High-level β-carotene production from xylose by engineered *Saccharomyces cerevisiae* without overexpression of a truncated *HMG1* (t*HMG1*)

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Abstract

β-carotene is a natural pigment and health-promoting metabolite, and has been widely used in the nutraceutical, feed and cosmetic industries. Here, we engineered a GRAS yeast *Saccharomyces cerevisiae* to produce β-carotene from xylose, the second most abundant and inedible sugar component of lignocellulose biomass. Specifically, a β-carotene biosynthetic pathway containing *crtYB*, *crtI* and *crtE* from *Xanthophyllomyces dendrorhous* were introduced into a xylose-fermenting *S. cerevisiae*. The resulting strain produced β-carotene from xylose at a titer three-fold higher than from glucose. Interestingly, overexpression of tHMG1, which has been reported as a critical genetic perturbation to enhance metabolic fluxes in the mevalonate (MVA) pathway and β-carotene production in yeast when glucose is used, did not further improve the production of β-carotene from xylose. Through fermentation profiling, metabolites analysis and transcriptional studies, we found the advantages of using xylose as a carbon source instead of glucose for β-carotene production to be a more respiratory feature of xylose consumption, a larger cytosolic acetyl-CoA pool, and up-regulated expression levels of rate-limiting genes in the β-carotene producing pathway, including ACS1 and HMG1. As a result, 772.81 mg/L of β-carotene was obtained in a fed-batch bioreactor culture with xylose feeding. Considering the inevitable production of xylose at large scales when cellulosic biomass-based bioeconomy is implemented, our results suggest xylose utilization is a promising strategy for overproduction of carotenoids and other isoprenoids in engineered *S. cerevisiae*.

Saccharomyces cerevisiae

INTRODUCTION

Carotenoids are diverse class of C_{40} isoprenoids widely produced by plants, bacteria, fungi and microalgae (Berman et al., 2015; Henríquez, Escobar, Galarza, & Gimpel, 2016). Of all known carotenoids, β-carotene is believed to be the most important due to its nutritional role as pro-vitamin A (Dowling & Wald, 1960) and health-promoting potential as an antioxidant (Palozza & Krinsky, 1992) and an anti-tumor agent (Williams, Boileau, Zhou, Clinton, & Erdman, 2000). Its wide applications in nutraceutical, feed and cosmetic industries lead to a fast-growing world market (Irwandi Jaswir, 2011). Currently, chemical synthesis remains the major route of commercial β-carotene production. Considering the safety concerns of chemical synthesis, and consumer preferences for natural additives, microbial production of β-carotene via metabolic engineering gains increasing interests and becomes an attractive alternative (Yoon et al., 2007; Zhao et al., 2013). The biological pathway of all isoprenoids use isopentenyl diphosphate (IPP) as precursor, which is synthesized through either MVA pathway in eukaryotes, or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in prokaryotes. Among potential microbial hosts, *Saccharomyces cerevisiae* has superior traits in industrial production of isoprenoids such as the GRAS (generally recognized as safe) status, ease of genetic manipulation, industrial robustness (Auesukaree et al., 2009), and the native MVA pathway which is generally considered as an effective supplier of isoprenoid precursor from acetyl-CoA (Vickers, Williams, Peng, & Cherry, 2017).

Researchers expend great efforts in heterologous production of carotenoids using engineered *S. cerevisiae* . Those efforts have far involved the optimization of metabolic flux, and balancing necessary cofactors by manipulating the expression levels of targeted genes (Das et al., 2007; Peralta-Yahya et al., 2011; Verwaal et al., 2007; Yan, Wen, & Duan, 2012). Among all the reported manipulation targets, overexpression of a truncated, soluble form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (t*HMG1*), a major rate-limiting enzyme of the MVA pathway, has been consistently recognized as an essential strategy for high-level production of carotenoids (Verwaal et al., 2007; Xie, Lv, Ye, Zhou, & Yu, 2015; Zhou et al., 2017) and other isoprenoids, such as artemisinic acid (Ro et al., 2006), farnesene (Meadows et al., 2016), squalene and amorphadiene (Kwak et al., 2017) in *S. cerevisiae* . In addition to t*HMG1* overexpression, up-regulation of the MVA pathway related genes such as*ERG8* , *ERG12* , *ERG19* , *IDI1* , *ERG20* (Y. Sun, Sun, Shang, & Yan, 2016) and down-regulation of the ergosterol pathway related genes such as *ERG9* (Yan et al., 2012) have been attempted to increase the production of carotenoids. However, extensive genetic manipulations usually increase metabolic burdens on the host and thus cause instable performance in industrial-scale fermentation (Hollinshead, He, & Tang, 2014).

More importantly, despite intensive genetic perturbations for driving metabolic fluxes towards carotenoids production, ethanol remains a major product due to the entirely fermentative metabolism of *S. cerevisiae* on glucose even in the presence of oxygen (Pfeiffer & Morley, 2014), which hindered the high-level production of carotenoids. This well-known metabolic regulation, termed the Crabtree effect, was not observed while using non-native sugar xylose as a carbon source (Y.-S. Jin, Laplaza, & Jeffries, 2004; Kwak et al., 2017; Matsushika, Goshima, & Hoshino, 2014). We, therefore, assumed that xylose fermentation by engineered *S. cerevisiae* might facilitate carotenoids production by alleviating glucose-dependent repression on respiratory metabolism. Additionally, xylose, comprising up to 30-40 % of lignocellulosic biomass, is the second most abundant sugar in nature that derived from non-edible sources (Kim, Ha, Wei, Oh, & Jin, 2012). Efficient production of value-added chemicals like carotenoids and vitamin A from xylose is an important step toward economically feasible and sustainable bioconversion processes of lignocellulosic biomass (Kwak, Jo, Yun, Jin, & Seo, 2019; L. Sun, Kwak, & Jin, 2019). However, carotenoids production from xylose in engineered *S. cerevisiae*has yet been reported.

As such, in this study, we sought to overproduce β-carotene from xylose in engineered *S. cerevisiae* . High-level production of β-carotene was achieved using xylose as a carbon source without t*HMG1* overexpression and other genetic perturbations. In order to explore the advantageous traits of xylose utilization for β-carotene production in engineered yeast, we assessed the differences in β-carotene production patterns from glucose and xylose via fermentation profiling, metabolites analysis and comparative transcriptional studies. To the best of our knowledge, the titer of β-carotene achieved in this study is among the highest reported in engineered *S. cerevisiae* (López et al., 2019; Xie, Ye, Lv, Xu, & Yu, 2015). This study demonstrated that using xylose as a carbon source would be a promising strategy potentially bypassing extensive genetic perturbations for high-level and stable production of carotenoids and other isoprenoids in *S.cerevisiae* .

MATERIALS AND METHODS

Strain Construction. The strains, plasmids and PCR primers used in this study are listed in Table S1 and Table S2, respectively. Standard molecular biology procedures were conducted as described previously (Green, Sambrook, & Sambrook, 2012). The integration plasmid YIplac211YB/I/E* was kindly provided by Verwaal et al. (Verwaal et al., 2007), which carries carotenoid biosynthesis genes from*Xanthophyllomyces dendrorhous* including *crtYB* , *crtI*and *crtE* . *URA3* was disrupted as an auxotrophic marker in the *S. cerevisiae* SR8 strain (Kim et al., 2013), using Cas9-based genome editing (G.-C. Zhang et al., 2014). A donor DNA for *URA3*disruption was amplified using primers URA3donor-U and URA3donor-D. Using the lithium acetate method, plasmid CAS9-NAT (Addgene#64329) was introduced into the SR8 strain first and the guide RNA plasmid gRNA-ura-HYB and the donor DNA were additionally introduced. Putative transformants were selected on YPD plate supplemented with 120 μg/mL nourseothricin and 300 μg /mL Hygromycin B (YPDNH). The positive colonies with *URA3* deletion were confirmed by sequencing using primers URA3-Seq-U and URA3-Seq-D and designated as the SR8U- strain. For yeast genomic integration,

the plasmid YIplac211YB/I/E* was linearized by *StuI* and transformed into the SR8U- strain using the lithium acetate method (Gietz, Schiestl, Willems, & Woods, 1995). After transformation, cells were plated on selective media (SCD-ura plate) and grew for 3 days. The most reddish colonies were picked and confirmed by diagnostic PCR. The resulted strain was designated as the SR8B strain. A CRISPR/Cas9 system was applied for overexpression of catalytic domain of *HMG1* (*tHMG1*) by genomic integration. The Cas9-NAT plasmid (Addgene#64329) was transformed into the SR8B strain before Cas9-based genetic modifications. The t*HMG1* gene flanked by a strong constitutive yeast promoter *TDH3* and terminator *CYC1* was amplified from the plasmid pRS425TDH-tHMG1 as a donor DNA. The plasmid pRS42H-CS5 coding for guide RNA which targets the intergenic site on Chr XV was co-transformed with the donor DNA fragments. Cells were selected on YPD plate supplemented with 120 µg/mL nourseothricin and 300 µg /mL Hygromycin B. Positive colonies were confirmed by diagnostic PCR and designated as the SR8BH strain.

Ψεαστ ὐλτυρε φορ τηε Προδυςτιον οφ β-ςαροτενε. To compare β-carotene production on glucose and xylose by engineered yeast, the engineered strains were inoculated from glycerol stocks into 5mL of a modified Verduyn medium (van Hoek, de Hulster, van Dijken, & Pronk, 2000) containing 20 g/L glucose or xylose as pre-cultures for glucose and xylose main cultures, respectively. The Verduyn medium contained per liter: $(NH_4)_2SO_4$, 15 g; KH_2PO4, 8 g; $MgSO_4$, 3 g; trace element solution, 10 mL; vitamin solution, 12 mL. The trace element solution contained per liter: EDTA, 15 g; $ZnSO_4$, 5.75 g; $MnCl_2$, 0.32 g; $CuSO_4$, 0.50 g; $CoCl_2$, 0.47 g; Na_2MoO_4, 0.48 g; $CaCl_2$, 2.90 g; $FeSO_4$, 2.80 g. The vitamin solution contained per liter: biotin, 0.05 g; calcium pantothenate, 1 g; nicotinic acid, 1 g; myoinositol, 25 g; thiamine hydrochloride, 1 g; pyridoxol hydrochloride, 1 g; p-aminobenzoic acid, 0.20 g. After pre-cultures for 2-3 days, cells were harvested and re-inoculated at an initial optical cell density of 1 at 600nm (OD_{600}) into main culture flasks, which were 250 mL baffled flasks with 50 mL of Verduyn medium containing either 40g/L glucose, or 40g/L xylose. Culture media were buffered with potassium hydrogen phthalate at a working concentration of 50 mM and pH of 5.5. We conducted aerobic batch fermentation experiments in a shaking incubator at 30 and 300 rpm. For a xylose fed-batch fermentation, the engineered strain was pre-cultured for 48 hours in 200 mL of Verduyn medium containing 40 g/L xylose at 30 and 300 rpm. The fed-batch fermentation was conducted in a 3-liter fermenter (New Brunswick Scientific-Eppendorf, Enfield, CT) with 1 L of Verduyn medium at 30 . Initial xylose concentration was 87.10 g/L and additional amounts of xylose were fed to reach 40 ± 5 g/L of xylose upon depletion. The pH was maintained at 5.5 by automatically pumping in 4M NaOH.

Quantitative Analysis. We monitored a cell density of each culture by measuring OD_{600} using a spectrophotometer (BioMate 5; Thermo Fisher Scientific, Waltham, USA). The dry cell weight (DCW) was then calculated from the measured OD_{600} by multiplying a conversion factor of 0.41 (1 OD_{600} =0.41 g DCW/L). To calibrate the conversion factor between optical density and dry cell weight, yeast cells were grown in the Verduyn medium, harvested by centrifugation at 10,000 rpm, and washed two times with distilled water. Washed cell pellets were resuspended in distilled water to various optical densities and filtrated via dried cellulose acetate membrane filters. After cell filtration, membrane filters were dried to constant weight in an 80°C convection oven and then weighed. Glucose, xylose, xylitol, glycerol, acetate and ethanol in the culture broth were quantified using high-performance liquid chromatography (HPLC, Agilent 1200 Series, Agilent Technologies, Wilmington, US) equipped with a refractive index detector and the Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc, Torrance, CA). The diluted culture supernatants were analyzed at 50 with 0.005 M H_2SO_4 as the mobile phase. The flow rate was set at 0.6 mL/min. β-carotene was extracted using acetone and quantified by measuring the absorbance at 453 nm (OD_{453}) with spectrophotometer as described previously (Yuan, Rouvière, LaRossa, & Suh, 2006). Specifically, cells were harvested from 1 mL culture broth by centrifugation. The cell pellets were resuspended with 1 mL acetone in a 2mL screwed cap tube and crushed by a BeadBeater (BioSpec, USA). Samples were then centrifuged, and colored supernatants were collected in a 5 mL tube for measuring OD_{453}. The extraction procedure was repeated for three times until the cell pellets turned white. A standard curve (**Fig. S1**) was obtained by measuring OD_{453} of a serial of β-carotene standard (Cat. No. C4582, Sigma, USA) solution with known concentration using spectrophotometer. The standard curve was then used to calculate the volumetric titer and specific content of β-carotene produced by engineered strains. For analysis of ergosterol production, 2 mL of fermentation

broth was centrifuged to separate the cells. The cell pellets were resuspended with 0.6 mL of extraction solution (50% KOH : C_2H_5OH = 2 : 3), and the mixture was saponified by incubating in 85 °C water bath for 2 hours. After chilling on ice, the saponified mixture was thoroughly mixed with 0.6 mL n-heptane to extract the sterol. After centrifugation, a total 0.5 mL of n-heptane layer was collected and dried in a centrifugal vacuum concentrator. Dried samples were dissolved in 0.5 mL of acetonitrile and analyzed using Shimadzu HPLC system equipped with UV detector (Shimadzu SPD-20A) and C18 column (Phenomenex Kinetex 5 μL C18). Ergosterol was separated with 100% acetonitrile at a flow rate of 2 mL/min and detected by UV absorbance at 280 nm. A standard curve (**Fig. S2**) was prepared using authentic ergosterol standard (Cat. No. 45480, Sigma, USA) for calculating ergosterol concentration from each sample. Lipid weight was determined as previously described (S. Zhang et al., 2016). Briefly, 2 mL cell cultures with OD_{600} adjusted at 10 were centrifuged at 15,000 rpm for 1 min. Cell pellets were transferred into 15-mL glass centrifuge tubes and were crushed using BeadBeater with 6 mL of chloroform/methanol (1:1 volumetric). The samples were then mixed with 1.5 mL water and vortexed for 1 min. After centrifugation, the organic layer was collected, washed with 1.5 mL of 0.1% (w/ v) NaCl water solution, and dried overnight at room temperature in a preweighed tube. The tube was further dried in an oven at 80 °C until they reached a constant weight to determine lipid content. Total lipid content was calculated from the final tube weight by subtraction of original tube weight and the corresponding β-carotene content for each sample.

Identification of Carotenoids Composition. To identify the carotenoids composition by HPLC, yeast extracts from glucose and xylose batch fermentation were separated on a reverse-phase C30 HPLC column (4.6 × 150 mm, 3 μm; YMC, Wilmington, NC) maintained at 18°C, and detected by a photodiode array detector (model 2996; Waters, Milford, MA) as previously described (Yeum et al., 1996). β-carotene, phytoene (Cat. No. 78903, Sigma, USA) and lycopene (Cat. No. SMB00706, Sigma, USA) standards were used for the identification.

Visualization of Lipid Bodies. Lipid bodies were visualized using confocal microscope after staining as described previously (Beopoulos et al., 2008). Fresh cells were harvested at exponential phase from a batch fermentation with either glucose or xylose as carbon source and resuspended at OD_{600} 20. Nile red (Cat. No. 72485, Sigma, USA) solution in acetone (1 mg/ml) was added to the cell suspensions (1/10 vol/vol) and incubating at room temperature for 1 hour to stain and identify lipids. After washing with saline, cells were resuspended to OD_{600} 20 in 50 mM potassium hydrogen phthalate buffer and immobilized using low melting-point agarose (Fisher scientific, Hampton, NH) on a Fluorodish (World Precision Instruments, USA) for viewing. Stained cells were viewed and photographed with a confocal microscope (Zeiss LSM 700, Carl Zeiss AG, Oberkochen, Germany) using an oil immersion objective (63×) at 633 nm radiation.

Real-time qPCR Quantification of mRNA. We conducted real-time qPCR analysis to investigate the expression levels of related genes. Total RNA was extracted and purified using MasterPure Yeast RNA Purification Kit (Epicentre, USA) following the attached protocol. RNA was reverse transcribed to cDNA using a cDNA synthesis kit (iScript, Bio-Rad, Canada). Real-time qPCR was performed in a LightCycler(r) 480 Real Time PCR system (Roche, Swiss) using SsoAdvanced^{TM} Universal SYBR(r) Green Supermix (Bio-Rad, Canada) and qPCR amplicon primers (**Table S3**). The housekeeping gene *ACT1* was used as the control. Relative gene expression of xylose condition *versus* glucose condition was calculated using the $2^{-\Delta\Delta^{\wedge}\tau}$ method and presented as fold change (Livak & Schmittgen, 2001).

RESULTS

ὃνστρυςτιον οφ α Ξψλοσε-φερμεντινγ, β-ςαροτενε-προδυςινγ *Σ. ςερεισιαε*. We engineered the GRAS yeast *S. cerevisiae*to functionally express the heterologous β-carotene synthetic pathway and produce β-carotene from xylose as well as glucose (**Fig. 1** ,**Fig. 2**). Specifically, *crtE* , *crtI* , *crtYB*genes from *X. dendrorhous* coding for GGPP synthase, phytoene desaturase, phytoene synthase and lycopene cyclase in the β-carotene biosynthesis pathway were integrated into the genome of the *S. cerevisiae* SR8, previously engineered to ferment xylose (Kim et al., 2013). The resulted strain was named as the SR8B strain.

Cοmparison of β-carotene Production Patterns on Glucose and Xylose. When xylose was used as a carbon source, we observed the SR8B cell cultures always exhibited an intense orange color while those using glucose appeared as light yellow (**Fig. 1B**). We suspected that carotenoids other than β-carotene might be produced and the composition of carotenoids in the cells grown on glucose and xylose might be different. To identify the carotenoids composition produced by engineered yeast cells grown on different carbon sources, cells cultured either on glucose or on xylose were harvested for carotenoids extraction and HPLC analysis. β-carotene was the predominant carotenoid produced on both conditions according to the chromatographs (**Fig. 2**), while the β-carotene peak in the chromatograph of xylose culture showed much higher intensity than that of glucose culture (**Fig. 2**). The intermediates phytoene and lycopene were also accumulated in xylose cultures, while only phytoene was detected in glucose condition with a lower peak intensity (**Fig. 2**).

The engineered SR8B strain was cultured aerobically on glucose and xylose to compare differences in fermentation profiles and β-carotene production patterns. When cultured on glucose, the engineered strain fermented glucose into a large amount of ethanol, and then started to consume ethanol as carbon source (**Fig. 3A**). In contrast, when cultured on xylose, the strain consumed xylose slower with negligible amounts of ethanol production but showed a higher cell mass titer and more glycerol accumulation (**Fig. 3B**). In terms of β-carotene production, a rapid fermentation of glucose, and subsequent ethanol consumption led to much less production of β-carotene as compared to xylose culture where xylose is consumed steadily with little ethanol production (**Fig. 3C** , **Fig.3D**). As a result, the SR8B strain accumulated β-carotene intracellularly with a specific content of 13.73 mg/g DCW and a volumetric titer of 96.42 mg/L from 40 g/L xylose. These are approximately two-fold and three-fold higher than 3.88 mg/g DCW and 24.22 mg/L of β-carotene produced from 42 g/L glucose (**Fig. 3C** , **Fig. 3D**).

Effect of t*HMG1* Overexpression on β-carotene Production using Xylose as the Source. To further enhance β-carotene production by engineered strain from xylose, HMG-CoA reductase, a well-known rate-controlling enzyme in the MVA pathway, was selected as the manipulation target (**Fig. 1A**). Specifically, an expression cassette containing a truncated *HMG1* (t*HMG1*) coding for the catalytic domain of HMG-CoA reductase under the control of*TDH3* promoter was integrated into the genome of the SR8B strain using Cas9-based genome editing. The resulting t*HMG1*overexpressing strain, named as the SR8BH strain, showed lower cell mass titers as compared to the SR8B strain on both glucose and xylose condition (**Fig. S3**). As expected, the SR8BH strain accumulated more β-carotene (6.49 mg β-carotene/g DCW) than the SR8B strain (3.88 mg β-carotene/g DCW) when glucose was used as a carbon source (**Fig. 4**). While, interestingly, under xylose fermentation, no improvement on β-carotene production was observed in the SR8BH strain (12.01 mg β-carotene/g DCW) compared to the SR8B strain (13.73 mg β-carotene/g DCW) (**Fig. 4**).

Effect of Xylose Utilization on the Production of Ergosterol and Lipids. In order to examine broader impacts of using xylose as a carbon source on other cytosolic acetyl-CoA derived products, cells were taken at the end of fermentation of the SR8B strain for ergosterol and lipid analysis. The SR8B strain accumulated ergosterol with a specific content of 16.99 mg/g DCW from xylose which was 28% more than using glucose, and a higher improvement of 69% in volumetric titer was observed due to the improved cell mass titer in xylose fermentation.

To investigate the effect of xylose utilization on lipids production by the SR8 strain, cells harvested from glucose and xylose cultures were stained and visualized under a confocal microscope. The cells grown on xylose were found to accumulate more lipid bodies (LB) with larger size than cells grown on glucose, leading to a bigger portion of the stained LB to the cell area (**Fig. 5A**). The enhanced LB formation suggested a greater lipids production capacity of engineered yeast on xylose fermentation, and this was confirmed by the lipids weight analysis. The engineered SR8B strain produced lipids through xylose utilization with a specific content 58% higher than glucose utilization (43.09 vs. 27.22 mg lipids/g DCW) (**Fig. S4**).

Comparison of Transcription Profiles of the Genes involved in β-carotene Biosynthesis in Engineered Yeast on Glucose and Xylose. To further reveal the potential mechanisms of improved

production by xylose utilization, transcriptional analysis was carried out on genes related to cytosolic PDH bypass (*ACS1* and*ACS2*), lipid biosynthesis pathway (*ACC1*), MVA pathway (*ERG10* , *ERG13* , *HMG1* , *HMG2* , *ERG12* ,*ERG8* , *ERG19* , *IDI1* , and *ERG20*) and ergosterol pathway (*ERG9*) (**Fig. 1C**). Cells for mRNA extraction and quantification were taken at the exponential phase of glucose and xylose fermentation by the SR8B strain, which is 19 hours and 31 hours, respectively. While most of the 13 genes studied did not show significant difference in transcriptional levels between glucose and xylose culture conditions, the expression levels of gene *ACS1*coding for acetyl-CoA synthase and gene *HMG1* coding for HMG-CoA reductase increased significantly in response to xylose substitution, where 2.72 ± 0.14 and 2.21 ± 0.20-fold differences were observed, respectively.

Ξψλοσε Φεδ-βατςη Φερμεντατιον φορ τηε Προδυςτιον οφ β-ςαροτενε. The capacity of β-carotene production from xylose by the engineered strain SR8B was assessed in a 1-L bioreactor via a fed-batch fermentation. Cells was inoculated at an initial cell density of OD_{600} = 3.11, and cultured with 87 g/L of xylose (**Fig. 6**). Upon the depletion of initially added xylose, additional xylose was provided to reach a concentration of 40 ± 5 g/L of xylose. The feeding was repeated seven times until the β-carotene titer ceased to increase. Despite the presence of high concentrations of xylose in the medium, ethanol accumulation was negligible during the fed-batch culture. Finally, cell density reached to OD_{600} of 165.90 (68.02 g DCW/L) and 772.81 mg/L of β-carotene was produced with a productivity of 5.40 mg/L·h (**Fig. 6**). The final β-carotene yield was 2.22 mg β-carotene/g xylose and specific content was 11.42 mg β-carotene/g DCW. In addition, a large amount of glycerol (30.07 g/L) and acetate (22.46 g/L) were accumulated at the end of fermentation.

DISCUSSION

Over the past 20 years, researchers have made great efforts in enabling efficient ethanol production from xylose, the second most abundant and inedible sugar component of lignocellulose biomass, in engineered yeast as an important step towards a robust second-generation biofuels industry (Y.-S. Jin, Lee, Choi, Ryu, & Seo, 2000; Kim et al., 2013). Recently, production of high-value metabolites, such as astaxanthin (Montanti, Nghiem, & Johnston, 2011), protopanaxadiol (Gao, Caiyin, Zhao, Wu, & Lu, 2018), squalene, and amorphadiene (Kwak et al., 2017), from xylose by engineered yeast has gained increasing interest due to the advantageous traits of xylose metabolism and the attracting economic profitability of biomass conversion (Kwak et al., 2019).

For the first time, we engineered a yeast *S. cerevisiae* to produce β-carotene from xylose. As compared to the conventional sugar glucose, xylose exhibited superior traits as a carbon source for the production of β-carotene in engineered *S. cerevisiae* . When cultured on xylose, the engineered strain SR8B produced remarkably less ethanol as compared when glucose was used as a carbon source (**Fig. 3**). This is attributed to the dysregulation effect of xylose on the glucose-dependent repression of the respiratory metabolism (Y.-S. Jin et al., 2004; Matsushika et al., 2014). As such, the engineered yeast produced β-carotene at a much higher yield from xylose (2.41 mg β-carotene /g xylose) than from glucose (0.39 mg β-carotene/ g glucose). As the xylose consumption was slower than glucose, the glucose cultures were extended to the ethanol consumption phase for a fair comparison. Nevertheless, the net production of β-carotene from sequential utilization of glucose and ethanol was still much lower than that from xylose culture regarding both volumetric titer and specific content (**Fig. 3**). In addition, a lower cell density was observed on glucose condition as compared to a corresponding xylose condition. This might be associated with the energetically high-cost conversion of ethanol into cytosolic acetyl-CoA in *S. cerevisiae*which restricts the yield of biomass or products that require ATP (Kok et al., 2012). The higher yield of cell biomass from xylose is another contributory factor of the enhanced β-carotene titer as cell concentration is important for the volumetric titers of intracellular metabolites.

Overexpression of t*HMG1* was critical to high-level production of β-carotene and other isoprenoids by engineered yeast, as described in previous reports (Verwaal et al., 2007; Xie et al., 2014). As such, we overexpressed t*HMG1* in the SR8B strain in order to further increase β-carotene production on xylose cultures. As expected, the newly constructed strain SR8BH produced β-carotene with a higher specific content than

the SR8B strain while cultured on glucose (**Fig. 4**). However, t*HMG1* overexpression did not result in any improvement of β-carotene production from xylose (**Fig. 4**). More interestingly, the beneficial effects of using xylose instead of glucose as a carbon source on β-carotene production (a 254% improvement in β-carotene specific content by SR8B strain) appeared to be much stronger than that of t*HMG1* overexpression on glucose condition (a 67% improvement in β-carotene specific content by SR8BH strain as compared to SR8B strain). These results suggested that using xylose as a carbon source in substitution for glucose is an effective strategy to increase β-carotene production in *S. cerevisiae* that could potentially bypass t*HMG1* overexpression and other genetic manipulations which often resulted in growth defects. Thus, we could not only avoid the cost of extra genetic perturbations eliciting reduced growth, but also ensure the stability of engineered strains.

The higher production of β-carotene and accumulation of intermediates (phytoene and lycopene) (**Fig. 2** , **Fig. 3**) suggested a better supply of precursors for the carotenogenic pathway when xylose was used as a carbon source as compared to glucose (Verwaal et al., 2007). To investigate the effects of xylose utilization on metabolic flux related to β-carotene biosynthesis, the accumulation of endogenous ergosterol and lipids were monitored as indicators for farnesyl pyrophosphate (FPP) supply and cytosolic acetyl-CoA pool, respectively (**Fig. 1A**). We observed that the engineered strain produced more ergosterol on xylose cultures as compared to glucose cultures (**Fig. 5A**). This is indirect evidence of a stronger metabolic flux through the MVA pathway that provides sufficient supply of FPP as a common precursor for isoprenoids and sterols. Additionally, cells grown on xylose were found to accumulate more lipids as compared to those grown on glucose (**Fig. 5B** , **Fig. S4**), indicating increased cytosolic acetyl-CoA pool which is also a key factor for high-level production of isoprenoids. Moreover, the increased lipids content might have promoted the accumulation of β-carotene by expanding cell-storage capacity for β-carotene as a lipophilic end product (Ma et al., 2019; Wei et al., 2018).

Previous reports demonstrated that xylose utilization in engineered*S. cerevisiae* leads to distinct transcriptional patterns of genes involved in various metabolic pathways as compared to glucose utilization (Y.-S. Jin et al., 2004; Kwak et al., 2017; Matsushika et al., 2014). Thus, we investigated the effect of xylose on expression levels of genes related to cytosolic PDH bypass, lipid synthesis, MVA pathway and ergosterol pathway via comparative real-time qPCR. Among all the genes studied, *ACS1* and *HMG1* were highly expressed when the cells were grown on xylose as compared to glucose, while others did not show significant difference in expression levels (**Fig. 1C**). It is known that the transcription of *ACS1* gene coding for acetyl-CoA synthase is subject to glucose repression (Berg et al., 1996). Therefore, we reason that using xylose instead of glucose as carbon source leads to the alleviation of the glucose-dependent repression on the transcription of *ACS1* , thus resulting in greater abundance of cytosolic acetyl-CoA as building blocks for lipids, ergosterol and β-carotene synthesis. As a key rate-limiting gene in the MVA pathway, *HMG1* was an essential target for manipulation in order to overproduce terpenes and sterols in *S. cerevisiae* . Overexpression of native or heterologous *HMG1* in engineered*S. cerevisiae* was shown to be beneficial for β-carotene production in previous studies (Li, Sun, Li, & Zhang, 2013; Yan et al., 2012). Accordingly, the improved transcriptional level of *HMG1* by xylose utilization could have further promoted the conversion of the abundant cytosolic acetyl-CoA into FPP as a precursor for β-carotene and ergosterol. This might be the reason why t*HMG1* overexpression was neither necessary nor desirable for β-carotene overproduction while xylose was used as a carbon source.

Owing to the peculiar physiologic characteristics of xylose fermentation, including low ethanol production and high cell mass yield, a high cell density culture of the SR8B strain was achieved through intermittent xylose feeding instead of further genetic perturbations, or sophisticated feeding algorithms. Consequently, a final β-carotene titer of 772.81 mg/L was achieved (**Fig. 6**), which, to our best knowledge, is one of the highest β-carotene titer reported to date in engineered *S. cerevisiae* (López et al., 2019; Xie, Ye, et al., 2015). However, the final yield (2.22 mg β-carotene/g xylose) and specific content (11.42 mg β-carotene/g DCW) of β-carotene was relatively lower than those of the batch fermentation (2.41 mg β-carotene/g xylose & 13.73 mg β-carotene/g DCW, respectively). This might be attributed to the large amount of glycerol and acetate accumulation which consumed noticeable carbon sources and energy. The

considerable accumulation of glycerol and acetate indicates that the engineered yeast cells might suffer from NADH/NAD^+redox and energy imbalance. A previous study also reported high-level glycerol accumulation in a xylose fed-batch fermentation (Kwak et al., 2017). The redox imbalance in xylose metabolism was known to be caused by the different cofactor dependences of XR (xylose reductase) and XDH (xylitol dehydrogenase) in xylose assimilation pathway (Kwak et al., 2019). Accordingly, strategies to eliminate the glycerol and acetate accumulation, such as using a NADH-preferred*Spathaspora passalidarum* Xyl1.2 in xylose assimilation pathway (Hou, 2012), replacing native NADPH-specific *HMG1* into a NADH-specific *Silicibacter pomeroyi HMG1* in the MVA pathway (Meadows et al., 2016), or rising the aeration by increasing the rate of agitation and supply of air, could lead to a further enhanced capacity of β-carotene production from xylose by our engineered yeast.

In conclusion, we constructed an engineered *S. cerevisiae* strain capable of producing β-carotene from xylose—the second most abundant and non-edible sugar in nature. As compared to the conventional sugar glucose, xylose displayed superior traits as a carbon source for the production of β-carotene in engineered *S. cerevisiae* , including a lower ethanol production, a higher cell mass yield, a larger cytosolic acetyl-CoA pool and up-regulated expression levels of rate-limiting genes. Hence, high-level β-carotene production in engineered *S. cerevisiae* was achieved in a fed-batch bioreactor simply through xylose feeding instead of further genetic perturbations or culture optimization. Our findings suggest xylose utilization is a promising strategy for overproduction of carotenoids and other isoprenoids in engineered *S. cerevisiae* .

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ASSOCIATED CONTENT

Supporting Information

Table S1. Strains and plasmids used in this study. Table S2. Primers for strain construction. Table S3. Primers for qPCR amplicons. Fig. S1. Standard curve for calculating β-carotene concentration. Fig. S2. Standard curve for calculating ergosterol concentration. Fig. S3. Batch fermentation profiles of engineered S. cerevisiae SR8BH on glucose and xylose. Fig. S4. Lipids production by engineered SR8B strain through glucose and xylose utilization.

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Y.-S.J., L.S. developed the idea of this work. Y.-S.J., L.S. designed the experiments. L.S. performed the experiments. L.S., C.A., Y.-G.L. and Y.-S.J. wrote the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

tHMGR , truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase gene; IPP, isopentenyl diphosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; MVA, mevalonate; MEP, 2-C-methyl-D-erythritol-4-phosphate; GRAS, generally recognized as safe; DCW, dry cell weight; LB, lipid body

REFERENCES

Auesukaree, C., Damnernsawad, A., Kruatrachue, M., Pokethitiyook, P., Boonchird, C., Kaneko, Y., & Harashima, S. (2009). Genome-wide identification of genes involved in tolerance to various environmental stresses in *Saccharomyces cerevisiae* . *Journal of Applied Genetics* , *50* (3), 301–310. doi: 10.1007/BF03195688

Beopoulos, A., Mrozova, Z., Thevenieau, F., Dall, M.-T. L., Hapala, I., Papanikolaou, S., ... Nicaud, J.-M. (2008). Control of Lipid Accumulation in the Yeast *Yarrowia lipolytica* . *Applied and Environmental Microbiology* , *74* (24), 7779–7789. doi: 10.1128/AEM.01412-08

Berg, M. A. van den, Jong-Gubbels, P. de, Kortland, C. J., Dijken, J. P. van, Pronk, J. T., & Steensma, H. Y. (1996). The Two Acetyl-coenzyme A Synthetases of *Saccharomyces cerevisiae* Differ with Respect to Kinetic Properties and Transcriptional Regulation. *Journal of Biological Chemistry* , *271* (46), 28953–28959. doi: 10.1074/jbc.271.46.28953

Berman, J., Zorrilla-López, U., Farré, G., Zhu, C., Sandmann, G., Twyman, R. M., ... Christou, P. (2015). Nutritionally important carotenoids as consumer products. *Phytochemistry Reviews* ,*14* (5), 727–743. doi: 10.1007/s11101-014-9373-1

Das, A., Yoon, S.-H., Lee, S.-H., Kim, J.-Y., Oh, D.-K., & Kim, S.-W. (2007). An update on microbial carotenoid production: Application of recent metabolic engineering tools. *Applied Microbiology and Biotechnology* , *77* (3), 505. doi: 10.1007/s00253-007-1206-3

Dowling, J. E., & Wald, G. (1960). The Biological Function of Vitamin A Acid. *Proceedings of the National Academy of Sciences of the United States of America* , *46* (5), 587–608.

Gao, X., Caiyin, Q., Zhao, F., Wu, Y., & Lu, W. (2018). Engineering*Saccharomyces cerevisiae* for Enhanced Production of Protopanaxadiol with Cofermentation of Glucose and Xylose. *Journal of Agricultural and Food Chemistry* , *66* (45), 12009–12016. doi: 10.1021/acs.jafc.8b04916

Gietz, R. D., Schiestl, R. H., Willems, A. R., & Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* , *11* (4), 355–360. doi: 10.1002/yea.320110408

Green, M. R., Sambrook, J., & Sambrook, J. (2012). *Molecular cloning: A laboratory manual* (4th ed). Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press.

Henríquez, V., Escobar, C., Galarza, J., & Gimpel, J. (2016). Carotenoids in Microalgae. In C. Stange (Ed.), *Carotenoids in Nature: Biosynthesis, Regulation and Function* (pp. 219–237). doi: 10.1007/978-3-319-39126-7_8

Hollinshead, W., He, L., & Tang, Y. J. (2014). Biofuel production: An odyssey from metabolic engineering to fermentation scale-up.*Frontiers in Microbiology* , *5* . doi: 10.3389/fmicb.2014.00344

Hou, X. (2012). Anaerobic xylose fermentation by *Spathaspora passalidarum* . *Applied Microbiology and Biotechnology* ,*94* (1), 205–214. doi: 10.1007/s00253-011-3694-4

Irwandi Jaswir. (2011). Carotenoids: Sources, medicinal properties and their application in food and nutraceutical industry. *Journal of Medicinal Plants Research* , *5* (33). doi: 10.5897/JMPRX11.011

Jin, Yong-Su, Lee, T. H., Choi, Y. D., Ryu, Y. W., & Seo, J. H. (2000). Conversion of xylose to ethanol by recombinant *Saccharomyces cerevisiae* containing genes for xylose reductase and xylitol dehydrogenase from Pichia stipitis. *Journal of Microbiology and Biotechnology* , *10* (4), 564–567.

Jin, Y.-S., Laplaza, J. M., & Jeffries, T. W. (2004).*Saccharomyces cerevisiae* Engineered for Xylose Metabolism Exhibits a Respiratory Response. *Applied and Environmental Microbiology* , *70* (11), 6816–6825. doi: 10.1128/AEM.70.11.6816-6825.2004

Kim, S. R., Ha, S.-J., Wei, N., Oh, E. J., & Jin, Y.-S. (2012). Simultaneous co-fermentation of mixed sugars: A promising strategy for producing cellulosic ethanol. *Trends in Biotechnology* ,*30* (5), 274–282. doi: 10.1016/j.tibtech.2012.01.005

Kim, S. R., Skerker, J. M., Kang, W., Lesmana, A., Wei, N., Arkin, A. P., & Jin, Y.-S. (2013). Rational and Evolutionary Engineering Approaches Uncover a Small Set of Genetic Changes Efficient for Rapid Xylose Fermentation in *Saccharomyces cerevisiae* . *PLOS ONE* ,*8* (2), e57048. doi: 10.1371/journal.pone.0057048

Kok, S., Nijkamp, J. F., Oud, B., Roque, F. C., Ridder, D., Daran, J.-M., Maris, A. J. A. (2012). Laboratory evolution of new lactate transporter genes in a jen1Δ mutant of *Saccharomyces cerevisiae*and their identification as ADY2 alleles by whole-genome resequencing and transcriptome analysis. *FEMS Yeast Research* , *12* (3), 359–374. doi: 10.1111/j.1567-1364.2011.00787.x

Kwak, S., Jo, J. H., Yun, E. J., Jin, Y.-S., & Seo, J.-H. (2019). Production of biofuels and chemicals from xylose using native and engineered yeast strains. *Biotechnology Advances* , *37* (2), 271–283. doi: 10.1016/j.biotechadv.2018.12.003

Kwak, S., Kim, S. R., Xu, H., Zhang, G.-C., Lane, S., Kim, H., & Jin, Y.-S. (2017). Enhanced isoprenoid production from xylose by engineered*Saccharomyces cerevisiae* . *Biotechnology and Bioengineering* , *114* (11), 2581–2591. doi: 10.1002/bit.26369

Li, Q., Sun, Z., Li, J., & Zhang, Y. (2013). Enhancing beta-carotene production in *Saccharomyces cerevisiae* by metabolic engineering.*FEMS Microbiology Letters* , *345* (2), 94–101. doi: 10.1111/1574-6968.12187

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-$\Delta\Delta$CT Method.*Methods* , *25* (4), 402–408. doi: 10.1006/meth.2001.1262

López, J., Cataldo, V. F., Peña, M., Saa, P. A., Saitua, F., Ibaceta, M., & Agosin, E. (2019). Build Your Bioprocess on a Solid Strain—β-Carotene Production in Recombinant *Saccharomyces cerevisiae* . *Frontiers in Bioengineering and Biotechnology* ,*7* . doi: 10.3389/fbioe.2019.00171

Ma, T., Shi, B., Ye, Z., Li, X., Liu, M., Chen, Y., Liu, T. (2019). Lipid engineering combined with systematic metabolic engineering of*Saccharomyces cerevisiae* for high-yield production of lycopene.*Metabolic Engineering* , *52* , 134–142. doi: 10.1016/j.ymben.2018.11.009

Matsushika, A., Goshima, T., & Hoshino, T. (2014). Transcription analysis of recombinant industrial and laboratory *Saccharomyces cerevisiae* strains reveals the molecular basis for fermentation of glucose and xylose. *Microbial Cell Factories* , *13* (1), 16. doi: 10.1186/1475-2859-13-16

Meadows, A. L., Hawkins, K. M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., Tsong, A. E. (2016). Rewriting yeast central carbon metabolism for industrial isoprenoid production. *Nature* ,*537* (7622), 694–697. doi: 10.1038/nature19769

Montanti, J., Nghiem, N. P., & Johnston, D. B. (2011). Production of Astaxanthin from Cellulosic Biomass Sugars by Mutants of the Yeast*Phaffia rhodozyma* . *Applied Biochemistry and Biotechnology* ,*164* (5), 655–665. doi: 10.1007/s12010-011-9165-7

Palozza, P., & Krinsky, N. I. (1992). [38] Antioxidant effects of carotenoids in Vivo and in Vitro: An overview. In *Carotenoids Part A: Chemistry, Separation, Quantitation, and Antioxidation* : *Vol.213* . *Methods in Enzymology* (pp. 403–420). doi: 10.1016/0076-6879(92)13142-K

Peralta-Yahya, P. P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J. D., & Lee, T. S. (2011). Identification and microbial production of a terpene-based advanced biofuel. *Nature Communications* ,*2* (1), 483. doi: 10.1038/ncomms1494

Pfeiffer, T., & Morley, A. (2014). An evolutionary perspective on the Crabtree effect. *Frontiers in Molecular Biosciences* , *1* . doi: 10.3389/fmolb.2014.00017

Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* ,*440* (7086), 940. doi: 10.1038/nature04640

Sun, L., Kwak, S., & Jin, Y.-S. (2019). Vitamin A Production by Engineered *Saccharomyces cerevisiae* from Xylose via Two-Phase in Situ Extraction. *ACS Synthetic Biology* , *8* (9), 2131–2140. doi: 10.1021/acssynbio.9b00217

Sun, Y., Sun, L., Shang, F., & Yan, G. (2016). Enhanced production of β-carotene in recombinant *Saccharomyces cerevisiae* by inverse metabolic engineering with supplementation of unsaturated fatty acids.*Process Biochemistry* , *51* (5), 568–577. doi: 10.1016/j.procbio.2016.02.004

van Hoek, P., de Hulster, E., van Dijken, J. P., & Pronk, J. T. (2000). Fermentative capacity in high-cell-density fed-batch cultures of baker's yeast. *Biotechnology and Bioengineering* , *68* (5), 7.

Verwaal, R., Wang, J., Meijnen, J.-P., Visser, H., Sandmann, G., Berg, J. A. van den, & Ooyen, A. J. J. van. (2007). High-Level Production of Beta-Carotene in *Saccharomyces cerevisiae* by Successive Transformation with Carotenogenic Genes from *Xanthophyllomyces dendrorhous* . *Applied and Environmental Microbiology.* ,*73* (13), 4342–4350. doi: 10.1128/AEM.02759-06

Vickers, C. E., Williams, T. C., Peng, B., & Cherry, J. (2017). Recent advances in synthetic biology for engineering isoprenoid production in yeast. *Current Opinion in Chemical Biology* , *40* , 47–56. doi: 10.1016/j.cbpa.2017.05.017

Wei, L.-J., Kwak, S., Liu, J.-J., Lane, S., Hua, Q., Kweon, D.-H., & Jin, Y.-S. (2018). Improved squalene production through increasing lipid contents in *Saccharomyces cerevisiae* . *Biotechnology and Bioengineering* , *115* (7), 1793–1800. doi: 10.1002/bit.26595

Williams, A. W., Boileau, T. W.-M., Zhou, J. R., Clinton, S. K., & Erdman, J. W. (2000). β-Carotene Modulates Human Prostate Cancer Cell Growth and May Undergo Intracellular Metabolism to Retinol. *The Journal of Nutrition* , *130* (4), 728–732. doi: 10.1093/jn/130.4.728

Xie, W., Liu, M., Lv, X., Lu, W., Gu, J., & Yu, H. (2014). Construction of a controllable β-carotene biosynthetic pathway by decentralized assembly strategy in *Saccharomyces cerevisiae* .*Biotechnology and Bioengineering* , *111* (1), 125–133. doi: 10.1002/bit.25002

Xie, W., Lv, X., Ye, L., Zhou, P., & Yu, H. (2015). Construction of lycopene-overproducing *Saccharomyces cerevisiae* by combining directed evolution and metabolic engineering. *Metabolic Engineering* , *30* , 69–78. doi: 10.1016/j.ymben.2015.04.009

Xie, W., Ye, L., Lv, X., Xu, H., & Yu, H. (2015). Sequential control of biosynthetic pathways for balanced utilization of metabolic intermediates in *Saccharomyces cerevisiae* . *Metabolic Engineering* , *28* , 8–18. doi: 10.1016/j.ymben.2014.11.007

Yan, G., Wen, K., & Duan, C. (2012). Enhancement of β-Carotene Production by Over-Expression of HMG-CoA Reductase Coupled with Addition of Ergosterol Biosynthesis Inhibitors in Recombinant *Saccharomyces cerevisiae* . *Current Microbiology* , *64* (2), 159–163. doi: 10.1007/s00284-011-0044-9

Yeum, K. J., Booth, S. L., Sadowski, J. A., Liu, C., Tang, G., Krinsky, N. I., & Russell, R. M. (1996). Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *The American Journal of Clinical Nutrition* , *64* (4), 594–602. doi: 10.1093/ajcn/64.4.594

Yoon, S.-H., Park, H.-M., Kim, J.-E., Lee, S.-H., Choi, M.-S., Kim, J.-Y., ... Kim, S.-W. (2007). Increased β-Carotene Production in Recombinant *Escherichia coli* Harboring an Engineered Isoprenoid Precursor Pathway with Mevalonate Addition. *Biotechnology Progress* , *23* (3), 599–605. doi: 10.1021/bp070012p

Yuan, L. Z., Rouvière, P. E., LaRossa, R. A., & Suh, W. (2006). Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli* . *Metabolic Engineering* ,*8* (1), 79–90. doi: 10.1016/j.ymben.2005.08.005

Zhang, G.-C., Kong, I. I., Kim, H., Liu, J.-J., Cate, J. H. D., & Jin, Y.-S. (2014). Construction of a Quadruple Auxotrophic Mutant of an Industrial Polyploid *Saccharomyces cerevisiae* Strain by Using RNA-Guided Cas9 Nuclease. *Applied and Environmental Microbiology* ,*80* (24), 7694–7701. doi: 10.1128/AEM.02310-14

Zhang, S., Skerker, J. M., Rutter, C. D., Maurer, M. J., Arkin, A. P., & Rao, C. V. (2016). Engineering *Rhodosporidium toruloides* for increased lipid production. *Biotechnology and Bioengineering* ,*113* (5), 1056–1066. doi: 10.1002/bit.25864

Zhao, J., Li, Q., Sun, T., Zhu, X., Xu, H., Tang, J., Ma, Y. (2013). Engineering central metabolic modules of *Escherichia coli* for improving β-carotene production. *Metabolic Engineering* ,*17* , 42–50. doi: 10.1016/j.ymben.2013.02.002

Zhou, P., Xie, W., Li, A., Wang, F., Yao, Z., Bian, Q., Ye, L. (2017). Alleviation of metabolic bottleneck by combinatorial engineering enhanced astaxanthin synthesis in *Saccharomyces cerevisiae* .*Enzyme and Microbial Technology* , *100* , 28–36. doi: 10.1016/j.enzmictec.2017.02.006

Figure captions

Fig. 1A. Biosynthetic pathway of β-carotene from glucose and xylose in engineered *S. cerevisiae* . A heterogolous xylose assimilation pathway containing xylose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) is connected with lower glycolytic pathway by pentose phosphate pathway (PPP). Pyruvate is produced from glucose and xylose and converted into cytosolic acetyl-CoA. Yeast synthesizes farnesyl pyrophosphate from cytosolic acetyl-CoA through the mevalonate pathway (MVA), as the common precursor for the biosynthesis of ergosterol and heterogolous β-carotene. HMG-CoA reductase (HMGR) is a key rate limiting enzyme in MVA. Cytosolic acetyl-CoA is also the precursor for yeast lipids synthesis. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldegyde-3-phosphate; X5P, xyllulose-5-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate. **Fig. 1B.** Pictures of glucose and xylose cultures of SR8B strain at 30 hour. **Fig. 1C.** Transcriptional levels of the genes related to the production of β-carotene in engineered *S. cerevisiae* SR8B on glucose and xylose. Samples were taken at exponetial phase from each condition for RNA extraction and expression level analysis. Fold changes were calculated by dividing genes expression levels on xylose by those on glucose. Three biological replicates and three technical replicates were performed for each gene, and the error bars represented standard deviations.

Fig. 2. The overlaid HPLC chromatograms of carotenoids extracted from the engineered *S. cerevisiae* SR8B cultured on glucose and xylose condition. Cells were cultured in a defined medium containing either 40 g/L glucose or 40 g/L xylose from initial OD_{600} 1. Same amount of cells were harvested at 90 hour from each condition to extract the carotenoids for HPLC chromotography.

Fig. 3. Batch fermentation profiles of the engineered *S. cerevisiae* SR8B on glucose (A) and xylose (B) conditions and the corresponding β-carotene production patterns on glucose (C) and xylose (D). Data are

presented as mean values and standard deviations of three independent biological replicates.

Fig. 4 . Comparison of β-carotene production by the engineered SR8B and SR8BH strains through glucose and xylose utilization. Cells were harvested at the end of fermentation for β-carotene extraction and quantification. Data are presented as mean values and standard deviations of three independent biological replicates

Fig. 5A . Ergosterol production by the engineered SR8B strain through glucose and xylose utilization. Cells were harvested at the end of fermentation for ergosterol extractiona and quantification. Data are presented as mean values and standard deviations of three independent biological replicates. **Fig. 5B.** Lipid bodies visualization of the engineered SR8B cells cultured in glucose and xylose condition. Cells were harvested at exponential phase and stained with Nile Red fluorescent dye. The stained cells were then viewd under a confocal microscope with 63× oil immersion objective at 633 nm.

Fig. 6 Xylose feb-batch fermentation of the SR8B strain in a 3-litter bioreactor. The bioreactor picture on the right was taken at 80 h.

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