Construction of a xylose metabolic pathway in Trichosporonoides oedocephalis ATCC 16958 for the production of erythritol and xylitol

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Abstract

Purpose

Erythritol is a valuable compound as sweetener and chemical material however cannot be fermented from the abundant substrate xylose.

Methods

The strain *Trichosporonoides oedocephalis* ATCC 16958 was employed to produce polyols including xylitol and erythritol by metabolic engineering approaches.

Results

The introduction of a substrate selective ribose-5-phosphate isomerase endowed *T. oedocephalis* with xylose-assimilation activity to produce xylitol, and eliminated glycerol production simultaneously. A more value-added product, erythritol was produced by further introducing a homologous xylulose kinase. The carbon flux was redirected from xylitol to erythritol by adding high osmotic pressure. The production of erythritol was improved to 46.5 g/L in flasks by fermentation optimization, and the process was scaled up in a 5-L fermentor, with 40 g/L erythritol production after 120 h, and a time-space yield of 0.56 g/L/h.

Conclusion

This study demonstrated the potential of *T. oedocephalis* in the synthesis of multiple useful products from xylose.

1 Introduction

Erythritol is a four carbon polyol, which exists ubiquitously in fruits, honey, mushrooms, fermented foods and seaweeds as a storage compound (Martău et al. 2020). Polyols including erythritol, sorbitol, mannitol and xylitol have been used extensively in food industry as non-nutritive sweeteners (Rice et al. 2020). Compared to other polyols, erythritol has gained increased interest for the cool and plain sweetness, multiple healthy effects, near zero calorific value and high safe dosage (den Hartog et al. 2010). Besides, erythritol is explored as a raw material in medical and chemical industry (Amada et al. 2012; Hentenaar et al. 2021; Wang et al. 2022). Correspondingly, the purchase price of erythritol is the highest among sweetener polyols, and the economic production of erythritol is a long-term researching focus of academia and industry. Currently, erythritol is the only polyol product which can be prepared by the fermentation of osmotolerant yeasts such as *Moniliella pollinis*, *Trichosporonoides megachiliensis* and *Yarrowia lipolytica* on industrial scale (Liang et al. 2023). For example, *M. pollinis* was used to produce 1.61 g/L/h erythritol from glucose with a yield of 40% (Burschäpers et al. 2002). Despite the successful

application of erythritol yeast fermentation, the development of more economical processes is in demand to alter the usage of glucose.

A variety of biomass derived substrates including glycerol, mucor-fermented okara, sugar beet molasses, oil crop waste and waste cooking oil have been tested for erythritol production (Khatape et al. 2022). However, disaccharides or complex substrates may not be hydrolyzed by osmotolerant yeasts, and mixed-strain fermentation becomes a potential choice. Co-cultivation of *Yarrowia lipolytica* on glycerol was developed to produce erythritol with the yield of 40 g/L erythritol (Zhang et al. 2021). In another aspect, osmotolerant yeasts tend to produce glycerol as the most common compound to resist osmotic stress, therefore metabolic engineering manipulation is needed to redirect carbon flux from glycerol to other polyols (Kayingo et al. 2001). In the case of erythritol, the flux is generally channeled to pentose phosphate pathway (PPP) and then erythritol pathway, and concurrently the erythritol catabolic pathway should be disrupted (Carly et al. 2017). Xylose is the second abundant biomass monosaccharide to glucose, and mostly enriched in the pretreatment process before cellulase hydrolysis (Yuhe et al. 2020). Microbial fermentation of *Rhodosporidium toruloides* was employed to produce D-arabitol with xylose and glucose (Jagtap and Rao 2018). Bioresource of sugarcane bagasse hemicellulosic hydrolysate (SCHH) was also used in the fermentation of *Cyberlindnera xylosilytica* to generate xylitol (Palladino et al. 2023). However, rare studies reveal approaches to produce erythritol from xylose, except UV mutagenesis of *Aureobasidium pullulans* CGMCC3.0837 leading to a mutant which produced 26.35 g/L erythritol from 120 g/L xylose (Guo et al. 2016). Nevertheless, the economic efficiency of the reported process is not satisfactory, and the underlying mechanism is not clear.

In our previous studies, the HOG1 gene of *Trichosporonoides oedocephalis* ATCC 16958 was deleted to enhance the erythritol productivity from glucose, and the fermentation conditions including metal ions and osmotic pressure were optimized (Li et al. 2016, 2017, 2018a; Kang et al. 2019). Notably, *Trichosporonoides* and *Moniliella* have a close affinity in taxonomy (Rosa et al. 2009), and *Moniliella megachiliensis* was reported to utilize less expensive substrates, nonrefined glycerol waste derived from palm oil and beef tallow to produce erythritol (Kobayashi et al. 2015a). In this study, a ribose-5-phosphate isomerase mutant from *Ochrobactrum* sp. (OsRpiB) with unique substrate scope was transformed into *T. oedocephalis* to endow it the xylose assimilation and xylitol production ability (Tang et al. 2021, 2022). Then the xylulose kinase (XK) and the xylitol dehydrogenase (XDH) genes were overexpressed to further redirect the carbon flux to erythritol (Fig. 1). After fermentation optimization, efficient erythritol production by engineered *T. oedocephalis* from xylose was achieved.

2 Materials And Methods

2.1 Strain and culture conditions

The wild type strain *T. oedocephalis* ATCC 16958 was obtained from the American Type Culture Collection (ATCC) and stored at 4°C. The seed grows in the medium consisting of 20 g/L glucose, 10 g/L yeast extract and 10 g/L peptone. Then *T. oedocephalis* strains were cultivated in flasks at 30°C and 200

rpm on an orbital shaker. For polyol production fermentation, the optimized medium consisted of 200 g/L xylose, 10 g/L yeast extract, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ and 40 g/L KCl.

2.2 Molecular biology methods

Restriction endonucleases were purchased from Takara Bio (Beijing, China). DNA polymerases and ligases were purchased from Vazyme (Nanjing, China). Primers synthesis, gene synthesis and DNA sequencing were performed by Tsingke (Shanghai, China). The plasmids and recombinant vectors which were stored in *E. coli* were extracted by Fastpure Plasmid Extraction Kit (Vazyme), and the PCR amplicons were purified from the agarose gels using FastPure Gel DNA Extraction Mini Kit (Vazyme). The yeast genome and the recombinant plasmids introduced into yeast were extracted by UNlQ-10 Column Yeast Plasmid Preps Kit and Rapid Yeast Genomic DNA Isolation Kit, respectively which were purchased from Sangon Biotech (Shanghai, China).

2.3 Recombinant plasmids and strains construction

All strains, plasmids and primers used in this study were listed in Table S1 and S2. Two plasmids of pET28a and pYES2 preserved by our laboratory were stored in *E. coil* DH5α. The OsRpiB gene with a double mutation of R94N/N137Q was generated from our previous study (Tang et al. 2022). Based on the genome sequencing data, the genes of XK and XDH were amplified by PCR using the genome DNA of *T. oedocephalis* as the template. The three genes of enzyme were ligated to the pYES2 plasmid with GAL1 promoter and CYC1 terminator, and constructed corresponding gene expression cassettes.

The recombinant plasmids of three enzymes were transformed into *T. oedocephalis* by a MicroPulser electro-transformation system from Bio-rad (Shanghai, China). The electric shock condition was 1500 V, 25 uF, and 300 Ω. The transformation mixtures were cultured with solid yeast nitrogen base (YNB) medium at pH 7.0, containing 20 g/L xylitol at 30℃ for 3–4 days for the selection of transformants.

2.4 Strain cultivation and polyol production

The transformants of *T. oedocephalis* were inoculated into 250 mL flasks containing 50 mL YPD medium with 50 mg/mL ampicillin, and cultured shaken at 30 ℃ and 200 rpm for 48 hours to activate the strains. Then the cultivation was inoculated with a 10% inoculation amount into 250 mL flasks containing 50 mL fermentation medium and cultured at the same condition for 168 h. The carbon source, nitrogen source and the concentration of KCl were optimized at different levels by single factor experiments. Fermentation broths of 10 mL were sampled at intervals and centrifuged (12,000×g, 10 min). The cell precipitations were dried to constant weight in an oven at 90 ℃ to determine the dry cell weight (DCW). The product content in the supernatants was analyzed by high performance liquid chromatography (HPLC). All erythritol fermentation tests were repeated three times independently to calculate mean values and standard errors.

2.5 Analytical methods

The determination of erythritol and other compounds were carried out by HPLC using a Sugar-Pak™ (6.5×300 mm, Waters) and a refractive index detector (RID, Agilent 1120). The samples were eluted with ultrapure water of 0.3 mL/min and column temperature was 80℃.

3 Results And Discussion

3.1 Construction of the xylose utilization pathway

The blueprint of genetic manipulation in this study was illustrated in Fig. 1. First, an expression cassette was constructed using OsRpiB gene and pYES2 plasmid for converting xylose to xylulose (Fig. S1). The cassette was transformed into *T. oedocephalis* and a transformant TOR was screened using xylose as substrate. TOR was first observed to assimilate xylose at the concentration of 200 g/L after 96 h fermentation (Fig. S2). The main fermentation product was identified as xylitol, at the concentration of 48.4 g/L, and no erythritol was observed. Then the XK and XDH genes were cloned from *T. oedocephalis* based on genome sequencing data to further develop an erythritol producing strain with xylose. An expression cassette containing the genes of three enzymes was constructed and transformed into *T. oedocephalis* to construct the engineered stain TORKD (Fig. S2). It should be noted that the purpose of introducing XDH was to facilitate the selection of TORKD using the substrate of xylitol instead of xylose, because the strain TOR could also assimilate xylose. Erythritol production of 10.6 g/L was achieved in the initial fermentation of TORKD with 200 g/L xylitol, and the concentration of xylitol was increased to 65.4 g/L, with a total xylose conversion rate of 83.3% at 96 h. Notably, neither TOR nor TORKD produced glycerol in fermentation with xylose, which was the main product when glucose was used as the substrate, and the product of xylose utilization performed superior purity (Fig. S3).

Biomass-derived carbon source is a promising alternative for biosynthesis industry with economic efficiency. Currently, the utilization of xylose in yeasts is mainly achieved through the non-oxidative pathway (relative to the oxidative pathway involving xylose reductase and xylitol dehydrogenase), in which isomerase plays an important role (Wasylenko and Stephanopoulos 2015). For strains which are engineered to utilize xylose, xylose isomerase (XI) is generally employed, for example in the host of *Saccharomyces cerevisiae* (Kim et al. 2013; Jo et al. 2017). However, XI, which is also named glucose isomerase has a wide substrate scope, which might lead to unfavorable effects in metabolic engineering. OsRpiB has the same function and efficiency as XI to convert xylose to D-xylulose, while shows no activity towards glucose (Tang et al. 2022). At 55℃ and 10 mM xylose concentration, the in vitro conversion rate of xylose reached 22% after 3 h. OsRpiB with unique substrate scope towards rare sugars has been leveraged in enzymatic cascade reactions to convert biomass hydrolysate to valuable products (Tang et al. 2023). In this study, the involvement of OsRpiB successfully endowed wild type *T. oedocephalis* with the ability to assimilate xylose. As the main product of strain TOR is xylitol, it is speculated that there is high activity of xylulose reductase in *T. oedocephalis*. Considering this activity is towards neither xylose nor xylitol, it might not been reported intensively. Then XK was further introduced aiming to increase the accumulation of D-xylulose phosphate. Previous instances had proved the

importance of XK in connecting xylose assimilation with PPP, and it is speculated that raised carbon flux to PPP led to the substantial production of erythritol in this case (Zhou et al. 2012; Lee et al. 2014).

3.2 Fermentation medium optimization

The effect of xylose concentration from 100 g/L to 400 g/L on the erythritol production was investigated in flask using TORKD. The fermentation with 200 g/L xylose obtained the highest erythritol concentration of 13.4 g/L at 72 h and then decreased rapidly (Fig. 2A and Fig. S4A), and both 100 g/L and 200 g/L tests exhausted xylose substrate within the fermentation after the fermentation of 168 h (Fig. 2C). The concentration peak value of erythritol showed no increase with the xylose concentration rising from 200 g/L to 400 g/L. Besides erythritol, the concentration of the main byproduct xylitol also reached the highest, 157.1 g/L at 200 g/L xylose, and then decreased in the trails of 300 g/L and 400 g/L xylose (Fig. 2A). The cell growth and metabolism might consume produced erythritol gradually through the erythritol pathway and PPP after 72 h, which can be verified by the DCW progress in Fig. 2B, while the production of xylitol continued in this period indicating the one-way generation catalyzed by active xylulose reductase. With the increase of xylose concentration, the DCW also increased from 16 g/L to 43 g/L, and the trend slowed when the xylose concentration was higher than 200 g/L. It can be deduced that the xylose inhibition effect on both cell growth and polyol production is significant under more than 200 g/L xylose, and most carbon source flows to xylitol production, stain growth and metabolism. The fermentation data of TORKD was summarized in Table 1, and the optimal erythritol production and yield were 0.19 g/L/h and 0.22 g/g, respectively. The fermentation pH generally decreased continuously to 4.0, which commensurate with the erythritol fermentation using glucose (Fig. S4C) (Li et al. 2018b).

Table 1
Data of erythritol production at 72 h under different xylose concentrations.

Xylose (g/L)	Erythritol (g/L)	Residual sugar (g/L)	Production (g/L/h)	Yield (g/g)
100	5.83 ± 0.25	43.28 ± 0.68	0.08	0.10
200	13.36 ± 0.60	139.91 ± 4.08	0.19	0.22
300	8.15 ± 0.30	205.54 ± 0.75	0.11	0.09
400	7.36 ± 0.45	297.99 ± 3.97	0.10	0.07

Different nitrogen sources including yeast extract, corn steep liquor, beef extract, soybean cake powder, and ammonium sulfate were tested aiming to make better utilization of the carbon source xylose. Yeast extract was found to produce the highest erythritol concentration of 14.1 g/L among five kinds of nitrogen sources, and the production of erythritol and xylitol (157.2 g/L) made no difference to previous experiments (Fig. 3A&B). Among nitrogen sources, the cultivation with corn steep liquor obtained the highest DCW, and xylose consumption rate was the highest when soybean cake powder was employed (Fig. 3C&D). The lowest productivity in ammonium sulfate medium might be due to the lowest pH of 2.3

during fermentation (data not shown), hence the pH control was be important when inorganic nitrogen source was used.

In the optimized medium, the introduction of OsRpiB switches the strains TOR and TORXD to produce xylitol from xylose efficiently. The 157.2 g/L xylitol concentration is higher than that of most reported instances, and the productivity of 1.16 g/L/h and the yield of 0.78 were competitive (Erian and Sauer 2022). Specially, high product purity was achieved by eliminating glycerol, which was a main product in fermentation with glucose. Despite the potential of TORXD to produce more valuable products from xylose, e.g. erythritol, however the redirection of carbon flux from xylitol generation remained a major hindrance.

3.3 Comparison of glucose and xylose fermentation

The wild type *T. oedocephalis* produced considerable erythritol with glucose, therefore the comparison of glucose fermentation and xylose-glucose cofermentation were conducted at the concentration of 200 g/L. Erythritol of 32.5 g/L was produced with pure glucose as the carbon source, which was the highest (Fig. 4A). A significant trend was higher glucose ratio led to more erythritol production, and higher xylose ratio benefitted xylitol production. In cofermentation, the best weight ratio of xylose and glucose was 1:2, producing to 23.6 g/L erythritol and 54.5 g/L xylitol. Notably, xylose fermentation could produce more DCW than glucose fermentation and cofermentation, demonstrating the high efficiency of introduced xylose metabolic pathway (Fig. 4B). The strain growth rate in xylose medium was even 29% higher than that in glucose medium in the first 24 h and obtained 137% DCW. During cofermentation, the glucose was rapidly consumed within 48 h, and then the consumption of xylose was obvious, hinting obvious carbon catabolite repression (CCR) (Fig. 4C&D).

Cofermentation of glucose and xylose has been investigated widely to utilize the two main lignocellulosic monosaccharide simultaneously and avoid tedious separation (Kim et al. 2022). Efforts of metabolic engineering has been taken to promote the utilization of xylose when the obstacle of CCR is severe, especially on the enhancement of xylose uptake channel (Fox and Prather 2020; Guo et al. 2022). In this study, the rapid consumption of glucose before xylose indicated significant CCR. The consumption rate of xylose maintained constant before and after the exhaustion of glucose, and suggested the xylose uptake channel of *T. oedocephalis* was unobstructed. The main obstacle might be minor xylose entering PPP, which was competed with the intense xylitol production pathway. Nevertheless, the economic efficiency of xylose fermentation is much higher than that of glucose fermentation, therefore the following optimization was still implemented with the carbon source of 200 g/L xylose.

3.4 Effect of high osmotic fermentation on erythritol production

In our previous research, KCl of more than 5 g/L promoted the erythritol production significantly by *T. oedocephalis* in glucose medium (Li et al. 2017). The effect of concentration of KCl on the erythritol production by xylose was then explored. With the increase of KCl from 0.5 to 40 g/L, the production of

erythritol boosted from 5.8 g/L to 46.5 g/L (Fig. 5A), and the concentration of xylitol decreased from 157.3 g/L to 130.2 g/L (Fig. 5B). Moreover, the DCW and xylose consumption rate also increased significantly (Fig. 5C&D). In the summary of Table 2, the addition of 40 g/L KCl achieved 2.53-fold erythritol productivity and 2.25-fold yield at the time point of 72 h.

Table 2
Data of erythritol production at 72 h under different KCl concentrations.

KCl (g/L)	Erythritol (g/L)	Residual xylose (g/L)	Productivity (g/L/h)	Yield (g/g)
0.5	13.56 ± 0.36	115.29 ± 1.95	0.19	0.16
5	19.47 ± 0.81	103.53 ± 1.69	0.20	0.20
10	25.24 ± 0.59	97.89 ± 1.37	0.26	0.24
20	38.78 ± 1.16	90.47 ± 0.40	0.40	0.35
40	46.51 ± 1.15	63.63 ± 1.60	0.48	0.36

Polyols can serve as osmoregulatory compounds in the various microbes (Kobayashi et al. 2015b). In this study, the addition KCl at 40 g/L activated the corresponding mechanism to produce more polyol. Notably, comparing to the decreased concentration of xylitol, only the production of erythritol was enhanced and no glycerol was found, indicating that the system upregulated gene expression in PPP and/or erythritol pathway in *T. oedocephalis*. Yang et al. reported the high osmotic pressure redirected the carbon flux to produce more erythritol and less mannitol in the fermentation of *Yarrowia lipolytica* CICC 1675 with the carbon source of glycerol (Yang et al. 2014). Therefore, the mechanism might downregulate the xylitol generating step, i.e. the xylulose reductase gene expression, which could be studied in future.

3.5 Production of erythritol in a 5-L fermentor

Based on the previous optimization, the best fermentation medium was set as 200 g/L xylose, 10 g/L yeast extract and 40 g/L KCl, and a scale-up was implemented in a 5-L fermentor. From the results shown in Fig. 6, the production of erythritol was 14% less than that in flask at the time point of 72 h, and the strain growth was 26% slower than that in flask at the time point of 120 h, which was supported by the lower xylose consumption rate. Mass transfer in larger scale led to variations in pH, dissolved oxygen and other factors between the fermentation tank and shaking flask (Hollinshead et al. 2014). The concentration and the productivity of erythritol reached the peak value of 40.1 g/L and 0.56 g/L/h at 72 h, and the concentration of by-product xylitol increased continuously to 131.5 g/L at 168 h.

4 Conclusions

In summary, through the co-expression of OsRpiB, TOXK and TOXDH, T. oedocephalis has the ability to convert xylose and produce erythritol. The best carbon source (200 g/L xylose), nitrogen source (10 g/L yeast extract) and inorganic salt (40 g/L KCl) were determined by single factor shaking flask experiment.

The yield of erythritol reached 46.5 g/L. In a 5-L fermentor, the erythritol production reached 40.06 g/L and 0.56 g/L/h after 120 h. This study provides the feasibility for the production of rare sugar alcohols from xylose by hypertonic tolerant yeast.

Declarations

Authorship contribution statement

Zhou Deng and Yinghui Mu: Investigation, Writing – original draft. **Zhi Chen** and **Lishi Yan**: Writing – review & editing. **Xin Ju**: Methodology, Supervision. **Liangzhi Li**: Funding acquisition, Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no competing financial interests.

Ethical approval This article does not contain any studies with human participants or animals.

Consent to participations Not applicable.

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Figures

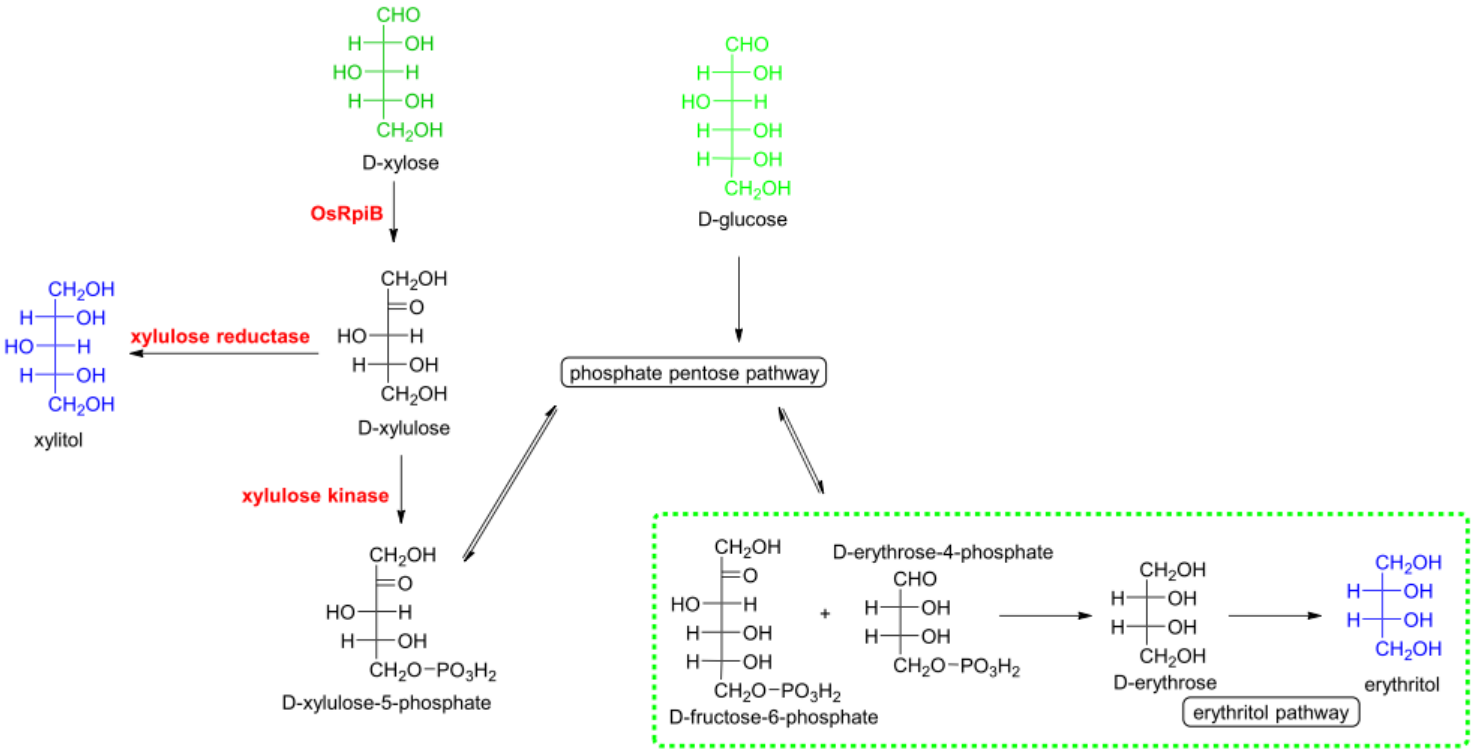

Figure 1

Schematic diagram for the erythritol fermentation pathway with xylose as the substrate. OsRpiB: ribose-5-phosphate isomerase from *Ochrobactrum* sp.

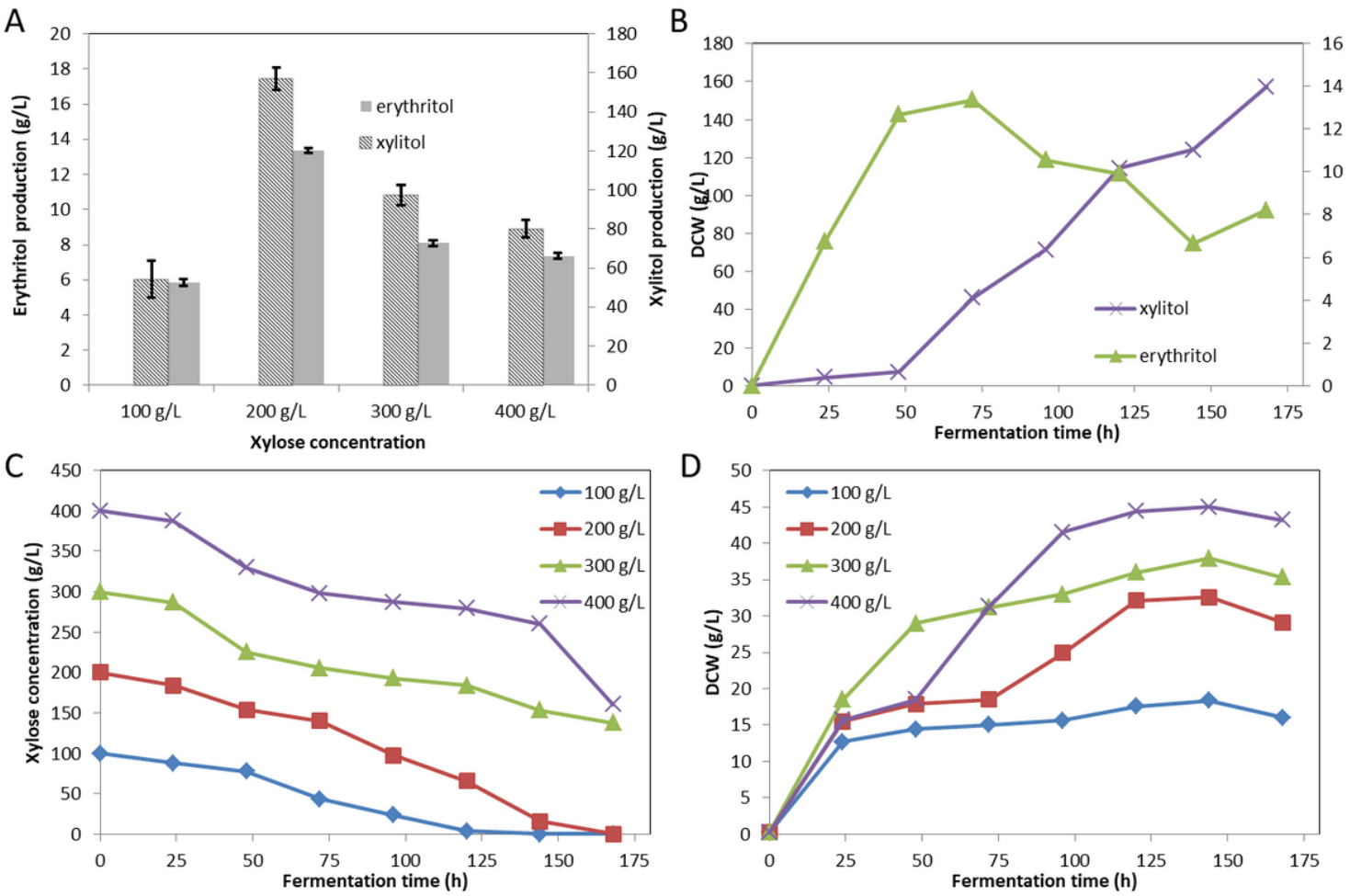

Figure 2

Effect of xylose concentration on the erythritol production. A) Production of polyols under different xylose concentrations. B) Production of polyols varied with fermentation time at a same xylose concentration. C) Different concentrations of xylose varied with fermentation time. D) DCW under different xylose concentrations.

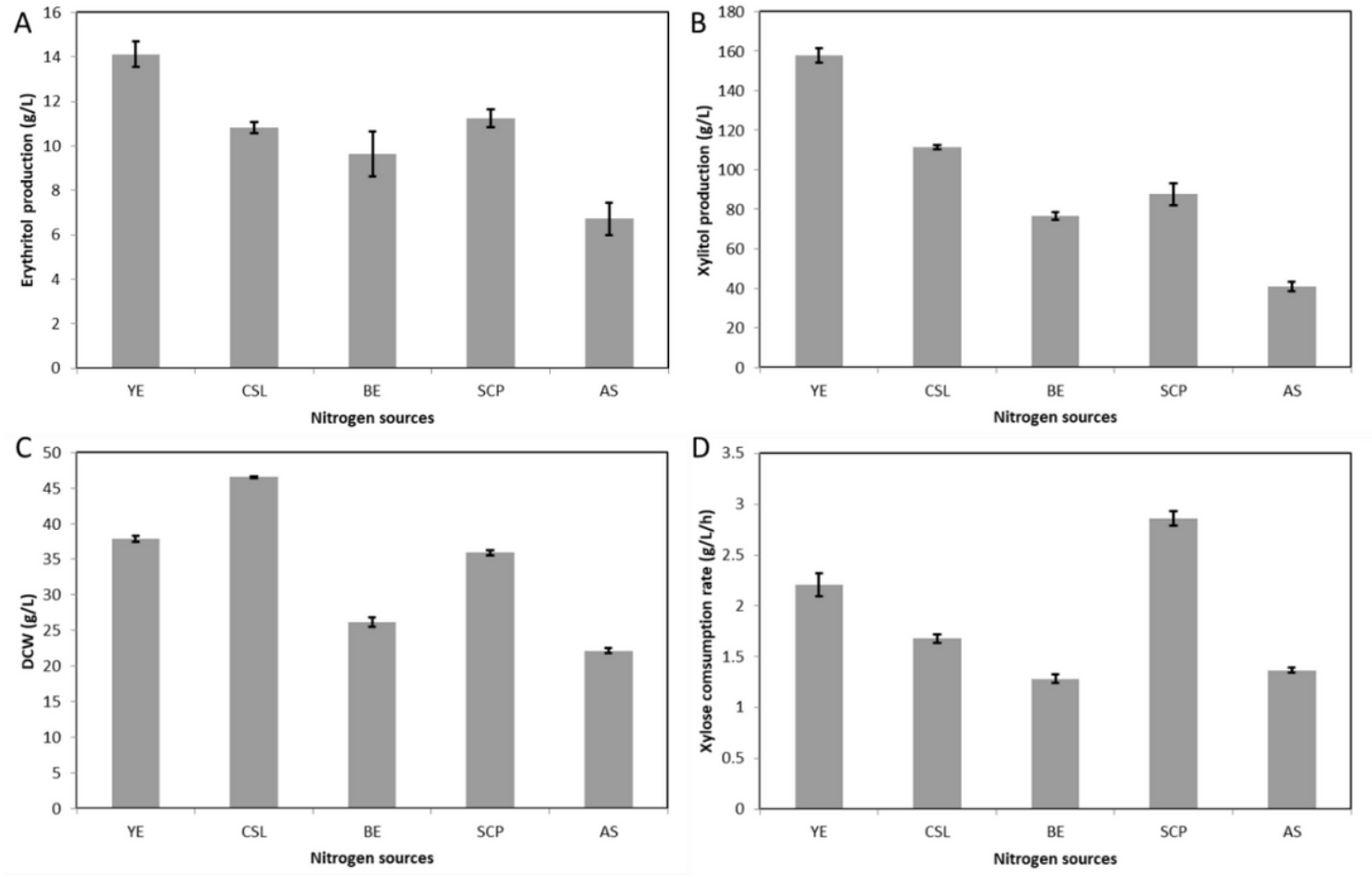

Figure 3

Effect of nitrogen source on the erythritol production.YE: yeast extract, CSL: corn steep liquor, BE: beef extract, SCP: soybean cake powder, and AS: ammonium sulfate. Production of A) Erythritol and B) Xylitol, C) DCW and D) Xylose concentration under different nitrogen sources.

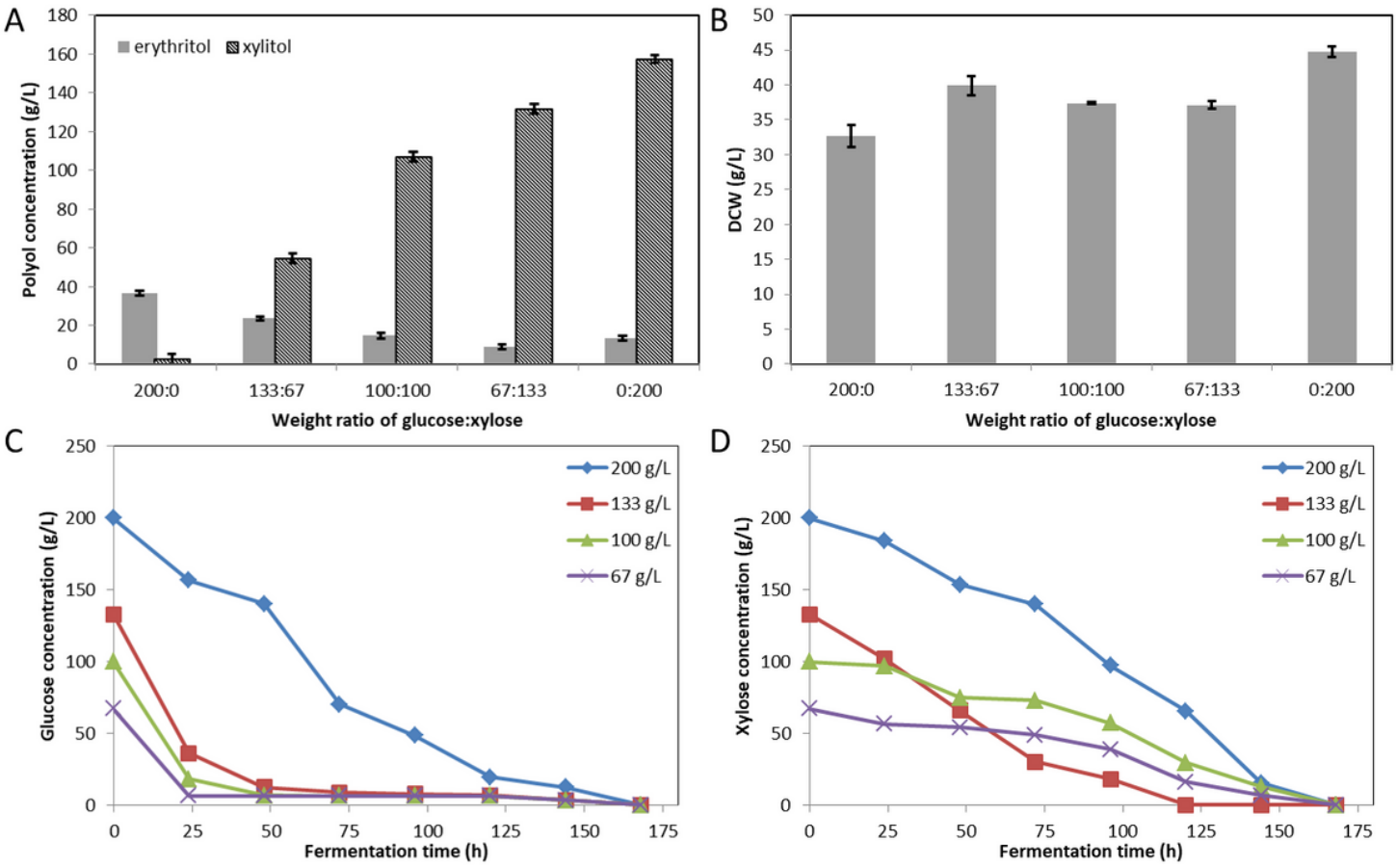

Figure 4

Effect of carbon source on the erythritol production. A) Polyol production and B) DCW at weight ratio of glucose-xylose. The concentration of C) Glucose and D) Xylose varied with fermentation time.

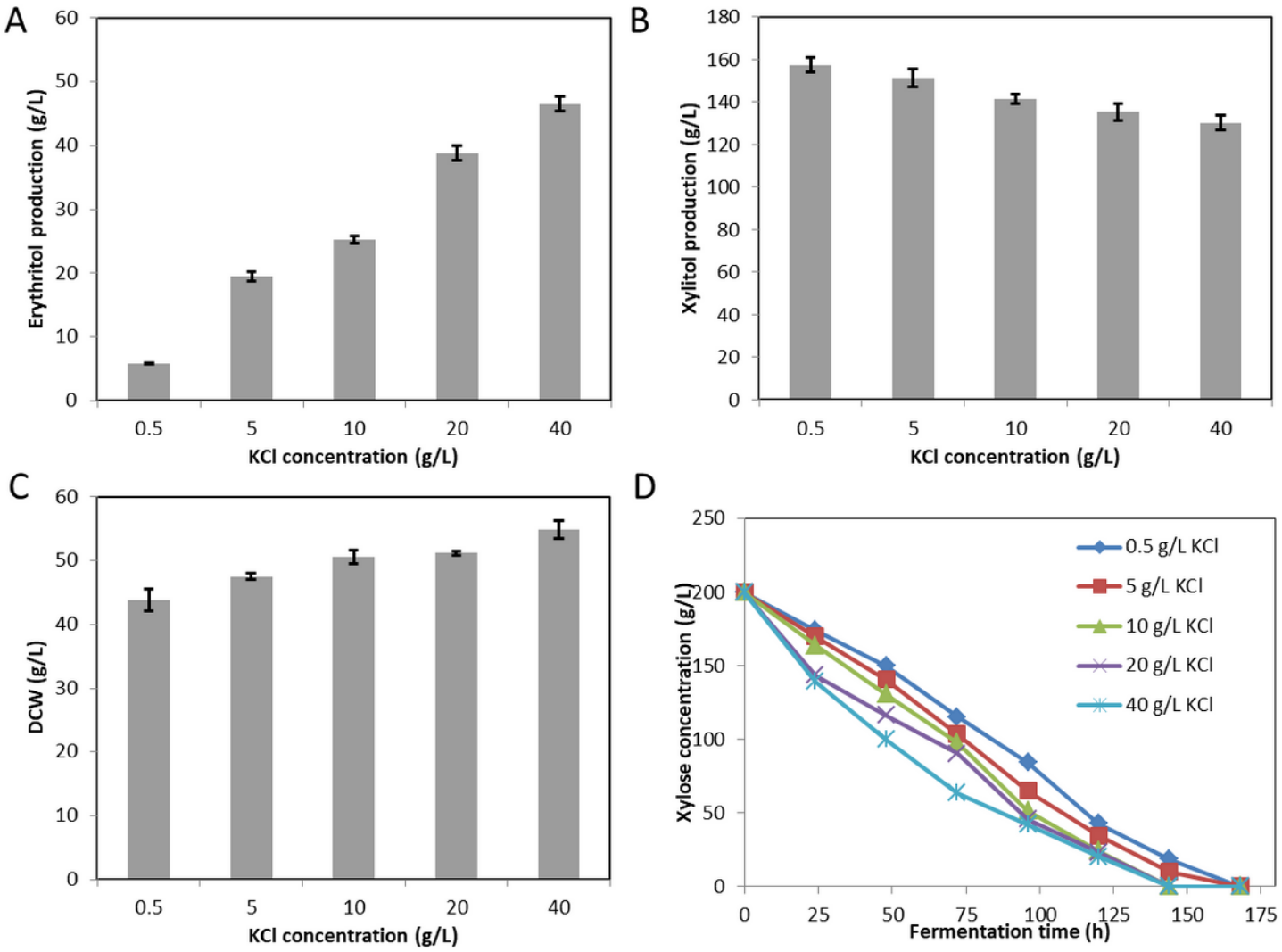

Figure 5

Effect of KCl on the erythritol production. A) Erythritol and B) Xylitol production, and C) DCW under different KCl concentrations. D) Concentration of xylose varied with fermentation time under different KCl concentrations.

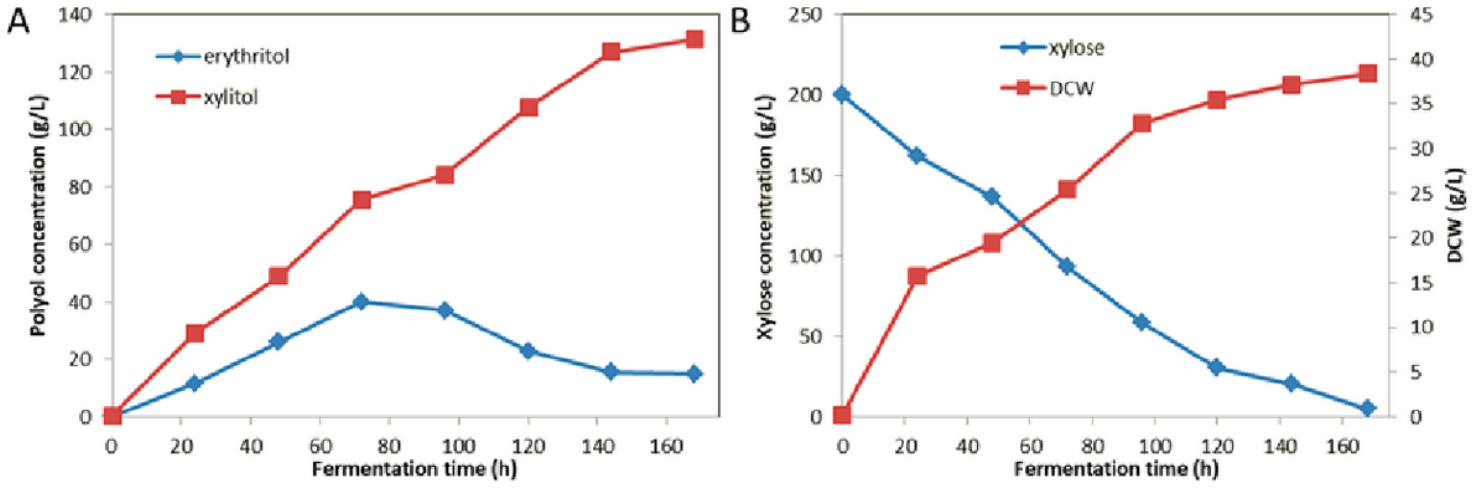

Figure 6

Production of erythritol in a 5-L fermentor with xylose. A) Production of polyols and B) Concentration of xylose varied with fermentation time.

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