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Original Research Article

High-level production of free fatty acids from lignocellulose hydrolysate by co-utilizing glucose and xylose in yeast

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ABSTRACT

Lignocellulose bio-refinery via microbial cell factories for chemical production represents a renewable and sustainable route in response to resource starvation and environmental concerns. However, the challenges associated with the co-utilization of xylose and glucose often hinders the efficiency of lignocellulose bioconversion. Here, we engineered yeast *Ogataea polymorpha* to effectively produce free fatty acids from lignocellulose. The non-oxidative branch of the pentose phosphate pathway, and the adaptive expression levels of xylose metabolic pathway genes *XYL1*, *XYL2* and *XYL3*, were systematically optimized. In addition, the introduction of xylose transporter and global regulation of transcription factors achieved synchronous co-utilization of glucose and xylose. The engineered strain produced 11.2 g/L FFAs from lignocellulose hydrolysates, with a yield of up to 0.054 g/g. This study demonstrated that metabolic rewiring of xylose metabolism could support the efficient co-utilization of glucose and xylose from lignocellulosic resources, which may provide theoretical reference for lignocellulose biorefinery.

1. Introduction

Lignocellulosic biomass represents a renewable CO_2 storehouse. Plants can efficiently fix carbon dioxide in the atmosphere through photosynthesis and convert it into organic carbon, generating lignocellulose. Compared with fossil fuels, the utilization of lignocellulosic biomass produces less greenhouse gas emissions, thus contributing to the green productions of various chemicals, materials, and other products due to its sustainability and relatively low costs [1–3]. The main lignocellulosic components cellulose (35–50 %) and hemicellulose (20–35 %) [4–6] can be enzymatically hydrolyzed to fermentative sugars like C6 sugars (glucose, D-mannose and galactose) and C5 sugars (xylose and arabinose), among which glucose and xylose are the main components [7–10]. Therefore, carbon efficient lignocellulose bioconversion to biofuels and chemicals requires the effective co-utilization of glucose and xylose, especially xylose. However, the lack of efficient metabolic elements for xylose uptake and catabolism leads to low efficiency in xylose utilization of many microorganisms [11,12]. Besides, the preferential utilization of glucose often inhibits the xylose metabolism, which causes the well-known glucose catabolite repression [13–15].

To overcome this issue, many endeavors have been made to improve xylose catabolism [11,16,17] and engineer xylose transporters [12,18,19], but the rate of xylose assimilation is still far behind that of glucose. Alternatively, researchers have attempted to enhance xylose utilization through adaptive laboratory evolution (ALE) in sole xylose medium [20–22], but limited targets have been identified to alleviate glucose repression. Despite lots of work focusing on *Saccharomyces cerevisiae* [11,12,20,23], other strains with natural ability of xylose metabolism are often neglected.

Native xylose-utilizing microbes may represent an ideal host for the effective co-fermentation of glucose and xylose [24,25], among which

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Ogataea polymorpha (*Hansenula polymorpha*) has a considerable capacity for xylose utilization [26,27], protein expression and chemical production [28,29]. Moreover, the thermo-tolerance (over 50 °C) and limited by-product accumulation of *O. polymorpha* also contributes to low costs in industrial production and high product yields. Our previous work indicated that enhancing xylose catabolism and uptake in *O. polymorpha* promoted the synchronous co-utilization of glucose and xylose [30], but the overall fermentative performance was not satisfactory, as only half of the glucose and xylose was used, especially in lignocellulosic hydrolysates. This phenomenon could be attributed to limited knowledge on xylose assimilation and transcriptional regulation. Therefore, it is necessary to systematically modify the xylose metabolism pathway of *O. polymorpha* to achieve efficient co-utilization of glucose and xylose.

Here, *O. polymorpha* was extensively engineered for the production of acetyl-CoA derivative free fatty acids (FFAs), a promising alternative of current gasoline, diesel and jet fuels [31,32]. By engineering the stepwise reactions of xylose to 5-phosphoxylulose (Xu5P), introducing xylose transporter, and knocking out some transcription factors that perturb xylose metabolism (Fig. 1), we obtained 1.4 g/L FFAs by co-utilizing 20 g/L glucose and 10 g/L xylose, 3.4-fold higher than that of the starting strain [30]. Furthermore, by using lignocellulose hydrolysates, 11.2 g/L of FFAs were produced from the shake flask fed-batch fermentation. These results indicate that appropriately strengthening the xylose metabolism pathway can alleviate the impact of glucose effect on xylose metabolism for the efficient co-utilization. *O. polymorpha*, as a cell factory, exhibits great potential of lignocellulose biorefineries to produce multifunctional value-added chemicals.

2. Materials and methods

2.1. Strains and media

All strains used in this study were listed in Table S2. All strains were stored in our lab or constructed in this study (Fig. S10). For cultivating yeast strains, YPD medium (20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract), or Delft minimal medium (2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 1 mL/L Vitamin solution, 2 mL/L Trace metal solution) containing the specific substrates. For strain screening, synthetic dropout (SD) media with 6.7 g/L yeast nitrogen base without amino acids and 20 g/L glucose was utilized, supplementing necessary amino acids. The *URA3* marker was removed on SD + URA + 5-fluoroorotic acid (FOA) plates, which consisted of 6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, 20 mg/L uracil, and 2 g/L 5-FOA (Sangon Biotech, Shanghai, China). *Escherichia coli* DH5α was used to construct plasmids, which was cultivated in LB medium (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract, supplemented with 100 mg/L ampicillin). All strains were cultivated at 37 °C, 220 rpm in a shake incubator (Zhichu Shaker ZQZY-CS8).

2.2. Genetic manipulation by CRISPR/Cas9 system

All strains were constructed via our previously established CRISPR/Cas9 system in *O. polymorpha*. Deletion and integration of genes were carried out following the standard procedure [33,34] with gRNA design, plasmids and donor DNA construction, transformation, colony verification, and selective marker removal. Plasmids, primers, constructs of donor DNA and the optimized heterologous genes were listed in Table S3 and Table S4, Table S5 and Table S6 respectively. DNA manipulation like PCR amplification, enzyme digestion, and ligation was performed according to the standard procedure [33]. Donor DNA and gene expression cassettes were constructed by overlap extension PCR (Table S5). For scarless gene deletion, upstream homologous arm was directly linked with downstream homologous arm via overlap extension PCR. The purified DNA fragments with an amount of 500 ng was applied in transformation, together with a specific gRNA plasmid. Similarly, for the site-specific integration, each DNA fragment like upstream and downstream homologous arms, promoter, gene, and terminator was prepared to construct the donor DNA by overlap extension PCR. Other steps were the same with those for scarless gene deletion. O. polymorpha was transformed by electroporation [33,35].

2.3. Detection of glucose and xylose by HPLC

The samples were centrifuged at 12,000 *g* for 10 min, and the supernatant was taken and filtered at 0.22 μm for detection. Concentrations of glucose, xylose and xylitol were analyzed in HPLC (Agilent 1260 HPLC, LC1260 Infinity II) by an Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad, Hercules) equipped with RID systems, which used 5 mmol/L H_2SO_4 as a mobile phase and was eluted at 50 °C with a flow rate of 0.5 mL/min. Data analysis was conducted using Openlab CDS (version 3.4).

2.4. FFA production in shake flasks

FFA production in shake flasks were carried out in Delft minimal medium containing 20 g/L glucose and 10 g/L xylose (G20X10

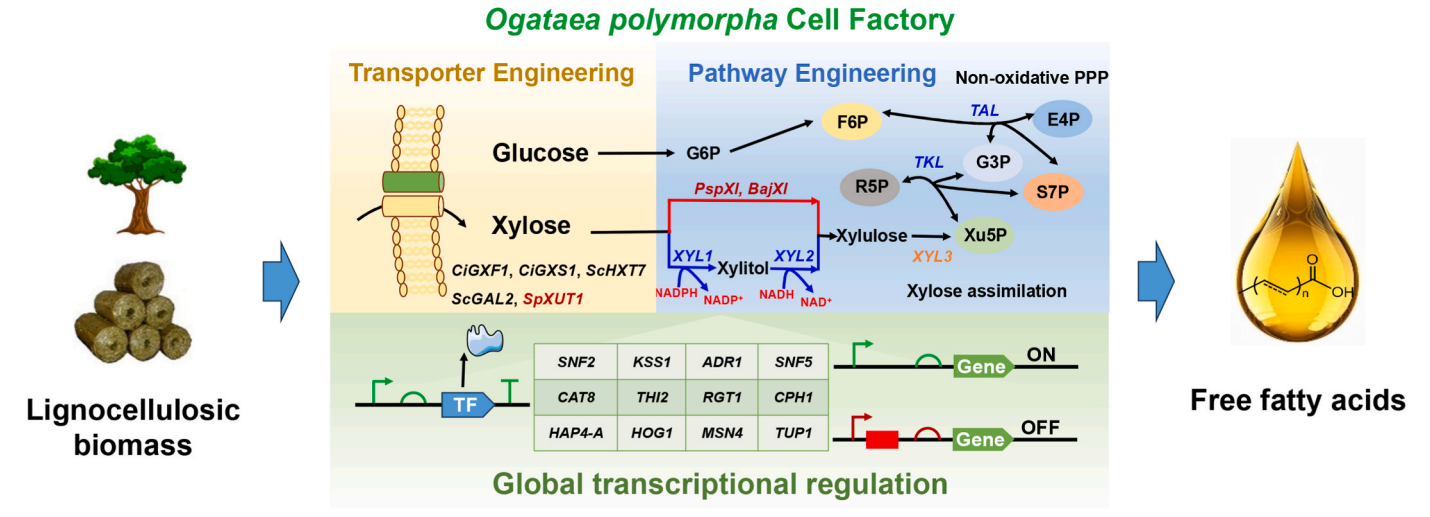

Fig. 1. A yeast platform for FFA production from lignocellulose by engineering the co-utilization of glucose and xylose. We systematically engineered xylose metabolic pathway and xylose transportation, and modified multiple global transcription factors involved in xylose metabolism to further promote the efficient co-utilization of xylose and glucose for FFA production from lignocellulosic biomass.

medium), 10 g/L xylose (X10 medium). Strains were pre-cultured in 15 mL tube with a working volume of 3 mL for 16–20 h, and were then transferred to 100 mL flasks with 20 mL medium at an initial OD_{600} of 0.2. After cultivation for 120 h or 96 h at 37 °C, 220 rpm, samples were collected to analyze the biomass (OD_{600}), sugar consumption, and FFA production.

2.5. FFA production from lignocellulosic hydrolysates

Lignocellulosic hydrolysates with approximately 37 g/L glucose and 19 g/L xylose were adopted and were prepared as previously described [8], containing formic acid, levulinic acid, acetic acid and phenols as the main inhibitors. First, the lignocellulosic hydrolysates supplemented with minimal medium (2.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 1 mL/L vitamin solution and 2 mL/L trace metal solution) was used for fermentation. Precultured strain NX53U were inoculated into 100 mL flasks with 20 mL of medium at an initial OD_{600} of 0.2. Samples were collected to analyze the biomass (OD_{600}), sugar consumption and FFA production within 168 h. On this basis, fed-batch fermentations using concentrated lignocellulosic hydrolysates (containing 72 g/L glucose and 38 g/L xylose) were performed in 200 mL flasks with an initial working volume of 20 mL. Strain NX53U with the initial OD_{600} of 0.5 was cultured for approximately 260 h for the measurements of OD_{600}, sugars and FFAs. The concentrated hydrolysates were fed into the flasks at 24 h (2 mL), 32 h (3 mL), 48 h (5 mL), 72 h (5 mL), 96 h (5 mL), 120 h (5 mL), 144 h (5 mL), 168 h (10 mL) and 216 h (5 mL). pH was adjusted with 4 M KOH according to the pH test strip to about 5–6.

2.6. FFA quantification

Total FFAs extraction was modified from previous reports [36]. Briefly, properly diluted cell cultures (100 μL) were mixed with 100 μL ddH_2O, and then 10 μL of 40 % tetrabutylammonium hydroxide (Sigma, Cat No. 86854) was added. Immediately, 200 μL of 200 mM iodomethane (Sigma, Cat No. 18507) in dichloromethane (Sigma) containing 0.1 mg/mL pentadecanoic acid (Sigma, Cat No. P6125) as internal standard was added, and the mixtures were shaken for 30 min using a vortex mixer (1200 rpm), and then centrifuged to promote phase separation at 2000 *g* for 10 min. The dichloromethane layer (150 μL) was transferred into a GC vial with glass insert and allowed to evaporate to dryness. The extracted methyl esters were then resuspended in pure hexane and analyzed by gas chromatography (Focus GC, Thermo Fisher Scientific) equipped with a Zebron ZB-5MS GUARDIAN capillary column (30 m * 0.25 mm * 0.25 μm, Phenomenex). The GC program was as follows: initial temperature of 40 °C, hold for 2 min; ramp to 180 °C at a rate of 30 °C per minute, then raised to 200 °C at a rate of 4 °C per min and hold for 1 min, then increased to 240 °C at a rate of 2 °C per minute, hold for 10 min. The injection volume was 1 mL. The flow rate of the carrier gas (nitrogen) was set to 1.0 mL/min.

Particularly, for samples of fed-batch fermentation with visible solids, the whole vessels of cell cultures were extracted with ethyl acetate, and the mixtures were shaken at 220 rpm for 30 min, and the upper layer was transferred to a new glass vial and allowed to evaporate to dryness. 200 μL ddH_2O was added, and the following procedure was exactly the same with the above protocol.

2.7. Statistical analysis

Continuous variables are expressed as mean of two or three biologically independent samples with displayed data-points. In all cases, significance was defined as $p \leq 0.05$. Statistical analysis was carried out using paired *t*-test with heteroscedasticity testing method by Office Excel Software.

3. Results

3.1. Non-oxidative pentose phosphate pathway promoted xylose utilization

To avoid the impaired cell growth and sugar utilization [37], gene *LEU2* (encodes 3-isopropylmalate dehydrogenase in leucine metabolism) was *in situ* restored in the previously constructed FFA-producing strain HpFA56 [30], generating strain NX1 (Fig. S1). The strain NX1 exhibited obviously higher rates of cell growth in both xylose (X10) and the mixed glucose and xylose (G20X10). Surprisingly, glucose consumption rate in NX1 was improved, while xylose utilization and FFA titers were significantly decreased, especially in the mixed glucose and xylose. Interestingly, the proportion of unsaturated fatty acids, especially for linoleic acid (C18:2), was significantly increased (Fig. S1G), which might be related to the changed levels of NADPH, as the enhanced NADPH supply provided an electron driving force for fatty acid desaturation [38], however, the production of total FFAs was significantly decrease. We supposed that the supplementation of the gene *LEU2* enhanced the glucose utilization for the increased biomass accumulation, which hindered xylose metabolism. Therefore, it is necessary to further enhance the capacity of xylose metabolism to achieve the efficient co-utilization of mixed sugars.

Xylose enters the central metabolism via the non-oxidative branch of the pentose phosphate pathway (PPP) in the form of Xu5P by the stepwise rearrangement reactions (Fig. 2A). Considering the strengthened xylose assimilation in the starting strain, the non-oxidative PPP was inferred as the rate-limiting step for efficient xylose utilization. The genes that play important roles in the rearrangement reactions are mainly transaldolase (*TAL*) and transketolase (*TKL*) [11]. Therefore, we first strengthened the expression of the *TAL* and *TKL* in strain NX1, generating strains NX2 and NX4, respectively, which was subsequently cultivated in both X10 and G20X10 media. In sole xylose, the overexpression of the *TKL* slightly promoted cell growth and xylose uptake in the early growth stage, while the overexpression of the *TAL* slowed down the uptake rate of xylose in the early growth stage (Fig. 2B and C). No obvious differences in FFA production in both engineered strains (Fig. 2F). However, a totally different result was observed in G20X10 medium. Strain NX2 with the overexpressed *TAL2* and *TAL3* could achieved higher biomass and more efficient co-utilization of 20 g/L glucose and 4.9 g/L xylose despite the longer lag phase (Fig. 2D and E). In contrast, only 1.7 g/L and 1.3 g/L of xylose was consumed in strain NX1 and NX4, respectively (Fig. 2E). In addition, strain NX2 produced 0.86 g/L FFAs with a yield of 0.033 g/g, which was 2.1 times higher than that of NX1 (0.41 g/L) (Fig. 2F). The strengthened non-oxidative PPP drove more metabolic flux to biomass and FFA production from xylose, but over 6 g/L xylose still remained. The combined *TAL* and *TKL* did not further promote xylose utilization (Fig. S2). In this case, we tried other strategies based on strain NX2 to balance cell growth and sugar consumption for the enhanced xylose metabolism.

3.2. XR/XDH and XI pathways synergistically promoted xylose utilization

The strengthened downstream non-oxidative PPP failed to pull all the xylose into central metabolism, which might be limited to the xylose assimilation pathway. Here, we systematically optimized the native XR (xylose reductase)/XDH (xylitol dehydrogenase) pathway and heterogenous XI pathway to further promote xylose metabolism in *O. polymorpha* (Fig. 3A). Firstly, we tested the effects of overexpressing *XYL1* (XR) and *XYL2* (XDH) on xylose metabolism in *O. polymorpha*. Among all engineered strains, NX18 with another copy of *XYL1* and *XYL2* performed best, which consumed 10 g/L xylose within 48 h (Figs. S3A and B), and no obvious xylitol accumulation was observed during the fermentation process (Fig. S4). However, introducing another copy of *XYL1*, *XYL2*, and *XYL3* (XK, xylulokinase) all failed to further improve cell growth in xylose, demonstrating the sufficient XR/XDH

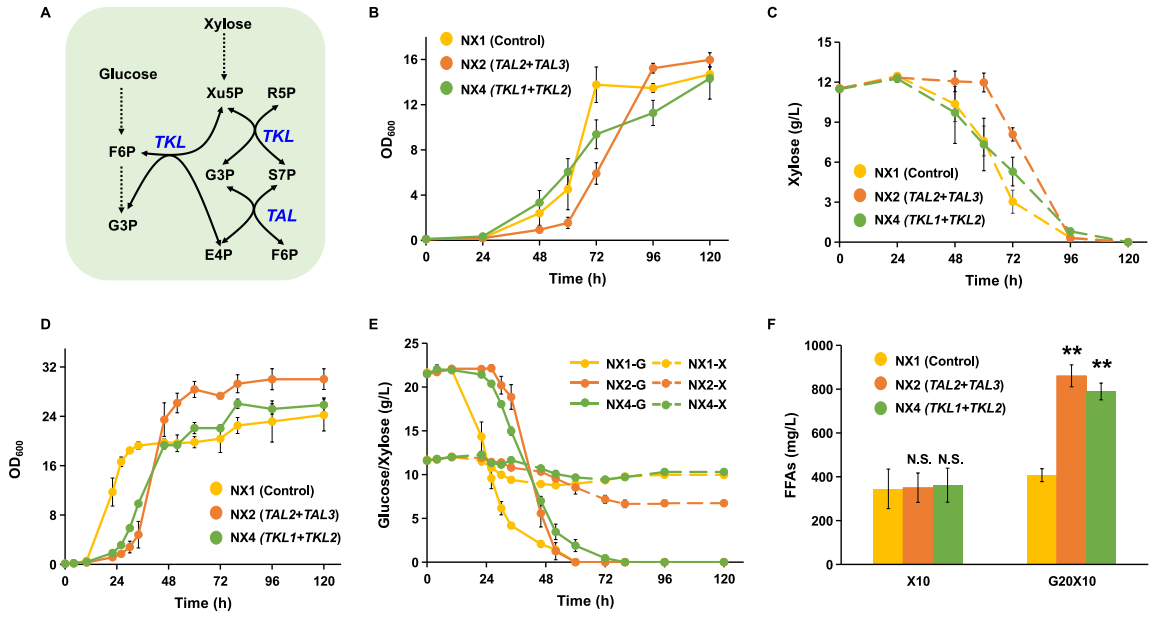

Fig. 2. Strengthening the non-oxidative pentose phosphate pathway to promote xylose metabolism. (A) Scheme of glucose and xylose metabolism through the pentose phosphate pathway. Cell growth (B) and xylose utilization (C) in 10 g/L xylose medium (X10) of strain NX2 (overexpressing gene *TAL2-TAL3*), NX4 (overexpressing gene *TKL1-TKL2*) and control strain. Cell growth (D) and utilization of xylose (dotted) and glucose (solid) (E) in mixed 20 g/L glucose and 10 g/L xylose medium (G20X10) of strain NX2, NX4 and control strain. (F) FFA production of strain NX2, NX4 and control strain in X10 and G20X10 medium. The data were presented as the mean ± s.e.m. (n = 4 biologically independent samples). *P value < 0.05, **P value < 0.01, N.S. represents no significant difference.

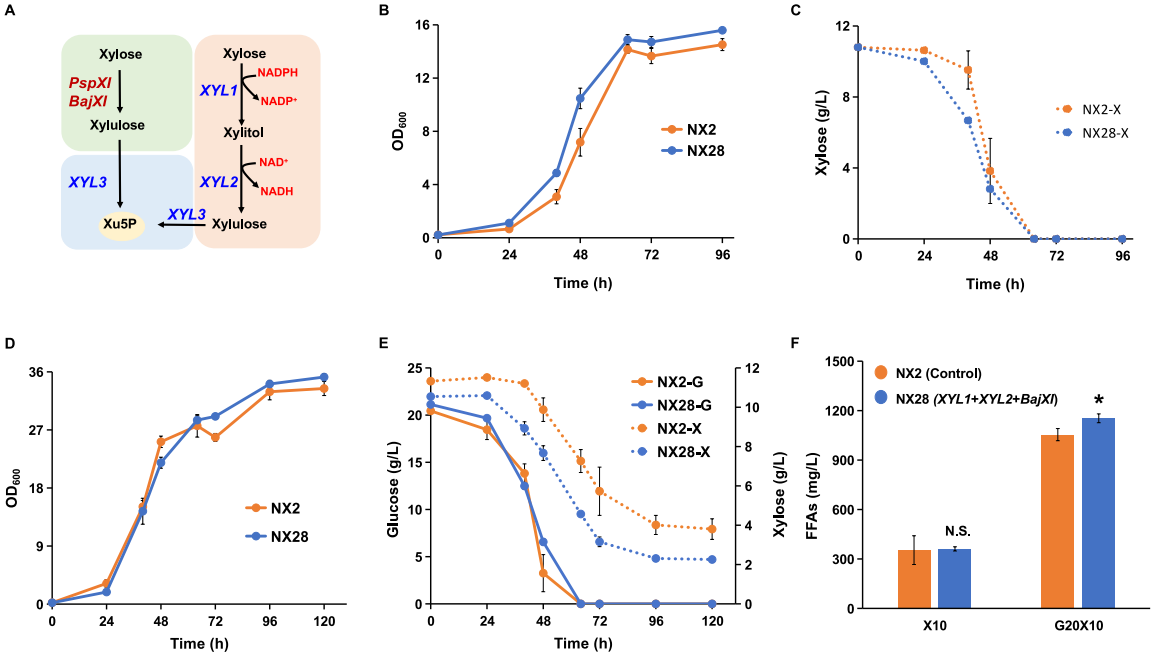

Fig. 3. The combined XR/XDH and XI pathway promoted co-utilization of glucose and xylose. (A) Scheme of xylose assimilation through the XR/XDH or XI pathway. Cell growth (B) and xylose utilization (C) of the control strain and NX28 with the overexpression of *XYL1*, *XYL2* and *BajXI* in X10 medium. Cell growth (D) and utilization of xylose (dotted) and glucose (solid) (E) of the control strain and NX28 in G20X10 medium. (F) FFAs production of strain NX2 and NX28 in X10 and G20X10 medium. The data were presented as the mean ± s.e.m. (n = 3 biologically independent samples). *P value < 0.05, **P value < 0.01, N.S. represents no significant difference.

pathway (Figs. S3C and D).

Alternatively, we attempted to use the XI (xylose isomerase) pathway to support xylose metabolism in *O. polymorpha*, since xylose isomerase has been reported to significantly enhance xylose utilization in *S. cerevisiae* and is immune to the cofactor imbalance [20,39]. Our previous results also showed that *Psp*XI* mutant could slightly promote xylose utilization [30]. However, the single XI pathway failed to support the cell growth in sole xylose despite the increased copy numbers and introducing other functional XI genes (Figs. S5A and B). As an auxiliary pathway, another two reported xylose isomerases, *Baj*XI and *Has*XI [20],

were evaluated in strain NX2. The introduction of gene *Baj*XI significantly improved both cell growth and xylose utilization (Figs. S5C and D), which proved XI pathway is useful in enhancing xylose utilization as it can directly converts xylose without the requirement of coenzyme involvement.

Finally, we conducted combinatorial optimization of XR/XDH pathway and XI pathway, generating strain NX28 with 2 copies of *XYL1/2*, 3 copies of *XYL3*, and *Psp*XI*-*Baj*XI (Fig. 3A). The capacity of xylose metabolism in strain NX28 was significantly improved, especially in the G20X10 medium (Fig. 3). A more efficient xylose utilization was detected, and approximately 6 g/L xylose was consumed when glucose is exhausted at 72 h. A total of 21.2 g/L glucose and 8.3 g/L xylose were co-utilized at 120 h, but there is still 2.3 g/L xylose unconsumed. Strain NX28 produced 1.15 g/L of FFAs (Fig. 3F) with a yield of 0.039 g/g, which was significantly higher than that of strain NX2 due to more xylose consumed. Our results demonstrated that the endogenous XR/XDH pathway is crucial for xylose metabolism in *O. polymorpha*, and the auxiliary XI pathway could synergistically improve strain performance for the efficient co-utilization of glucose and xylose.

3.3. Transporter engineering enhanced sugar co-utilization

In addition to xylose metabolic pathway, xylose transporter is often a decisive factor to control the efficiency of synchronous sugar co-utilization. Therefore, we first evaluated five different xylose transporters, including *CiGXF1* and *CiGXS1* [40,41] from *Candida intermedia*, *ScHXT7** and *ScGAL2* from *S. cerevisiae* [39], and *SpXUT1* from *S. passalidarum* [19]. To balance the cell stress caused by these transporters, strong (P_{GAP}) and moderate (P_{PDH}) constitutive promoters were adopted to drive the gene expression, respectively (Fig. 4A). In X10 medium, 4 engineered strains, NX37 (P_{PDH}-*ScHXT7**), NX39 (P_{PDH}-*ScGAL2*), NX40 (P_{GAP}-*SpXUT1*), and NX41 (P_{PDH}-*SpXUT1*), showed the improved cell growth and xylose utilization, but all strains with the overexpressed *CiGXF1* and *CiGXS1* possessed the compromised capacity of xylose metabolism (Figs. S6A and B). In addition, in G20X10 medium, only strains NX37 and NX40 obviously promoted the synchronous co-utilization of glucose and xylose (Figs. S6E and F). Although the strain NX37 was able to quickly utilize xylose, the massive accumulation of xylitol (5.7 g/L) (Fig. S7) caused a waste of carbon sources (Fig. S6C). Comprehensively considering cell growth, sugar consumption, and FFA production, we found the expression of *SpXUT1* under control of P_{GAP} (NX40) was more effective. As shown in Fig. 4C, about 8.2 g/L of xylose were synchronously utilized with glucose, obtaining 1.30 g/L of FFAs with a yield of 0.041 g/g (Fig. 4D), which was significantly higher compared to those of strain NX28. More importantly, the introduction of xylose transporter *SpXUT1* synergistically promoted the utilization efficiencies of both glucose and xylose (Fig. 4C).

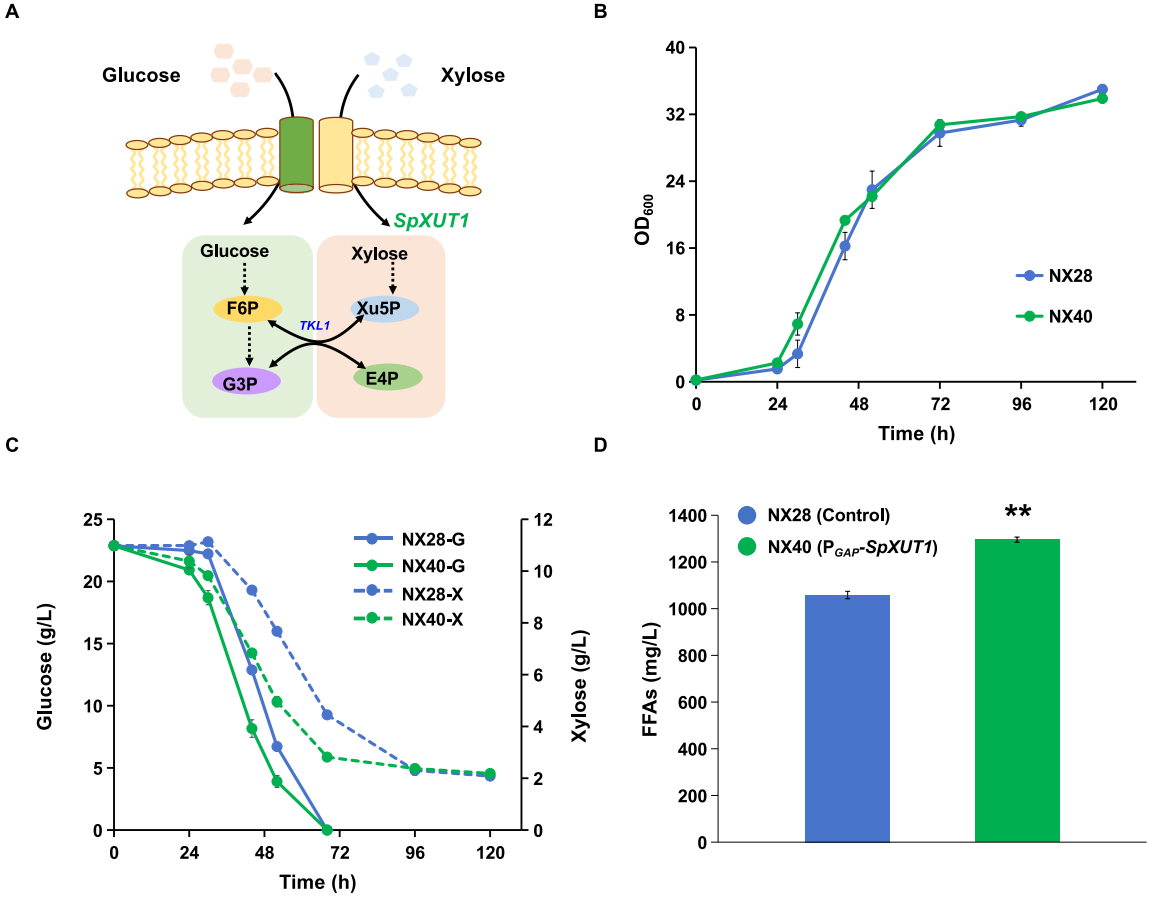

Fig. 4. The introduction of xylose transporters further promotes the synchronous co-utilization of xylose and glucose. (A) To achieve synchronous co-utilization of xylose and glucose, a hexose transporter mutant with a higher affinity to xylose (*SpXUT1*) was introduced. (B) Cell growth of strain NX40 and control strain in G20X10 medium. (C) Glucose utilization (solid) and xylose utilization (dotted) of strain NX40 and control strain in G20X10 medium. (D) FFA production of strain NX40 and control strain in G20X10 medium. The data were presented as the mean ± s.e.m. (n = 3 biologically independent samples). *P value < 0.05, **P value < 0.01, N.S. represents no significant difference.

3.4. Engineering global transcriptional regulation factors to achieve synchronous utilization of glucose and xylose

Rational design helped to optimize xylose catabolism and uptake, but some inhibitors may exist to regulate xylose metabolism at the global transcriptional level in *O. polymorpha*. A total of 12 transcription factors [39,42–44] were screened (Fig. 1), and their functions mainly involved in stress response, MAPK cascade, respiration, and SWI/SNF chromatin remodeling complex. Except for *MSN4* and *TUP1*, we had successfully deleted the other 10 transcription factors, but the absence of most transcription factors did not further enhance the synchronous co-utilization of glucose and xylose in *O. polymorpha* (Fig. S8). The disruption of transcription factor *HAP4-A* [43] that controls the expression of respiratory genes (strain NX53), showed a significant improvement in the synchronous utilization of glucose and xylose (Fig. 5C). Almost all xylose (10.6 g/L) was consumed synchronously with glucose (22.6 g/L) within 96 h, and a better growth behavior was also observed (Fig. 5B). This phenomenon could be attributed to the upregulated gene *RKI* and downregulated gene *RPE* owing to the absence of *HAP4-A*, which promoted Xu5P supply from xylose and maintained the stable PPP (Fig. 5A) [43]. In addition, strain NX53 can produce 1.33 g/L of FFAs with a yield of 0.04 g/g, which was significantly higher than NX40 (Fig. 5D).

It is worth noting that knocking out the transcription factor *SNF2* and *SNF5* related to chromatin remodeling increased the uptake rates of glucose and xylose in the early stage of fermentation (Figs. S8C and D). Therefore, we attempted to knock out *SNF2* and *SNF5* based on *HAP4-A*Δ strain to shorten the fermentative lag phase. However, the disrupted *SNF2* (NX55) and *SNF5* (NX56) both resulted in a significant decrease in biomass and FFA production (Figs. S9A and C). Although the glucose uptake in strain NX55 and NX56 was slightly accelerated, the residual xylose was also correspondingly increased (Fig. S9B), which was supposed to compromise further fermentations using real lignocellulosic hydrolysates.

3.5. FFA production from lignocellulosic hydrolysates

To promote fermentative behaviors, the prototrophic strain NX53U was obtained by supplementing the gene *URA3* (codes orotidine-5′-phosphate decarboxylase) into strain NX53, which exhibited obviously higher rates of cell growth and similar sugar utilization in G20X10 medium (Fig. 6A). Subsequently, the lignocellulosic hydrolysates were adopted to carry out FFAs fermentation. Lignocellulosic hydrolysates used in this study contained 70 g/L of total reducing sugars, including approximately 37 g/L glucose and 19 g/L xylose. Besides, formic acid, levulinic acid, acetic acid and phenols were detectable as the main inhibitors [8]. To improve cell growth, minimum medium components without sugars (delft) were added. The results showed that the engineered *O. polymorpha* also simultaneously utilized xylose and glucose in the hydrolysates (Fig. 6B), producing 3.61 g/L FFAs with a yield of

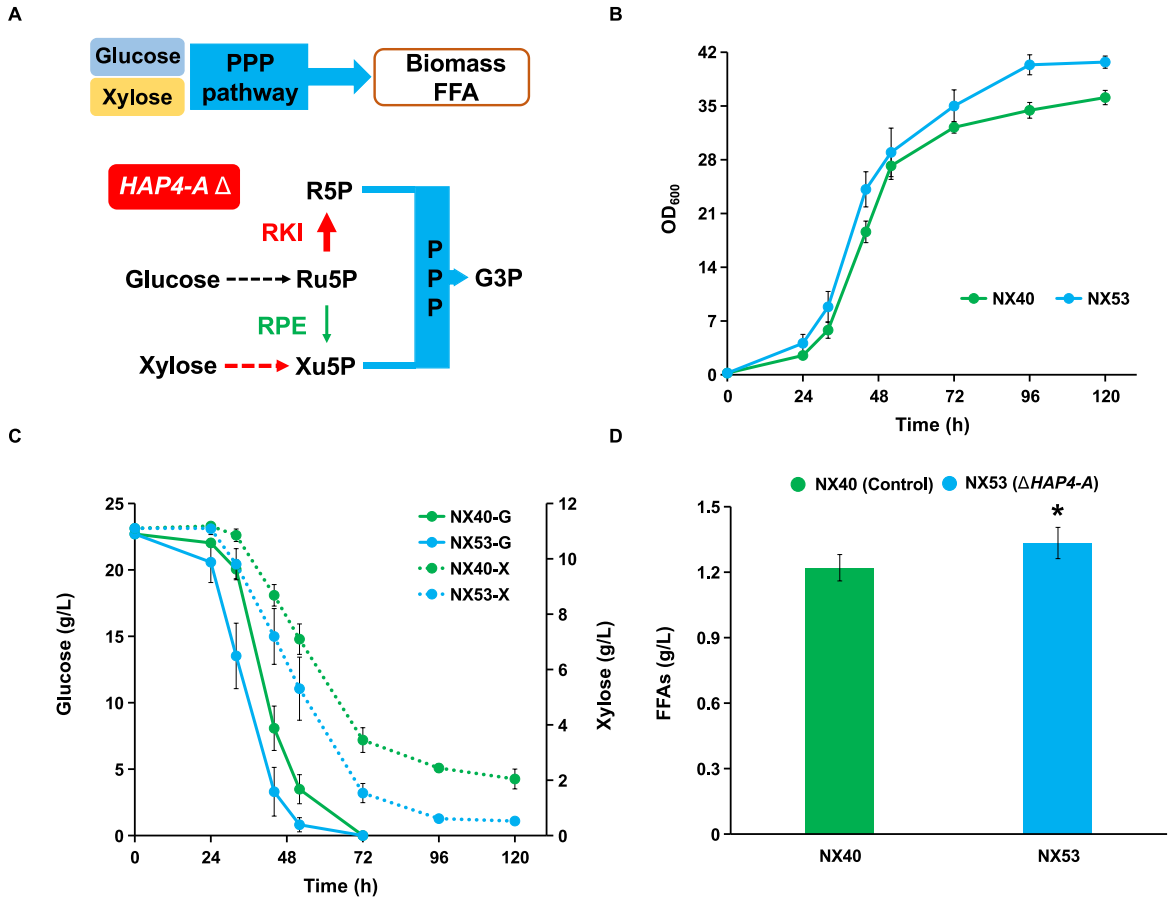

Fig. 5. Global transcriptional regulation promoted the co-utilization of glucose and xylose. (A) In order to further achieve synchronous co-utilization of glucose and xylose, the main transcription factor *HAP4-A* that controls the expression of respiratory genes was knocked out. The absence of transcription factor *HAP4-A* leads to the significant upregulation of *RPI* gene and the significant downregulation of *RPE* gene [43]. (B) Cell growth of strain NX53 and control strain in G20X10 medium. (C) Glucose utilization (solid) and xylose utilization (dotted) of strain NX53 and control strain in G20X10 medium. (D) FFA production of strain NX53 and control strain in G20X10 medium. The data were presented as the mean ± s.e.m. (n = 3 biologically independent samples). *P value < 0.05, **P value < 0.01, N.S. represents no significant difference.

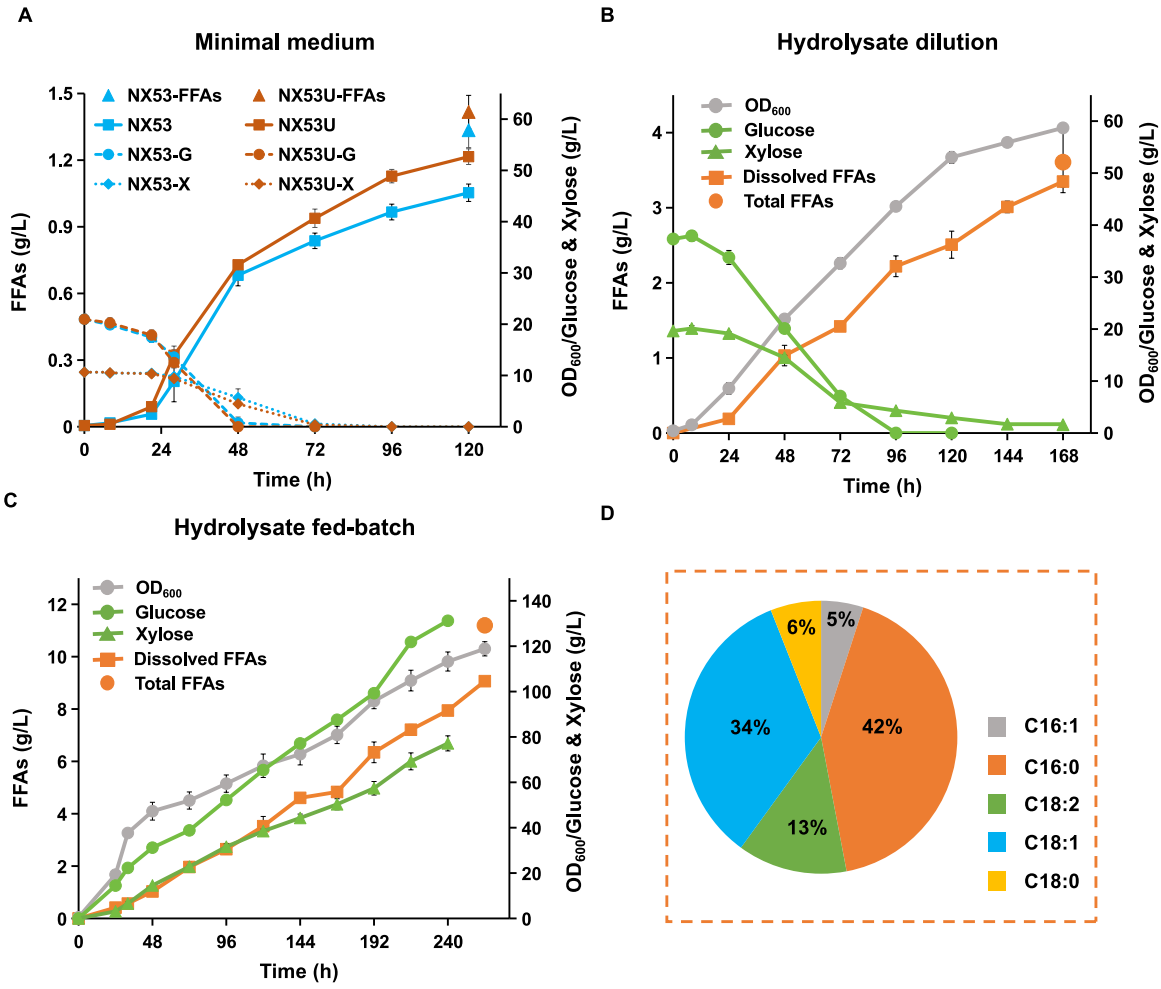

Fig. 6. FFA production from lignocellulosic hydrolysates. (A) Fermentation of strains NX53 and NX53U with the restored auxotrophic gene *URA3* in G20X10 medium. (B) Fermentation of strains NX53U in lignocellulosic hydrolysates, supplemented with minimal medium. (C) Fed-batch fermentation of strain NX53U for FFAs production by feeding raw lignocellulosic hydrolysates. Fermentation in flasks was conducted at 37 °C and 220 rpm. (D) The composition of FFAs. The data were presented as the mean ± s.e.m. (n = 4 biologically independent samples).

0.067 g/g (Fig. 6B). The increased osmotic stress and inhibitors partially hindered cell growth and sugar consumption, resulting in a prolonged fermentative process of 168 h with 2.4 g/L residual xylose (Fig. 6B).

We also attempted to increase the sugar concentrations (72 g/L glucose and 38 g/L xylose) by concentrating the lignocellulosic hydrolysates, which led to the extremely slow cell growth due to the doubled osmotic stress and inhibitors. In this case, fed-batch fermentation using the concentrated lignocellulosic hydrolysates was carried out. Fortunately, this step-feeding strategy greatly promoted fermentative behavior with partially relieved cellular stress. A total of 131 g/L glucose and 77 g/L xylose from the hydrolysates were consumed in a synchronous manner, generating 11.20 g/L FFAs with a yield of 0.054 g/g, which is higher than strain HpFA56 (Table S1) [30]. The ratios of FFAs composition in these two strains were similar (Fig. S1G), and the palmitic acid (C16:0) and oleic acid (C18:1) accounted for nearly 80 % of total FFAs (Fig. 6C and D). More importantly, our results turned *O. polymorpha* into a kind of oil producing yeast, reaching a comparable level to those of the oleaginous yeast, such as *Yarrowia lipolytica* [45], and *Lipomyces starkeyi* [46], and our engineered strain also could effectively utilize more sugars in a relatively short time (Table S1). Therefore, this study provided a feasible route for lignocellulose biorefinery using non-oleaginous yeast to produce FFAs.

4. Discussion

Lignocellulosic biomass is one of the most abundant resources [1–3], but its biotransformation faces huge challenges in regard of coordinated utilization of multiple carbon sources [7]. Therefore, the efficient co-utilization of glucose and xylose is an important prerequisite for the industrial applications of lignocellulosic biomass [47]. In this study, we proposed an efficient yeast platform to produce FFAs by systematically engineering xylose metabolism and transcriptional regulation.

Even if *O. polymorpha* is a natural host for xylose assimilation, it is still a challenge for synchronous utilization of glucose and xylose. We previously enhanced xylose utilization by strengthening the xylose isomerase (*Psp*XI*), xylulose kinase (*XYL3*) and xylose transporter (*HXT1**) in *O. polymorpha* [30], which however could not support the efficient co-utilization of glucose and xylose. In particular, the restored auxotrophic gene *LEU2* that has been reported to promote cell growth and FFA production [48–50], aggravated glucose repression instead.

In this case, to achieve the efficient co-utilization of glucose and xylose, our first strategy focused on the xylose metabolism. Non-oxidative branch of the pentose phosphate pathway often becomes a rate-limiting step in xylose metabolism [11]. Interestingly, we found that the overexpression of *TAL*, rather than *TKL*, can significantly improve the co-utilization of glucose and xylose, suggesting the relatively low distribution of PPP toward central metabolism in

O. polymorpha. More importantly, considering the bidirectional reactions catalyzed by *TAL* and *TKL*, the balanced strength of non-oxidative PPP may play an essential role in xylose metabolism. Interestingly, the enhanced metabolic flux to PPP generated sufficient Xu5P, which proved to be beneficial for biomass accumulation and FFA production.

Another limitation lies in xylose assimilation pathway. A great difference in xylose assimilation was observed between the natural and non-natural hosts. In natural xylose-utilizing hosts like *O. polymorpha*, XI pathway alone cannot support the xylose metabolism, but the combined XI and endogenous XR/XDH pathway further enhanced the co-utilization of glucose and xylose without significant accumulation of xylitol. On the contrary, XI pathway could achieve efficient xylose assimilation in non-natural hosts like *S. cerevisiae* [20,39]. We suspected that the long-term natural selection causes the high adaptability between the xylose assimilation and endogenous metabolic pathways in *O. polymorpha*, which is very difficult to beat by exogenous pathways. Therefore, the balanced xylose assimilation toward central metabolism vis the intermediate Xu5P is essential for efficient co-utilization of glucose and xylose. The efficient xylose assimilation relied on the fast xylose uptake. Therefore, multiple transporters with high xylose affinity were further screened and introduced, among which *SpXUT1* significantly increased the utilization of both glucose and xylose, but about 2 g/L of residual xylose remained, which reminds us that the metabolism of xylose seems to be somewhat restricted.

Engineering the transcription factors can help to rewire the central metabolism toward xylose assimilation by relieving the catabolite repression from glucose. We here found that deletion of *HAP4-A* [43] related to respiration greatly improved the co-utilization of glucose and xylose for FFAs production. Previous results showed that *HAP4-A* was involved in the transcriptional regulation of xylose metabolism by up-regulating gene *XYL1* and *XYL3* [43]. Particularly, the absence of *HAP4-A* resulted the upregulation of *RKI* genes and downregulation of *RPE*, which further promoted Xu5P supply from xylose and maintained the stable PPP. The final strain NX53 produced 1.8-fold more FFAs, probably owing to the enhanced sugar consumption, especially xylose for sufficient supply of precursors and NADPH [30]. Interestingly, the strengthened xylose metabolism is generally considered as the repressor of glucose metabolism. However, our results showed that the adaptive metabolic distribution between glucose and xylose could synergistically promoted the utilization efficiency of both sugars. All these modifications enabled the complete co-utilization of glucose and xylose in glucose-xylose mixture and real lignocellulose hydrolysates. Consequently, to achieve efficient co-utilization of glucose and xylose in *O. polymorpha*, the systematically enhanced xylose metabolism, coupled with global transcriptional regulation, can provide guidance for the biorefinery of lignocellulosic materials.

CRediT authorship contribution statement

Xin Ni: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jingjing Li:** Methodology, Investigation, Data curation. **Wei Yu:** Writing – review & editing, Methodology, Investigation. **Fan Bai:** Methodology, Investigation. **Zongbao K. Zhao:** Formal analysis, Data curation. **Jiaoqi Gao:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Fan Yang:** Validation, Supervision, Methodology, Investigation. **Yongjin J. Zhou:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis.

Declaration of competing interest

Yongjin Zhou is an Associate Editor for Synthetic and Systems Biotechnology and was not involved in the editorial review or the decision to publish this article. Other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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