 3.6% of glucose converted to xylitol alongside ribitol byproducts [14]. The second pathway, the D-arabitol-dependent pathway, converts Ru5P to D-arabitol, which is subsequently catalyzed into D-xylulose. Expression of the *DalD* gene from *Klebsiella Pneumoniae* and *Xyl2* from *Pichia stipitis* in *Zygosaccharomyces rouxii* yielded 15 g/L of xylitol from 400 g/L of glucose, with a yield of 0.038 g xylitol/g glucose [15]. Further, expression of the *DalD* gene from *Klebsiella pneumoniae* and *Xyl2* from *Gluconobacter oxydans* in *P. pastoris* resulted in a xylitol yield of 0.078 g/g glucose [16]. Despite these advances, low productivity remains a significant hurdle for industrial application of microbial xylitol production from glucose.

P. pastoris exhibits higher metabolic fluxes through the PPP, leading to enhanced NADPH production and an increased supply of key precursors critical for efficient xylitol production [17]. Additionally, *P. pastoris* has a natural ability to efficiently utilize cost-effective and sustainable carbon sources, such as methanol and glycerol [18, 19]. Therefore, *P. pastoris* was selected as the chassis cell to develop efficient microbial cell factories for xylitol production.

In this study, we aimed to enhance xylitol production from various carbon sources in methylotrophic yeast *P. pastoris* through pathway optimization and enzyme engineering (Fig. 1). Initially, we constructed the XU5P-dependent pathway, significantly improving xylitol production. Introducing the D-arabitol-dependent pathway further enhanced xylitol production, although it led to the accumulation of xylulose. Additionally, we engineered NADPH-dependent *Xyl2* mutants and deleted the endogenously characterized xylitol dehydrogenase, resulting in further improvements. The optimized strain produced a xylitol titer of 2.8 g/L with a yield of 0.14 g/g glucose in shake flask fermentation. When glycerol or methanol were used as sole carbon sources, xylitol production reached 7000 mg/L with a yield of 0.35 g/g glycerol and 0.25 g/L with a yield of 0.015 g/g methanol, representing the highest reported xylitol production from glucose, glycerol and methanol to date.

Methods

Strains, plasmids and reagents

All plasmids and strains used in this study are listed in Supplementary Tables 1 and 2. The 2× Phanta® Max Master Mix was sourced from Vazyme (Nanjing, China). PrimeSTAR® HS DNA Polymerase, Premix Taq™ (Ex Taq™ Version 2.0 plus dye), and PrimeSTAR® Max DNA Polymerase were obtained from Takara. Phusion™ High-Fidelity DNA Polymerase, CutSmart Buffer (10X), BpiI (BbsI-HF), T4 DNA Ligase, and 10 mM ATP were purchased from NEB. Hygromycin was procured from Yeasen (Shanghai, China). DNA purification kits (Cycle Pure Kit, Plasmid Purification Kit, and Gel Purification Kit) were obtained from Vazyme. Other chemicals, such as D-Sorbitol, DL-Dithiothreitol (DTT), Ethylene Glycol, and Dimethyl Sulfoxide (DMSO), were purchased from Sigma-Aldrich.

Strain cultivation

Escherichia coli strain DH5α was used for plasmid construction. *E. coli* strains were cultivated in Luria–Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) with or without 100 μg/mL hygromycin, at 37 °C with shaking at 200 rpm. *P. pastoris* strain were cultivated in yeast extract peptone medium (YP) consisting of 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose

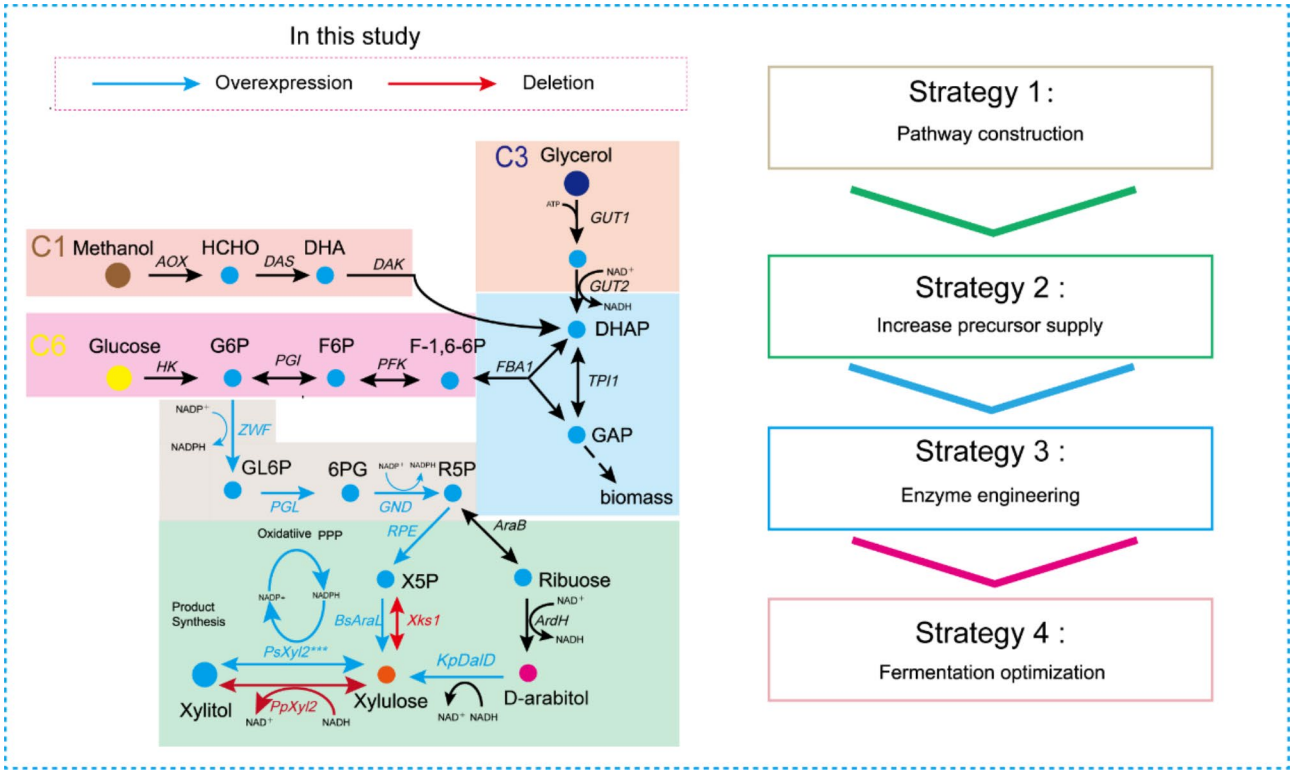

Fig. 1 Metabolic engineering for xylitol biosynthesis from various carbon sources in *P. pastoris*. A high-yield xylitol-producing cell factory was constructed by introducing two synthetic pathways, enhancing the pentose phosphate pathway, performing enzyme engineering, and optimizing fermentation conditions. The engineered strain is capable of producing xylitol from C_1, C_3, and C_6 carbon sources, including methanol, glycerol, and glucose. The key enzymes encoded by the genes used in this study are: *ZWF*, glucose6-phosphate dehydrogenase; *PGL*, phosphogluconolactonase; *GND*, Phosphogluconate dehydrogenase (decarboxylation); *RPE*, ribulose-phosphate-3-epimerase; *Xks1*, xylulose kinase; *AraL*, xylose phosphatase from *B. subtilis*; *KpDalD*, D-arabitol dehydrogenase from *K. pneumoniae*; *PsXyl2*, xylitol dehydrogenases from *P. stipitis*

(YPD), at 30 °C with shaking at 200 rpm, for routine growth and preparation of competent cells.

Shake flask fermentations for xylitol production were carried out in either YP or minimal medium (7.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 10 mg/L histidine, trace metal and vitamin solutions), supplemented with 20 g/L glucose, methanol, or glycerol as the carbon sources [20, 21]. Initially, single colonies were inoculated into 1 mL of YPD to establish 24 h pre-cultures. These pre-cultures were then inoculated into 100-mL non-baffled flasks containing 20 mL of liquid medium at an initial OD_{600} of 0.2 and cultivated at 30 °C with shaking at 200 rpm for 96 h.

Genetic manipulation

The strain GS115 was used as the base strain for all genetic manipulations. All primers used in this study are listed in Supplementary Tables 3, and all codon-optimized gene sequences are provided in Supplementary Table 4, synthesized by GenScript. To delete genes and integrate expression cassettes, we employed the CRISPR–Cas9 system [22]. Guide RNAs (gRNAs) for gene targeting were identified using the CRISPR Direct webtool (http://crispr.dbcls.jp). All gRNA plasmids were constructed based on the BB3cH_pGAP_23*_pLAT1_Cas9 backbone plasmid, generously provided by Professor Gao, and their accuracy was verified by sequencing. All sites for gene insertion were referenced by a previous work [23]. Native promoters, genes, homology arms, and terminators were amplified from GS115 genomic DNA. Gene deletion and expression cassette construction were carried out using fusion PCR. DNA transformation was performed using a modified electroporation method [24], and transformed cells were selected on YPD plates containing 100 μg/mL hygromycin after three days of incubation.

Metabolite extraction and analysis

At the end of shake flask cultivation, all samples were centrifuged to collect the supernatant. The supernatant was filtered through a 0.22 μm membrane and stored at − 20 °C for subsequent quantification of extracellular xylitol, xylulose, and D-arabitol.

Xylitol, xylulose, and D-arabitol concentrations were quantified using a Agilent HPLC system equipped with a refractive index detector (RID). Separation was achieved

using an Aminex HPX-87 H column (Bio-Rad, Hercules, CA; 300 × 7.8 mm; 10 µL injection). Ultrapure water containing 5 mM H_2SO_4 was used as the mobile phase, with a flow rate of 0.6 mL/min. The oven temperature was maintained at 50 °C, and the total run time was approximately 30 min.

Result

Construction and optimization of Xu5P-dependent pathway

In *P. pastoris*, glucose is metabolized into Ru5P via the PPP, which is subsequently converted into Xu5P and xylulose. Xylulose is then reduced to xylitol through the catalytic action of *XDH* (Fig. 2a). To facilitate xylitol production, two xylitol dehydrogenases, *Sc*Xyl2 from *S. cerevisiae* and *Ps*Xyl2 from *P. stipitis*, were overexpressed under the control of a constitutive promoter *TEF1* in the gsy002 strain [25]. The strains were cultivated in minimal

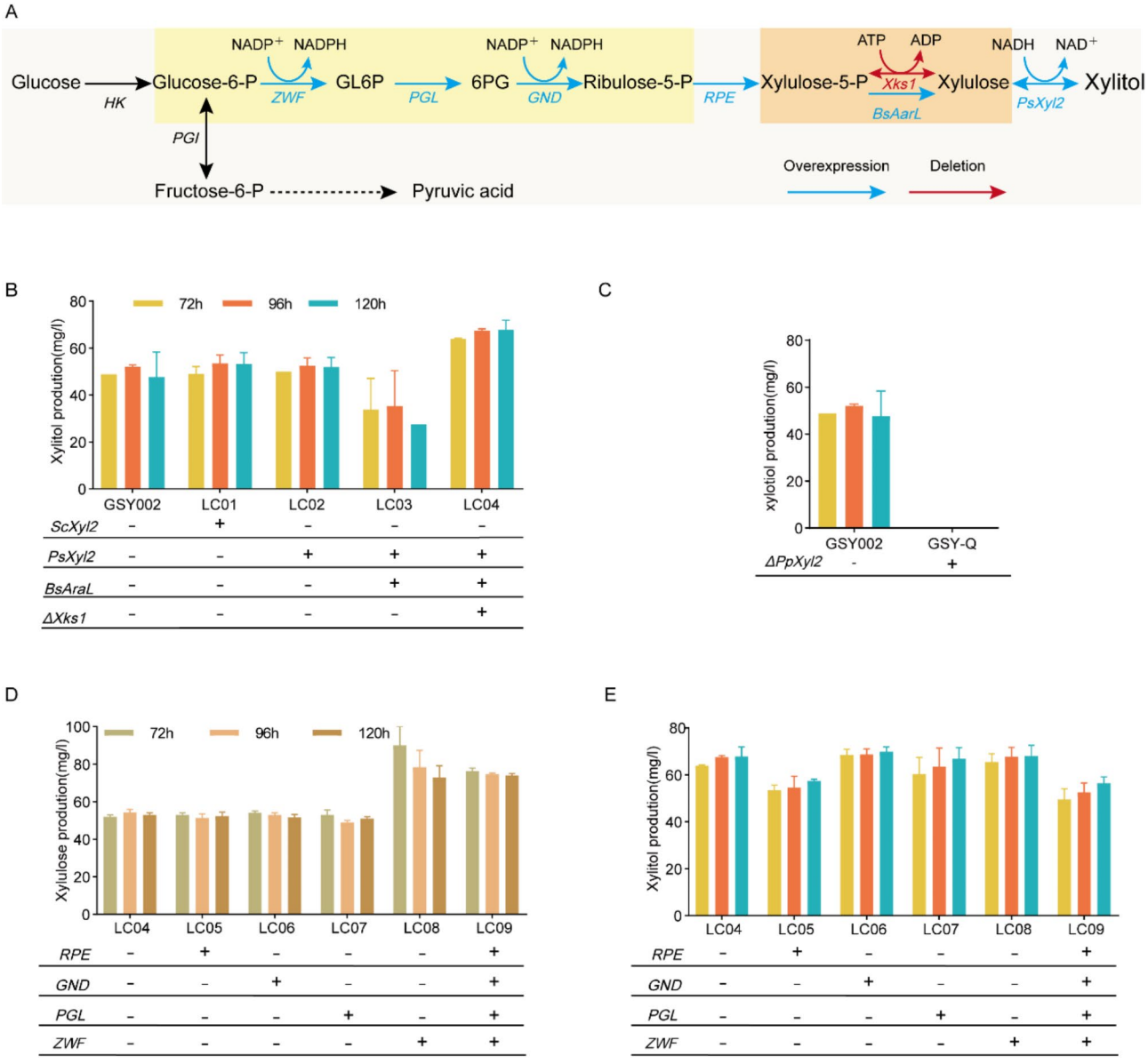

Fig. 2 Construction of XU5P-dependent pathway for xylitol production. (**A**) Metabolic engineering of XUMP-dependent pathway. (**B**) Xylitol production in engineered *P. pastoris* strains expressing heterologous xylitol dehydrogenases and replacing xylulose kinase. (**C**) Deletion of native xylitol dehydrogenase encoding gene *PpXyl2* in the wild-type strain abolishes xylitol production. (**D**) Xylulose production in engineered *P. pastoris* after enhancing the PPP. (**E**) Xylitol production by enhancing the PPP. Cells were cultured in 20 mL of minimal medium with 2% glucose as the sole carbon source. Samples were taken at 72 h, 96 h and 120 h for xylitol detection. Data represent the mean ± SD from biological triplicates

medium containing 2% glucose, and samples were analyzed at three fermentation time points: 72 h, 96 h and 120 h. As illustrated in Fig. 2b, the parental strain gsy002 naturally produced 50 mg/L of xylitol, indicating the presence of an endogenous *XDH* in *P. pastoris* that facilitates xylitol production [16]. The deletion of the NAD^+-dependent *XDH*, essential for xylose metabolism in *P. pastoris* [26], resulted in a strain incapable of producing xylitol with increase of xylulose accumulation (Fig. 2c and Fig. S2a), confirming the enzyme's function in xylitol biosynthesis. Heterologous expression of *Sc*Xyl2 and *Ps*Xyl2, generating strains LC01 and LC02 (Fig. 2b), did not significantly increase xylitol production, suggesting that the native *XDH* activity is sufficient for converting xylulose into xylitol. Given its widespread use in xylose and xylitol metabolism, as well as the extensive research on mutants for more efficient conversion, *Ps*Xyl2-expressing strains were selected for further investigation [27–29]. Therefore, xylulose generation likely represents the rate-limiting step in xylitol synthesis.

Xylulokinase, a reversible enzyme in yeast, can also competitively convert xylulose and ATP into Xu5P, thereby reducing the accumulation of xylulose for xylitol production. AraL, a xylulokinase from *Bacillus subtilis* with phosphatase activity towards D-xylulose-5-phosphate [11], was expressed in LC02 to increase xylitol production. Surprisingly, xylitol production in LC03 as well as D-arabitol production was lower compared to the parent strain LC02, despite similar cell growth and xylulose production (Fig. 2b and Fig. S3a), suggesting that xylulose may be reversibly converted by *Xks1*. Deleting *Xks1* in LC03 significantly enhanced xylitol production by 40% relative to gsy002 (Fig. 2b) and the xylulose production was also increased (Fig. S3b).

To further increase the supply of xylulose, genes involved in the pentose phosphate pathway—*ZWF*, *PGL1*, *GND1*, and *RPE* were overexpressed individually and in combination (Fig. 1a) [11],. The engineered strains LC08 and LC09, overexpressing *ZWF* and all four genes, respectively, showed a nearly 65% and 60% increase in xylulose production compared to LC04 after 96 h (Fig. 2d). Despite slightly improved cell growth (Fig. S4), xylitol production decreased (Fig. 2e). This suggests that enhancing the PPP increased NADPH availability [30], while xylitol synthesis depends on NADH, leading to a cofactor imbalance that limits further xylitol production. LC09 may have a stronger carbon flux toward the PPP, resulting in more NADPH production compared to LC08. This increased NADPH may contribute to a more pronounced metabolic imbalance, leading to reduced xylitol production. However, supporting precursor supply through the PPP is critical for xylitol production, thus LC09 was selected for further study. Additionally, the production of xylitol plateaued after 96 h; therefore, the sample taken from the 96 h fermentation will be analyzed in subsequent experiments.

Introduction of a D-arabitol-dependent pathway

P. pastoris can synthesize D-arabitol from ribulose-5-phosphate (Ru5P). In our study, D-arabitol production was observed in both the parent strain and the engineered strains (Fig.S2b and S3c). D-arabitol can be converted to xylulose by D-arabitol dehydrogenase, contributing to the xylitol precursor poolFig. . 3a). To enhance xylulose availability, we expressed the D-arabitol dehydrogenase gene *KpDalD* from *K. pneumoniae* in strain LC09, generating strain LC10. This modification resulted in a significant increase in xylitol production to 620 mg/L, a 930% improvement compared to LC09Fig. . 3b). Xylulose accumulation increased by 300%, while D-arabitol levels slightly decreasedFig. . 3c, 3d).

The accumulation of xylulose and D-arabitol suggested a limitation in DalD and XDH activity. Overexpression of *Ps*Xyl2 and *Kp*DalD in strains LC11 and LC12, respectively, did not improve xylitol production relative to LC10. Additionally, a decrease in D-arabinose and xylulose production was observed (Fig. 3e and Fig. S5). (Fig. 3e and Fig. S5). These results imply that the bottleneck is not due to deficiencies in these enzymes.

NADPH-dependent xylitol dehydrogenase increased xylitol production

The PPP generates NADPH, which suggests that using NADPH-dependent xylitol dehydrogenase could resolve the NADH/NAD^+ cofactor imbalance and reduce byproduct formation. Previous studies have engineered *Ps*Xyl2 mutants with altered coenzyme specificity toward $NADP^+$/NADPH, improving xylose utilization [29]. In this study, we evaluated three *Ps*Xyl2 mutants (*Ps*Xyl2*, *Ps*Xyl2**, and *Ps*Xyl2***) for their impact on xylitol production (Fig. 4a). Among these, *Ps*Xyl2*** expression, alongside *Kp*DalD, led to the highest increase in xylitol production in the gsy002 strain, while expression of other variants did not result in significant improvements (Fig. 4b). Moreover, xylulose and D-arabitol production decreased when *Ps*Xyl2*** was expressed, indicating more efficient conversion to xylitol with sufficient NADPH availability.

We expressed *Ps*Xyl2*** in the LC10 strain to determine if its expression could enhance the conversion of xylulose and D-arabitol. Overexpression of *Ps*Xyl2*** led to a 37% increase in xylitol yield, reaching 850 mg/L, compared to LC10, whereas overexpression of wild-type *Ps*Xyl2 did not significantly impact xylitol production (Fig. 4c). As expected, xylulose and D-arabitol levels in the *Ps*Xyl2***-expressing strain LC14 remained unchanged (Fig. 4c). Additionally, overexpression of native *Pp*Xyl2 resulted in a 27% increase in xylitol production, coupled with a

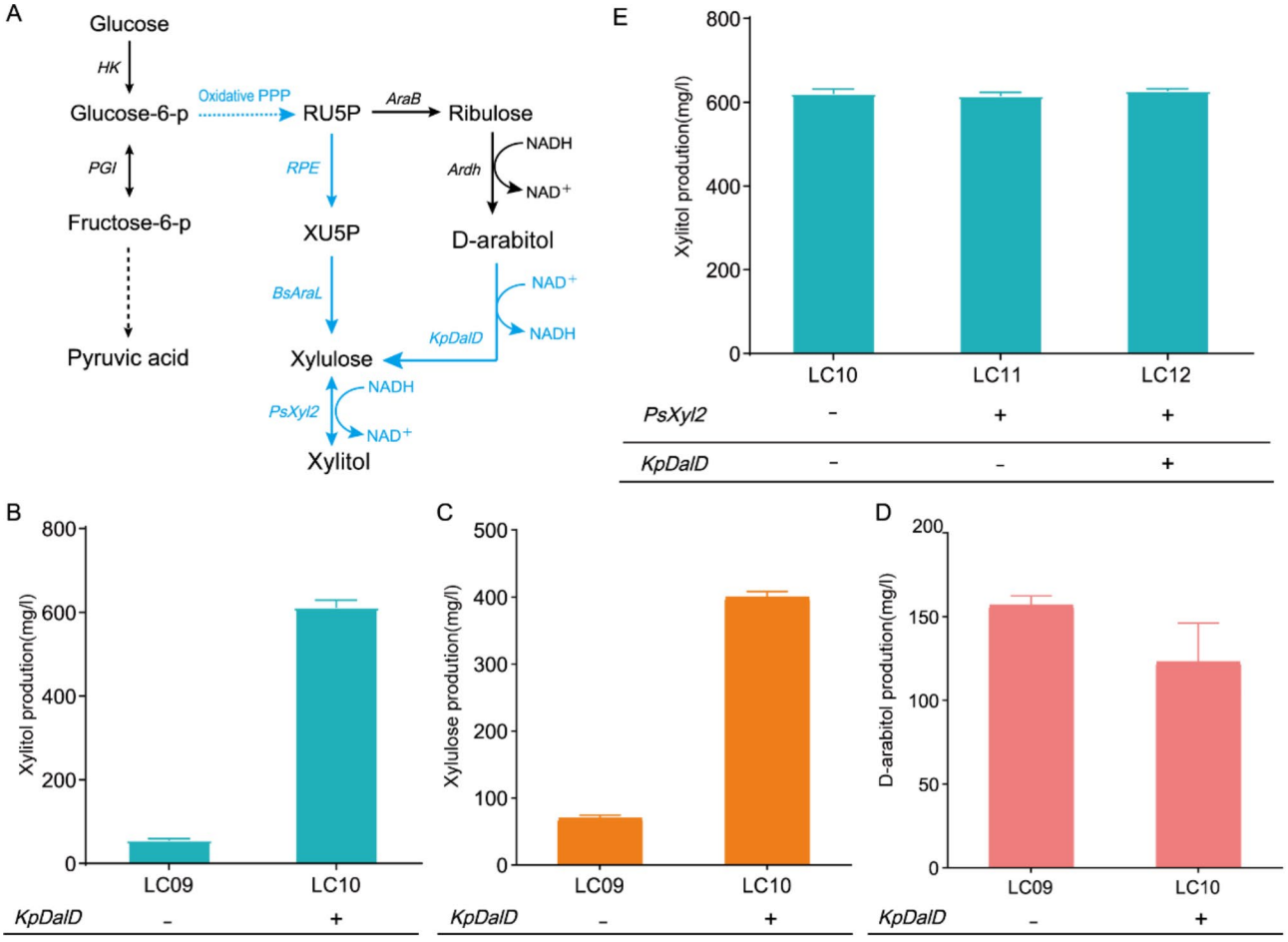

Fig. 3 Introduction of D-arabitol-dependent pathway increased xylitol production. (**A**) Metabolic engineering of D-arabitol-dependent pathway. (**B**) Xylitol production in engineered strain LC10 by expressing *KpDalD*. (**C**) Xylulose production in LC10. (**D**) D-arabitol production in LC10. (**E**) Xylitol production by copy number optimization of *KpDalD* and *PsXyl2*. Cells were cultured in 20 mL of minimal medium with 2% glucose as the sole carbon source. Samples were collected at 96 h for xylitol detection. Data represent the mean ± SD from biological triplicates

decrease in both xylulose and D-arabitol levels (Fig. 4c). These findings suggest that *Ps*Xyl2*** overexpression enhances the consumption of xylulose and D-arabitol, potentially increasing NADPH utilization to drive pentose phosphate pathway flux for sustained xylulose and D-arabitol supply. In contrast, native *Pp*Xyl2 overexpression primarily accelerates the conversion of these precursors, leading to their depletion.

To explore whether further increasing NADPH-dependent *Ps*Xyl2*** and NADH-dependent *Pp*Xyl2 could enhance xylitol production, both enzymes were expressed in the LC14 strain. However, the resulting strain, LC15, showed a significant decrease in xylitol production (Fig. 4d). This reduction was likely due to excess native *Pp*Xyl2 catalyzing the reverse reaction, converting xylitol back into xylulose, which noticeably accumulated. In contrast, deleting *PpXYL2* in LC14 led to a 28% increase in xylitol production, reaching 1050 mg/L (Fig. 4d). These results suggest that co-expression of NADPH-dependent *Ps*Xyl2*** with NADH-dependent *Pp*Xyl2 does not contribute to xylitol production and may even be counterproductive.

Systematic optimization for efficient xylitol production

To develop a NADPH-dependent synthetic pathway aimed at further increasing xylitol production, we integrated several positive engineering strategies into the XP04 strain. The deletion of *Xks1* had no significant effect on xylitol, xylulose, or D-arabitol. In contrast, co-overexpression of *BsAraL* alongside the *Xks1* deletion led to a remarkable 170% increase in xylitol production, reaching 1300 mg/L (Fig. 5a). Additional enhancement of the PPP was achieved by co-overexpressing *ZWF*, *PGL1*, *GND1*, and *RPE*, which resulted in a slight increase in both xylitol and xylulose production. Finally, deletion of the native *Pp*Xyl2 was performed to establish a pathway that exclusively relies on NADPH-dependent xylitol dehydrogenase. This modification further increased xylitol production to 1520 mg/L in minimal medium, while xylulose levels decreased accordingly (Fig. 5a). Although

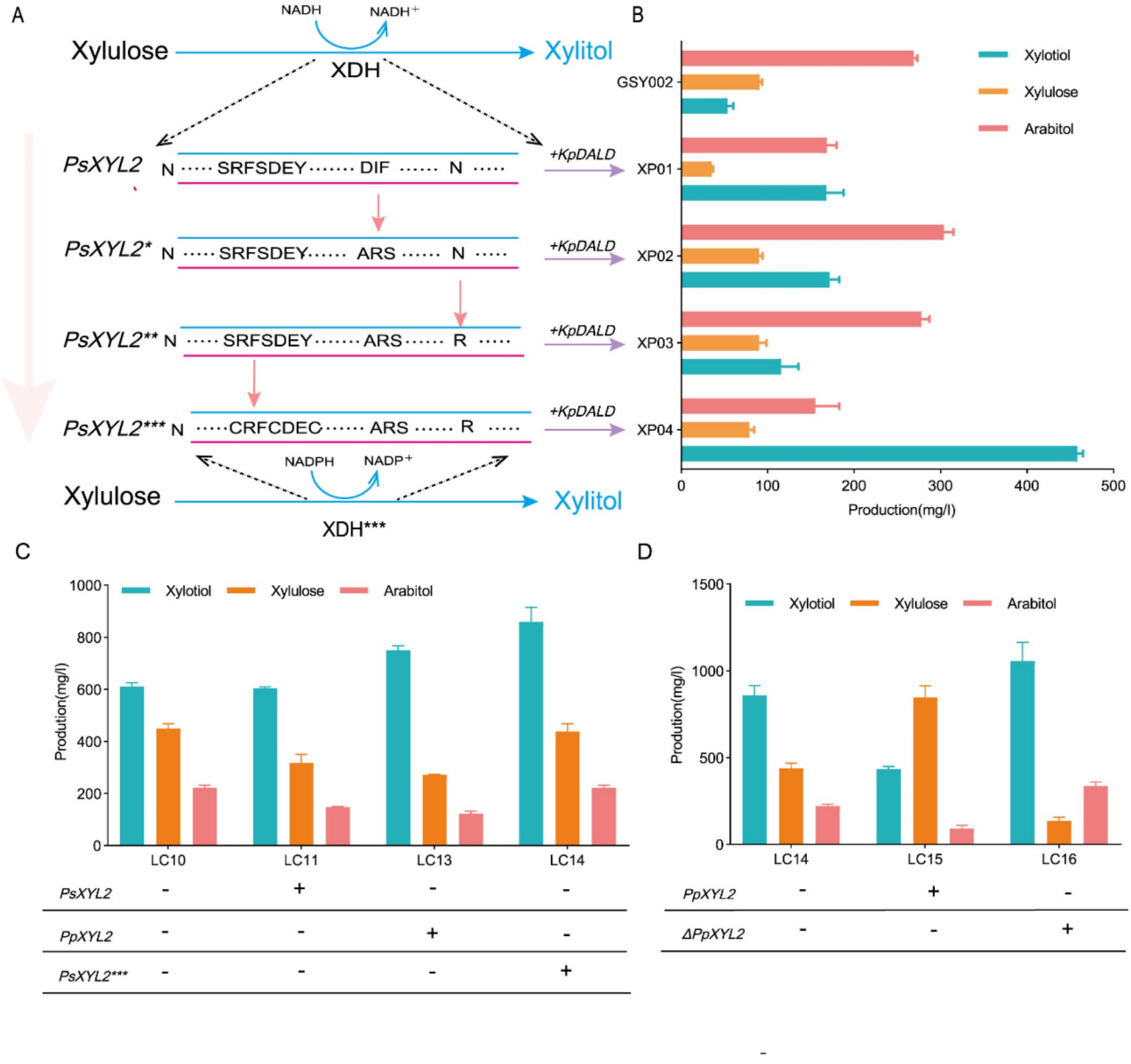

Fig. 4 The effect of NADPH-dependent xylitol dehydrogenase via protein engineering on xylitol production. (**A**) Conversion of NADH-dependent xylitol dehydrogenase to NADPH-dependent xylitol dehydrogenase through site-specific mutations. (**B**) Yields of xylitol, xylulose, and D-arabitol by expressing of *PsXyl2* mutants with *KpDalD* in the gsy002 strain. (**C**) The effect of positive *Ps*Xyl2 mutant on xylitol production in the engineered LC10 strain. (**D**) Deletion of native *PpXyl2* increased xylitol production in *Ps*Xyl2 mutant expressing strain. All data represent the mean ± SD of biological triplicates

this engineering strategy significantly improved xylitol yields, cell growth was impacted, with a 28% reduction observed in strains overexpressing *BsAraL* alongside the *Xks1* deletion (Fig. S6).

To assess xylitol production in a rich medium, strain XP10 was cultivated in YPD, a nutrient-rich medium that supports yeast growth. As shown in Fig. 5b, xylitol production significantly increased under these conditions, reaching 2800 mg/L, with a yield of 0.14 g xylitol/g glucose in shake flask fermentation—representing the highest yield reported to date.

Production of xylitol from alternative feedstocks

Cheaper and more sustainable carbon sources, such as glycerol and methanol, hold great potential for xylitol production but have been relatively underexplored. In this study, we investigated the use of glycerol and methanol as sole carbon sources for xylitol production. As shown in Fig. 6, when glycerol was used as the sole carbon source, xylitol production significantly increased, reaching 7.0 g/L with a yield of 0.35 g/g glycerol—2.5 times higher than when glucose was used. However, when methanol was employed as the sole carbon source, xylitol production was limited to only 250 mg/L. In

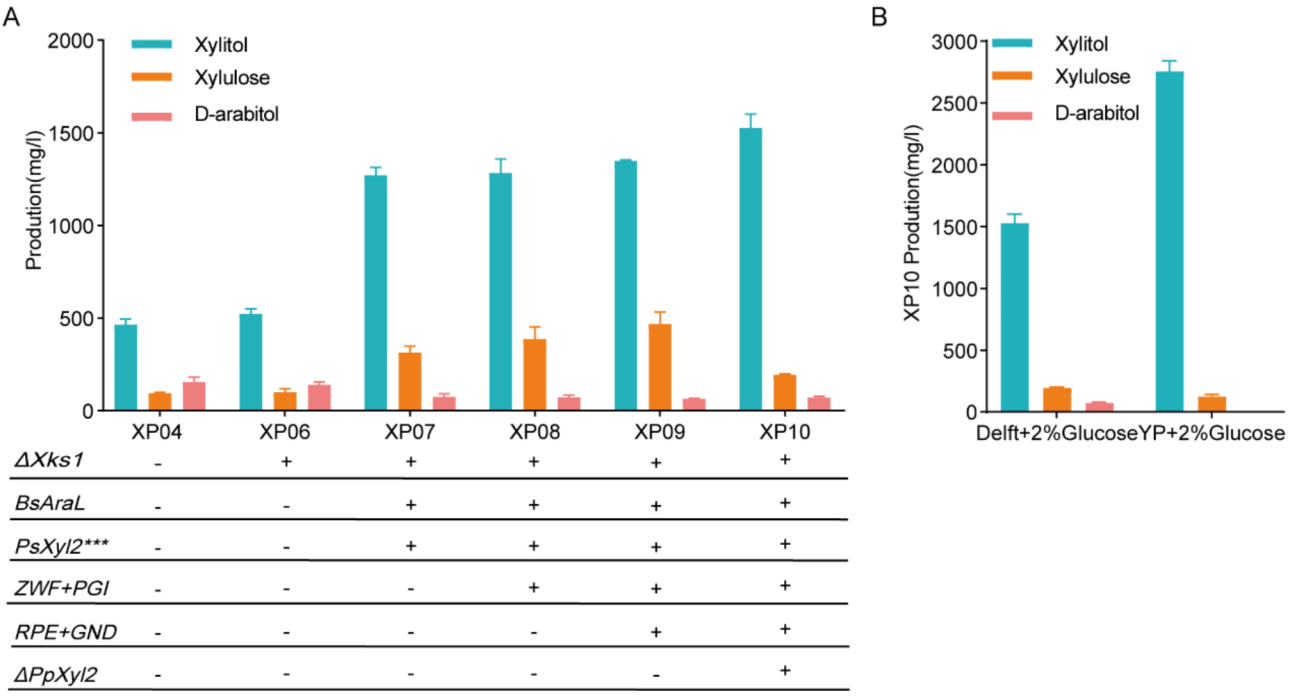

Fig. 5 Integrated positive engineering for efficient xylitol production. (**A**) Xylitol, xylulose, and D-arabitol production in the optimized strains. (**B**) Xylitol production is elevated in rich medium. Cells were cultured in 20 mL of minimal medium or YP medium containing 2% glucose as the sole carbon source. Data represent the mean ± SD from biological triplicates

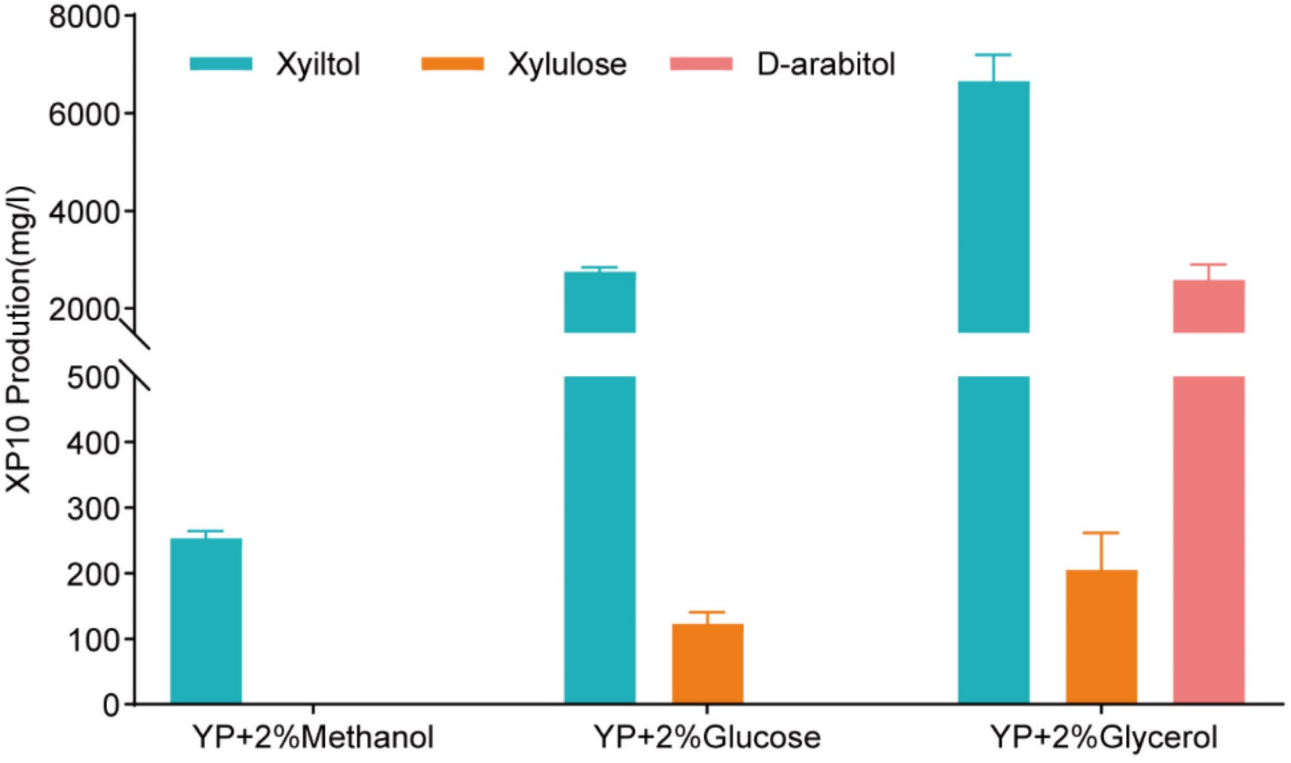

Fig. 6 Xylitol production using different carbon sources. Cells were cultured in 20 mL of YP medium supplemented with 2% of methanol, glucose, glycerol. Data represent the mean ± SD from biological triplicates

addition, the by-products were analyzed under these cultivation conditions. We observed that acetic acid and xylulose were produced when glucose was used as a carbon source, while xylulose and D-arabitol were produced when glycerol was used. No by-products were detected under methanol conditions (Fig. 6 and Fig. S7). These findings highlight glycerol as a more favorable carbon source for xylitol production, while further metabolic engineering efforts are required to improve methanol's conversion efficiency into xylitol.

Discussion

Xylitol is valued with numerous applications in the food and pharmaceutical industries for its health benefits. The increasing demand for xylitol, however, has traditionally been met through chemical methods, which are often unsustainable for large-scale production. To address these issues, there has been growing interest in utilizing microbial cell factories engineered through synthetic biology to produce xylitol from more affordable and sustainable carbon sources, such as glucose and ethanol [14, 16, 25]. Despite these advances, the low yield of xylitol from biological processes remains a significant barrier to its widespread industrial application. Previous studies have attempted to enhance xylitol production by constructing synthetic pathways using either the XU5P-dependent pathway [14] or the D-arabitol-dependent pathway. In this study, the XU5P-dependent pathway was optimized in *Pichia pastoris* by introducing the sugar-phosphatase *Bs*AraL to replace the native xylulokinase, enabling the irreversible conversion of D-xylulose-5-phosphate into xylulose [11]. This modification resulted in an increased xylitol yield. Additionally, the D-arabitol-dependent pathway was introduced by expressing the D-arabitol dehydrogenase *Kp*DalD, further enhancing xylitol production. The simultaneous integration of these two pathways demonstrated a combinatorial positive effect on xylitol yield.

Different heterologous xylitol dehydrogenases have been used in *P. pastoris* for xylitol production [16]. Interestingly, we found that native NADH-dependent xylitol dehydrogenase *(Pp*Xyl2) also plays a critical role in xylitol production (Fig. 2c). This finding underscores the native xylitol dehydrogenase shows outstanding activity on xylitol biosynthesis from glucose.

The PPP was strengthened by overexpressing key genes to produce the necessary precursors. However, the PPP also generates NADPH, whereas most xylitol dehydrogenases are NADH-dependent. This mismatch can lead to an imbalance of cofactors, thereby limiting xylitol production. To address this issue, we selected an NADPH-dependent xylitol dehydrogenase (*Ps*Xyl2***) from several mutants of *Ps*Xyl2, which had been engineered for altered coenzyme specificity towards $NADP^+$/NADPH. The expression of this NADPH-dependent dehydrogenase significantly increased xylitol production (Fig. 4a). Furthermore, the deletion of the native NADH-dependent *Pp*Xyl2 in favor of the NADPH-dependent xylitol dehydrogenase pathway led to further improvements in xylitol yield, achieving a concentration of 2800 mg/L, with a xylitol yield of 0.14 g/g glucose, which is much higher than the 0.078 g/g glucose reported previously [16], and is the highest yield in *P. pastoris* recorded to date (Fig. 5b). These results indicate that NADPH-dependent xylitol dehydrogenase is more suitable for creating microbial cell factories aimed at biotechnological xylitol production. To further enhance xylitol synthesis in the future, there are several promising metabolic engineering strategies, including increasing the copy number of key pathway enzymes, introducing xylitol-phosphate dehydrogenase, which has been shown to function effectively in *Bacillus subtilis* to achieve a xylitol yield of 0.23 g/g glucose [31], reducing glycolysis by downregulating enzymes like phosphoglucose isomerase and phosphofructokinase, boosting gluconeogenesis through overexpression of fructose-1,6-bisphosphatase, and enhancing glucose transport and phosphorylation. While glucose is a relatively low-cost feedstock for xylitol production, its use presents a sustainability challenge due to competition with food resources. This underscores the need for developing alternative, more sustainable feedstocks. Previous studies have demonstrated that ethanol and glycerol can serve as feedstocks, though with a notably low xylitol yield (less 5 mg/L) and 0.045 g/g glycerol [25, 32]. In this study, we investigated glycerol, an industrial by-product, and methanol, which can be synthesized from CO_2, as alternative carbon sources. Xylitol production from glycerol reached 7000 mg/L, which is 2.5-fold higher than from glucose. In contrast, methanol as a sole carbon source yielded only 250 mg/L of xylitol. This study is the first to successfully demonstrate xylitol production from both glycerol and methanol, with glycerol proving to be the more efficient feedstock. However, further metabolic engineering is needed to enhance methanol's conversion efficiency for xylitol production.

Conclusion

In conclusion, xylitol production was significantly enhanced through the combined use of two synthetic pathways. The introduction of NADPH-dependent xylitol dehydrogenase, a novel approach for xylitol production, further boosted yield, achieving the highest recorded yield to date at 0.14 g xylitol/g glucose and 0.35 g xylitol/g glycerol. Additionally, the successful use of sustainable feedstocks, such as glycerol and methanol, underscores the potential for environmentally friendly and economically viable xylitol production. Our engineering strategies

provide novel insights and a foundation for enabling commercial-scale biotechnological production of xylitol.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02683-3.

Supplementary Material 1

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Author contributions
XL and MC designed and performed main experiments, analyzed data and wrote the manuscript. XYL performed main experiments. WC、ZZ and QW analyzed partial data. TY and AY provided resources. HT supervised the project and revised the final version of the manuscript. All authors read and approved the final manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have read and agreed to the published version of the manuscript.

Competing interests
The authors declare no competing interests.

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References

1. Kumar S, Dheeran P, Taherzadeh MJ, Khanal SK. fungal biorefineries. In *Fungal Biology*. 2018.
2. Vishal A, Markéta M, Daniela E, Manoj S, Subhasish S, Kumar S. Biological and pharmacological potential of xylitol: a molecular insight of unique metabolism. Foods. 2020;9:1592–1592.
3. Group IB. US billion ton update: biomass supply for a bioenergy and bioproducts industry (executive summary). Ind Biotechnol. 2011;7:375–80.
4. Xylitol Market Size. &ShareAnalysis - Growth Trends & Forecasts (2024–2029). [https://www.mordorintelligence.com/industry-reports/xylitol-market]
5. Katsimpouras C, Dedes G, Bistis P, Kekos D, Kalogiannis KG, Topakas E. Acetone/water oxidation of corn stover for the production of bioethanol and prebiotic oligosaccharides. Bioresour Technol. 2018;270:208–15.
6. Albuquerque TLd S, IJd M, GRd, Rocha MVP. Biotechnological production of xylitol from lignocellulosic wastes: a review. Process Biochem. 2014;49:1779–89.
7. Yong SJ, Jose C, Jeffries TW. Xylitol production by a *Pichia stipitis* D-xylulokinase mutant. Appl Microbiol Biotechnol. 2005;68:42–5.
8. Meinander NQ, Hahn-Hägerdal B. Influence of cosubstrate concentration on xylose conversion by Recombinant, XYL1-expressing *Saccharomyces cerevisiae*: a comparison of different sugars and ethanol as cosubstrates. Appl Environ Microbiol. 1997;63:1959–64.
9. Bae SM, Park YC, Lee TH, Kweon DH, Choi JH, Kim SK, Ryu YW, Seo JH. Production of xylitol by Recombinant *Saccharomyces cerevisiae* containing xylose reductase gene in repeated fed-batch and cell-recycle fermentations. Enzyme Microb Technol. 2004;35:545–9.
10. Cirino PC, Chin JW, Ingram LO. Engineering *Escherichia coli* for xylitol production from glucose-xylose mixtures. Biotechnol Bioeng. 2006;95:1167–76.
11. Yin W, Cao Y, Jin M, Xian M, Liu W. Metabolic engineering of *E. coli* for xylose production from glucose as the sole carbon source. ACS Synth Biol. 2021;10:2266–75.
12. Onishi H, Suzuki T. Microbial production of xylitol from glucose. Appl Microbiol. 1969;18:1031–5.
13. Sugiyama M, Suzuki S-i, Tonouchi N, Yokozeki K. Cloning of the xylitol dehydrogenase gene from *Gluconobacter oxydans* and improved production of xylitol from D-arabitol. Biosci Biotechnol Biochem. 2003;67:584–91.
14. Toivari MH, Ruohonen L, Miasnikov AN, Richard P, Penttilä M. Metabolic engineering of *Saccharomyces cerevisiae* for conversion of D-glucose to xylitol and other five-carbon sugars and sugar alcohols. Appl Environ Microbiol. 2007;73:5471–6.
15. Harkki AM, Myasnikov AN, Apajalahti JHA, Pastinen OA. Manufacturing of xylitol using Recombinant microbial hosts. United States United STATES Patent US19950368395. 1995.
16. Cheng H, Lv J, Wang H, Wang B, Li Z, Deng Z. Genetically engineered *Pichia pastoris* yeast for conversion of glucose to xylitol by a single-fermentation process. Appl Microbiol Biotechnol. 2014;98:3539–52.
17. Nie Y, Huang M, Lu J, Qian J, Lin W, Chu J, Zhuang Y, Zhang S. Impacts of high β-galactosidase expression on central metabolism of Recombinant *Pichia pastoris GS115* using glucose as sole carbon source via (13)C metabolic flux analysis. J Biotechnol. 2014;187:124–34.
18. Schwarzhans JP, Luttermann T, Geier M, Kalinowski J, Friehs K. Towards systems metabolic engineering in *Pichia pastoris*. Biotechnol Adv. 2017;35:681–710.
19. Jiang W, Hernández Villamor D, Peng H, Chen J, Liu L, Haritos V, Ledesma-Amaro R. Metabolic engineering strategies to enable microbial utilization of C1 feedstocks. Nat Chem Biol. 2021;17:845–55.
20. Wang X, Li Y, Jin Z, Liu X, Gao X, Guo S, Yu T. A novel CRISPR/Cas9 system with high genomic editing efficiency and recyclable auxotrophic selective marker for multiple-step metabolic rewriting in *Pichia pastoris*. Synth Syst Biotechnol. 2023;8:445–51.
21. Yu T, Zhou YJ, Wenning L, Liu Q, Krivoruchko A, Siewers V, Nielsen J, David F. Metabolic engineering of *Saccharomyces cerevisiae* for production of very long chain fatty acid-derived chemicals. Nat Commun. 2017;8:15587.
22. Gassler T, Heistinger L, Mattanovich D, Gasser B, Prielhofer R. CRISPR/Cas9-mediated homology-directed genome editing in *Pichia pastoris*. Methods Mol Biol. 2019;1923:211–25.
23. Cai P, Duan X, Wu X, Gao L, Ye M, Zhou YJ. Recombination machinery engineering facilitates metabolic engineering of the industrial yeast *Pichia pastoris*. Nucleic Acids Res. 2021;49:7791–805.
24. Lin-Cereghino J, Wong WW, Xiong S, Giang W, Luong LT, Vu J, Johnson SD, Lin-Cereghino GP. Condensed protocol for competent cell preparation and transformation of the Methylotrophic yeast *Pichia pastoris*. Biotechniques. 2005;38(44):46.
25. Tang H, Wu L, Guo S, Cao W, Ma W, Wang X, Shen J, Wang M, Zhang Q, Huang M, et al. Metabolic engineering of yeast for the production of carbohydrate-derived foods and chemicals from C1–3 molecules. Nat Catal. 2023;7:21–34.
26. Li P, Sun H, Chen Z, Li Y, Zhu T. Construction of efficient xylose utilizing *Pichia pastoris* for industrial enzyme production. Microb Cell Fact. 2015;14:22.
27. Kim SR, Ha SJ, Kong II, Jin YS. High expression of XYL2 coding for xylitol dehydrogenase is necessary for efficient xylose fermentation by engineered *Saccharomyces cerevisiae*. Metab Eng. 2012;14:336–43.
28. Meinander NQ, Boels I, Hahn-Hägerdal B. Fermentation of xylose/glucose mixtures by metabolically engineered *Saccharomyces cerevisiae* strains expressing XYL1 and XYL2 from *Pichia stipitis* with and without overexpression of TAL1. Bioresour Technol. 1999;68:79–87.
29. Watanabe S, Kodaki T, Makino K. Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc. J Biol Chem. 2005;280:10340–9.

30. Anna ME, Michael S. Utilizing yeasts for the conversion of renewable feedstocks to sugar alcohols - a review. Bioresour Technol. 2021;346:126296–126296.
31. Povelainen M, Miasnikov AN. Production of xylitol by metabolically engineered strains of *Bacillus subtilis*. J Biotechnol. 2007;128:24–31.
32. Cheng H. Construction method and recombinant yeast stain *Yarrowia lipolytica* for xylitol synthesis. United States Patent Application 20220259622. 2022.

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