Microbial Cell Factories

RESEARCH Open Access

Altered sterol composition mediates multiple tolerance of *Kluyveromyces marxianus* for xylitol production

Lili Ren[1†], Hao Zha[1†], Qi Zhang[1†], Yujie Xie[1], Jiacheng Li[1], Zhongmei Hu[1], Xiurong Tao[1], Dayong Xu[1], Feng Li[1*] and Biao Zhang[1*]

Abstract

Background Currently, the synthesis of compounds based on microbial cell factories is rapidly advancing, yet it encounters several challenges. During the production process, engineered strains frequently encounter disturbances in the cultivation environment or the impact of their metabolites, such as high temperature, acid-base imbalances, hypertonicity, organic solvents, toxic byproducts, and mechanical damage. These stress factors can constrain the efficiency of microbial fermentation, resulting in slow cell growth, decreased production, significantly increased energy consumption, and other issues that severely limit the application of microbial cell factories.

Results This study demonstrated that sterol engineering in *Kluyveromyces marxianus*, achieved by overexpressing or deleting the coding genes for the last five steps of ergosterol synthase (*Erg2-Erg6)*, altered the composition and ratio of sterols in its cell membrane, and affected its multiple tolerance. The results suggest that the knockout of the *Erg5* can enhance the thermotolerance of *K. marxianus*, while the overexpression of the *Erg4* can improve its acid tolerance. Additionally, engineering strain overexpressed *Erg6* improved its tolerance to elevated temperature, hypertonic, and acid. YZB453, obtained by overexpressing *Erg6* in an engineering strain with high efficiency in synthesizing xylitol, produced 101.22 g/L xylitol at 45°C and 75.11 g/L xylitol at 46°C. Using corncob hydrolysate for simultaneous saccharification and fermentation (SSF) at 46°C that xylose released from corncob hydrolysate by saccharification with hemicellulase, YZB453 can produce 45.98 g/L of xylitol, saving 53.72% of the cost of hemicellulase compared to 42°C.

Conclusions This study elucidates the mechanism by which *K. marxianus* acquires resistance to various antifungal drugs, high temperatures, high osmolarity, acidity, and other stressors, through alterations in the composition and ratio of membrane sterols. By employing sterol engineering, the fermentation temperature of this unconventional thermotolerant *K. marxianus* was further elevated, ultimately providing an efficient platform for synthesizing high-value-added xylitol from biomass via the SSF process at temperatures exceeding 45 °C.

†Lili Ren, Hao Zha and Qi Zhang contributed equally to this work.

*Correspondence:
Feng Li
lifeng@chnu.edu.cn
Biao Zhang
zhangbiao@chnu.edu.cn

Full list of author information is available at the end of the article

Keywords *K. marxianus*, Elevated temperature, Sterol engineering, Xylitol, Simultaneous saccharification and fermentation

Background

Microorganisms serve as excellent cell factories for developing industrial biotechnology, contributing to environmental friendliness and sustainable economics. The favorable physiological properties of industrial microorganisms, such as metabolic capacity and robustness, play crucial roles in successful biomanufacturing [1]. Conventional metabolically-directed strategies often fail to achieve the desired phenotypes because they focus solely on target metabolic functions while neglecting the physiological responses of microorganisms to environmental stress [2]. To address the new challenges posed by the biotechnological production of fuels, chemicals, natural products, and bulk chemicals, microorganisms must not only possess robust metabolic capabilities but also exhibit strong robustness and stress resistance. This enables them to achieve excellent characteristics such as energy saving, emission reduction of carbon, consumption reduction, efficiency enhancement, and quality improvement in practical bioconversion processes [2–4]. The main problem faced by the production of chemicals with microorganisms using biomass such as cellulose and lignocellulose is that the optimal temperature for cellulase or hemicellulase hydrolysis is 45-50°C, while the optimal fermentation temperature is only 28-33°C, generally not exceeding 36°C [5]. This limits the application of simultaneous saccharification and fermentation (SSF) technology at high temperatures. Furthermore, microorganisms also face multiple stresses such as high osmolarity, acidity, and inhibitors produced during biomass hydrolysis when fermenting biomass hydrolysates. Therefore, strains with resistance to multiple stresses are crucial for industrial fermentation [6].

Xylitol is a natural five-carbon sugar alcohol and one of the important functional food additives [7]. Xylitol has a sweetness relative equivalent to sucrose and one-third lower caloric content. Therefore, xylitol is widely used as a sweetener for diabetics and in chewing gum [7, 8]. Besides, studies have shown that xylitol also promotes calcium absorption in the intestine, reduces bone loss, and maintains normal bone density [9–11]. The global xylitol market size reached US$ 976.7 Million in 2023. Looking forward, IMARC (The International Market Analysis Research and Consulting) Group expects the market to reach US$ 1,429.1 Million by 2032, exhibiting a growth rate (CAGR) of 4.2% during 2024–2032. The market is experiencing steady growth driven by expanding applications in the food and beverage industry, regulatory support and favorable policies promoting natural sweeteners over artificial sweeteners, and the rising prevalence of diabetes and obesity [12]. The chemical catalytic hydrogenation of xylose is currently the main method for industrial production of xylitol [13]. However, this production process is complex and less safe due to separate hydrogen production and reaction under high temperature and pressure [14]. It also requires high-priced pure xylose as raw material to avoid mixed alcohols affecting product quality, leading to higher costs [14, 15]. The biological fermentation method, however, uses xylose reductase within cells to produce xylitol, eliminating separate hydrogen production. It operates at normal temperature and pressure, resulting in low energy consumption, simple equipment, and safe operation [15]. The enzyme's substrate specificity allows for less refined raw materials, making it a promising development direction [15]. However, microbial production of xylitol also faces the problem of decreased fermentation efficiency due to multiple stresses during the fermentation process.

K. marxianus, commonly known as thermotolerant yeast, is widely found in cheese and kefir grains and is a GRAS (Generally Recognized as Safe) level microorganism certified by the US FDA (Food and Drug Administration) [16, 17]. *K. marxianus* can grow at elevated temperatures above 45°C and has a wide range of substrate sources, making it a platform for developing high-value compound synthesis from various inexpensive substrate sources [18]. *K. marxianus* has proven to be an ideal platform for the biological synthesis of xylitol, as previous reports have shown that, compared to other yeast strains, the engineered *K. marxianus* strains produced 322.07 g/L xylitol at 42 °C. Furthermore, the substrate sources for xylitol production were highly diverse, encompassing xylose alone, glucose plus xylose, and glycerol plus xylose [13, 19, 20]. However, under fermentation conditions, the fermentation temperature is still less than 45°C, which is still a distance from the temperature required for SSF [13, 19, 20]. Previous studies have shown that nonsense mutations of *Erg3* have been found in seven evolved strains of *S. cerevisiae* with enhanced thermotolerance [1]. Further studies have shown that knockout of the *Erg5* gene also enhanced the high-temperature tolerance of *S. cerevisiae* and *Penicillium oxalicum* [21], indicating that the type and proportion of sterols in the cell membrane can affect the strength of the yeast cell membrane, thereby regulating the thermotolerance of the yeast.

Therefore, this study investigated the effects of different types and proportions of sterols in the cell membrane constructed by knockout and overexpression of ergosterol last five steps synthase coding genes (*Erg2-Erg6*) on

the multiple tolerance of *K. marxianus* (Fig. 1A), based on which a more tolerant engineered strain was constructed, achieving the biological synthesis of xylitol from corncob hydrolysate through SSF at 46°C.

Methods

Microorganisms and media

The following items were purchased from Sangon Biotech Co. (Shanghai, China): D-glucose, glycerol, yeast nitrogen base without amino acids (YNB), restriction enzymes,

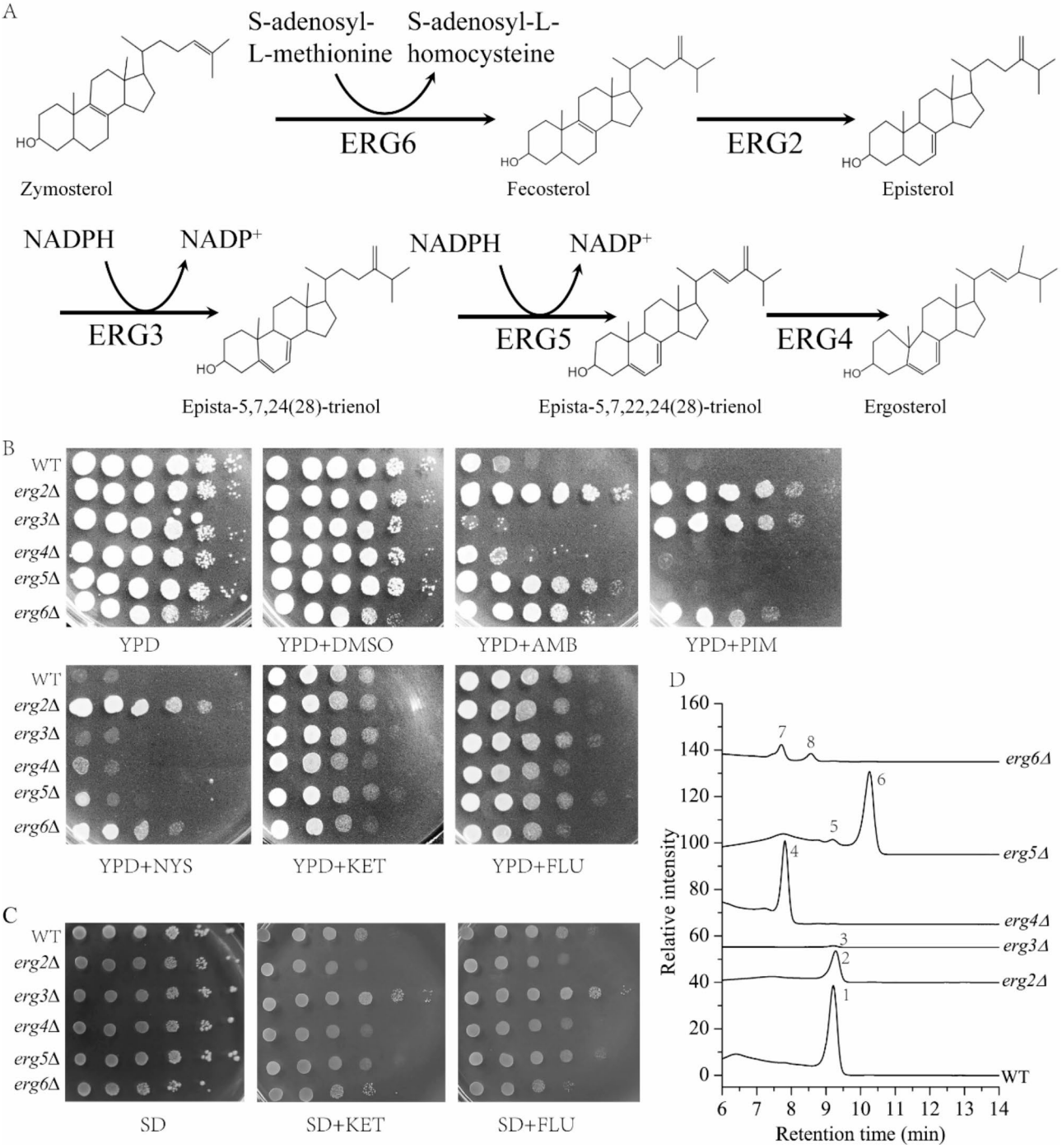

Fig. 1 (**A**) The conversion of zymosterol to ergosterol as the last 5 steps in ergosterol biosynthesis of *K. marxianus*. (**B**) The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under antifungal agents' stress on the YPD plate. (**C**) The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under azole drugs stress on SD plate. (**D**) Sterol production of strains WT, *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* analyzed by HPLC assays

and modifying enzymes (Phosphatase, T4 DNA Ligase, and T4 polynucleotide kinase). Additionally, yeast extract and peptone were obtained from Oxoid Ltd. (Basingstoke, Hampshire, England). Corncob (30–60 mesh) was purchased from Huanghe Co. (Puyang, Henan, China), and the proportion of xylan in the corncob is approximately 38%. Hemicellulose was purchased from Longkete Co. (Jinan, Shandong, China). *K. marxianus* NBRC1777 (NITE Biological Resource Center, Tokyo, Japan), a haploid yeast strain. YZB100, a *KU70* disruptive strain of *K. marxianus* NBRC1777, was used as a wild type in this study. YZB101 is a *URA3* defective strain of YZB100 [22]. YNBD plates (6.7 g/L YNB and 20 g/L glucose) supplemented with appropriate amino acids were used to select transformants and compare the growth of strains with different genotypes. Yeast extract-peptone (YP) medium (10 g/L yeast extract, 20 g/L peptone) with different carbon sources was used to culture *K. marxianus* strains aerobically or anaerobically. Solid plates were prepared by adding 15 g/L agar to each medium. *Escherichia coli* DH5α was used for cloning and grew in a lysogeny broth (LB) medium.

Plasmids

Table S1 provides a summary of the plasmids and strains used in this study. The primers utilized in this research, which were purchased from General Biotech Co. (Chuzhou, China), are listed in the supplementary material (Table S2). The plasmid p414-TEF1p-Cas9-CYC1t was obtained from Addgene (Watertown, MA, USA). For the amplification of the Snr52 promoter, pZB089 was used, while pZB090 was employed to amplify the gRNA. CAN1.Y-Sup4t-Cyc1t fragment [23]. pZB043, pZB080, pZB094, pZB095, and pZB104 were constructed for the recombinant expression of *Erg6*, *Erg3*, *Erg2*, *Erg4*, and *Erg5*, respectively (Table S1) [24]. The plasmid pMD18T-ΔScURA3 was utilized to amplify the fragment of a truncated *ScURA3*, which served as the genetic selection marker for regeneration (Table S1) [20]. Plasmid pZB074 pZB082, pZB097, pZB099, and pZB107 were constructed for *Erg6*, *Erg3*, *Erg2*, *Erg4*, and *Erg5* disruption by replacing the *Ura3* expression cassette with part of each gene (Fig. S1).

Construction of Erg2, *Erg3*, *Erg4*, *Erg5*, and *Erg6*deletion and overexpression strains of *K. marxianus*

The disruption cassettes of *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* were amplified from corresponding plasmids with specific primers and transformed into YZB101 using the lithium acetate method [25]. Transformants were selected on a YNB-glucose plate. The *Erg* genes in YZB101 were disrupted via homologous recombination and verified by genomic DNA PCR. The obtained strain was named YZB207, YZB190, YZB208, YZB226, and, YZB156 (Table S1 and Fig. S2A). The *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* overexpression strains were constructed through a transient CRISPR/Cas9 method in a previous study and strains were named YZB250, YZB251, YZB244, YZB252, and YZB253, respectively (Table S1 and Fig. S2A) [24].

YZJ074 is a previously reported *K. marxianus* strain that efficiently utilizes xylose and glycerol to produce xylitol, and YZJ080 is the *Ura3*-deficient strain of YZJ074 [20]. Similarly, *Ku70* in YZJ080 was disrupted to obtain strain YZB152 by amplifying the disruption cassette with pZB061 as a template. After that, The *Ura3* in YZB152 was disrupted and selected on an SD plate supplemented with 0.1% 5′-fluoroorotic acid (5′-FOA) and uracil, and the obtained strain was named YZB229. The *Erg5* in YZB229 was disrupted using homologous recombination to obtain strains YZB414. The *Ura3* in YZB414 was disrupted to regenerate the selection marker and obtained strain YZB426. The *Erg6* overexpression cassettes were integrated into YZB229 and YZB426 at the *Xyl2* gene site using the transient CRISPR/Cas9 method [19] and obtained strain were named YZB447 and YZB440. As the *Xyl2* in YZJ080 was disrupted using homologous recombination with the *Trp1* gene as a selection marker [26], and the integrating of *Erg6* overexpression cassettes marking strains YZB447 and YZB440 to the *Trp1* defective type. Thus, the *Trp1* gene overexpression cassettes were re-integrated into YZB440 and YZB447 to obtain strains YZB452 and YZB453 at the original *Trp1* site (Fig. S2B).

The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under antifungal agents stress

The strains were pre-cultured in 5 ml YPD medium for 18–24 h at 30 °C. After harvesting, the cells were washed three times and resuspended in sterile deionized water to an optical density at 600 nm (OD600nm) of 10. The suspensions were then serially diluted by 10-fold. Subsequently, 2 µL of each dilution was spotted onto YPD plates containing various antifungal agents to create different stress conditions. The antifungal agents used were Amphotericin (AMB) at 20 µg/mL, pimaricin (PIM) at 16 µg/mL, nystatin (NYS) at 4 µg/mL, ketoconazole (KET) at 20 µg/mL, and fluconazole (FET) at 20 µg/mL. A plate containing 880 µg/mL dimethyl sulfoxide (DMSO) was set as a control, as it was the solvent for the antifungal agents. Additionally, the growth performance of the strains on SD plates containing 20 µg/mL of KET and FET was also tested. The phenotypes were recorded after incubating the plates for 1–3 days at 30°C.

The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under different temperatures

The strains were pre-cultured in 5 ml YPD medium for 18–24 h at 30 °C. After harvesting, the cells were washed three times and resuspended in sterile deionized water to an OD600nm of 10. The suspensions were then serially diluted by 10-fold. Subsequently, 2 μL of each dilution was spotted onto YPD, SD, and SD plates containing 10 μg/mL ergosterol. The phenotypes were recorded after incubating the plates for 1–3 days at 4 °C, 30°C, 37°C, and 45°C, respectively.

The performance of *K. marxianus* strains overexpressed *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* under elevated temperatures

The strains were pre-cultured in 5 ml YPD medium for 18–24 h at 30 °C. After harvesting, the cells were washed three times and resuspended in sterile deionized water to an OD600nm of 10. The suspensions were then serially diluted by 10-fold. Subsequently, 2 μL of each dilution was spotted onto SD plates. The phenotypes were recorded after incubating the plates for 1–5 days at 30°C and 47 °C, respectively.

The growth profiles of *K. marxianus* strains deleted or overexpressed *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* under elevated temperatures

Strains YZB100, *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* were pre-cultured in YPD overnight and then inoculated into 250-mL flasks containing 50 mL of YPD media. The initial OD600nm was adjusted to 0.2, and the cultures were incubated at 30 °C and 46 °C with shaking at 220 rpm. Additionally, the wild-type (WT) strain and strains overexpressing *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* were also pre-cultured in YPD overnight and inoculated into 250-mL flasks containing 50 mL of YPD media. The initial OD600nm was adjusted to 0.2, and the cultures were incubated at 30°C, 45°C, and46 °C with shaking at 220 rpm. The experiments were performed in triplicates, and results shown as the mean values. The bars in the figures indicate the ranges of standard deviation.

The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under multiple chemical stress

The strains were pre-cultured in 5 ml of YPD medium for 18–24 h at 30°C. After harvesting, the cells were washed three times and resuspended in sterile deionized water to an $OD_{600nm}$of 10. The suspensions were then serially diluted by 10-fold. Subsequently, 2 μL of each dilution was spotted onto SD plates containing various chemicals to create different stress conditions. The chemicals used were NaCl at 0.75 M, acetic acid at 1 g/L, ethanol at12% (v/v), furfural at 1 g/L, 5-hydroxymethylfurfural (5-HMF) at 1 g/L, phenol at 1 g/L, SDS at 0.5 mg/L, H_2O_2 at 3 mM, and Congo red (CR) at 150 μg/mL. The phenotypes were recorded after incubating the plates for 1–5 days at 30°C. The experiments were performed in triplicates, and results shown as the mean values. The bars in the figures indicate the ranges of standard deviation.

Xylitol produced at 46°C with YZB152, YZB414, YZB452, and YZB453

The strains were pre-cultured in 50 ml of YPD medium for 18–24 h at 37°C. After harvesting, the cells were resuspended into 250 mL Erlenmeyer flasks containing 50 mL of YPGX medium (composed of 10 g/L yeast extract, 20 g/L peptone, 5 g/L glycerol, and 12.5 g/L xylose) to an OD600nm of 1. The fermentations were carried out with shaking at 220 rpm at 46°C. The experiments were performed in triplicates, and results shown as the mean values. The bars in the figures indicate the ranges of standard deviation.

Xylitol produced at 46°C with YZB152, YZB414 (YZB152, *ΔErg5*), YZB452 (YZB152 + *Erg6*, *ΔErg5*) and YZB453 (YZB152 + *Erg6*)

Strains were pre-cultured in YPD overnight and inoculated into 250-mL flasks containing 50 mL YP medium (10 g/L yeast extract, 20 g/L peptone) containing 12.5 g/L xylose and 5 g/L glycerol) with an optical density at 600 nm (OD_{600nm}) of 0.2. The fermentations were performed with 220 rpm shaking at 46°C. The experiments were performed in triplicates, and results shown as the mean values. The bars in the figures indicate the ranges of standard deviation.

Batch fermentation with YZB152 and YZB453 for xylitol production using a fermenter

A seed culture (250 mL) was obtained after culturing the yeast in YPD medium at 37 °C and then inoculated into a 5-L modular benchtop fermenter (Baoxing Corp., Shanghai, China) containing 2.5 L of fermentation medium at its natural pH. Fermentation experiments were conducted using YZB152 and YZB453 strains with YP medium containing either 40 g/L glycerol and 100 g/L xylose or 60 g/L glycerol and 150 g/L xylose at 45 °C, 450 rpm, and 1 vvm. Subsequently, fermentation experiments were performed using YZB152 and YZB453 strains with YP medium containing 1 g/L glycerol and 2.5 g/L xylose at 46 °C, 450 rpm, and 1 vvm. Finally, fermentation experiments were conducted using YZB453 strain with YP medium containing varying concentrations of glycerol and xylose: 2 g/L glycerol and 5 g/L xylose, 3 g/L glycerol and 7.5 g/L xylose, and 4 g/L glycerol and 10 g/L xylose, all at 46 °C, 450 rpm, and 1 vvm. The experiments were performed in triplicates, and results shown as the mean values. The bars in the figures indicate the ranges of standard deviation.

Xylitol producing with YZB453 at elevated temperatures through SSF

The corncob hydrolysate was prepared as previously reported [26]. Acid hydrolysis of corncobs was performed at 127 °C using a dilute acid mixture consisting of 0.5% (w/w) H_2SO_4 and 1.5% (w/w) H_3PO_4 for a duration of 1 h. A solid: liquid ratio of 1:3 (quantity: volume) was used. The resulting corncob residue and hydrolysate were neutralized by adding lime cream until the pH reached 6.0. The neutralized mixture was then used directly for fermentation without sterilization. The medium composition for SSF consisted of corncob residue and hydrolysate derived from 150 g/L of corncob, supplemented with 20 g/L glycerol, 1% yeast extract, and 2% peptone. After sterilization, 5 FPU/g of lignocellulose was added. The strain YZB453 was pre-cultured and inoculated into the fermenter at a volume of 10%. The fermentation conditions were set at 450 rpm with an aeration rate of 1 vvm and temperatures of 42–46 °C, respectively. The concentrations of D-glucose, xylitol, D-xylose, and glycerol were analyzed as described previously [27]. The experiments were performed in triplicates, and results shown as the mean values. The bars in the figures indicate the ranges of standard deviation.

Results

Effects of *Erg2-Erg6* gene knockouts on antifungal drug resistance in *K. marxianus*

As shown in Fig. 1B, strains with knockouts of *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* exhibit different phenotypes under the stress of different drugs, with some developing resistance to the drugs and others becoming more sensitive. Specifically, strain with the *Erg2* gene knocked out show increased resistance to amphotericin B, pimaricin, and nystatin. Strains with the *Erg3* gene knocked out exhibit increased sensitivity to amphotericin B and nystatin but significantly enhanced resistance to pimaricin. Strain with the *Erg4* gene knocked out was very similar to the wild type. Strain with the *Erg5* gene knocked out showed significantly enhanced resistance to amphotericin B, slightly increased resistance to nystatin, and the same resistance to pimaricin as the wild type. Strain with the *Erg6* gene knocked out exhibited significantly enhanced resistance to amphotericin B, pimaricin, and nystatin compared to the wild type, similar to the *erg2Δ* strain. Additionally, all strains demonstrate strong resistance to azole drugs (ketoconazole and fluconazole) (Fig. 1C). To determine whether this resistance is due to the yeast's ability to absorb sterols from natural media, which confers tolerance to azole drugs, the effects of natural and synthetic media on yeast tolerance to azole drugs were further investigated. YPD medium is a natural medium that contains various sterols, which the yeast can absorb if it cannot synthesize sterols itself. SD medium is a synthetic medium that only contains a carbon source and does not contain various sterols, so the yeast must synthesize sterols on their own. As shown in Fig. 1C, both on YPD and SD media, wild-type and knockout strains exhibit strong resistance to fluconazole and ketoconazole, with strain with the *Erg3* gene knocked out showing enhanced resistance to fluconazole and ketoconazole. However, there is no significant difference in resistance to fluconazole and ketoconazole among strains with the *Erg2*, *Erg4*, *Erg5*, and *Erg6* genes knocked out.

The varying resistances of yeast strains to amphotericin B, pimaricin, and nystatin are attributed to changes in sterol composition in the yeast cell membrane due to gene knockouts leading to altered sterol levels. Specifically, knocking out *Erg4* enhances resistance to amphotericin by accumulating epista-5,7,22,24(28)-trienol instead of ergosterol, its target. Similarly, *Erg5* knockout results in the accumulation of epista-5,7,24(28)-trienol, enhancing resistance to amphotericin B. After *Erg3* knockout, yeast can still synthesize ergosterol via a compensatory pathway, remaining sensitive to amphotericin but resistant to pimaricin due to inability to synthesize its target, epista-5,7,24(28)-trienol. Strains with *Erg2* and *Erg6* knocked out cannot synthesize targets of these three drugs, exhibiting strong resistance. All strains show strong resistance to azole drugs due to *K. marxianus*'s robust sterol synthesis ability, because antifungal drugs like ketoconazole and fluconazole target key enzymes on the fungal cell membrane, inhibiting ergosterol biosynthesis.

The effects of *Erg2-Erg6* deletion on the thermotolerance of *K. marxianus* strains

As shown in Fig. 2A, phenotypic experiments at different temperatures on three types of media—YPD, SD, and SD + ergosterol—with strains WT, *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* indicate that the deletion of genes other than *Erg6* has no effect on the growth of *K. marxianus* at 4°C, 30°C, and 37°C. However, the deletion of the *erg2*, *erg3*, *erg4*, and *erg6* significantly reduces the thermotolerance of *K. marxianus* at 45°C (Fig. 2A). This phenomenon is consistent on both YPD and SD plates, and the addition of ergosterol does not alter this high-temperature sensitivity, indicating that it is not caused by the blockage of ergosterol synthesis due to the deletion of these genes. The *Erg5* deletion strain exhibits high-temperature tolerance similar to that of the WT strain, and it is difficult to determine whether there are any differences. Therefore, growth curves in liquid cultures were also performed. As shown in Fig. 2B, the deletion of *Erg2*, *Erg3*, *Erg4*, and *Erg5* nearly did not affect the growth *K. marxianus* under normal temperature, while the deletion of *Erg6* decreased the growth of *K. marxianus* for about 50% under normal condition. When the culture temperature was improved to 46°C, the growth of *Erg2*, *Erg3*, *Erg4*, and *Erg6* deleted

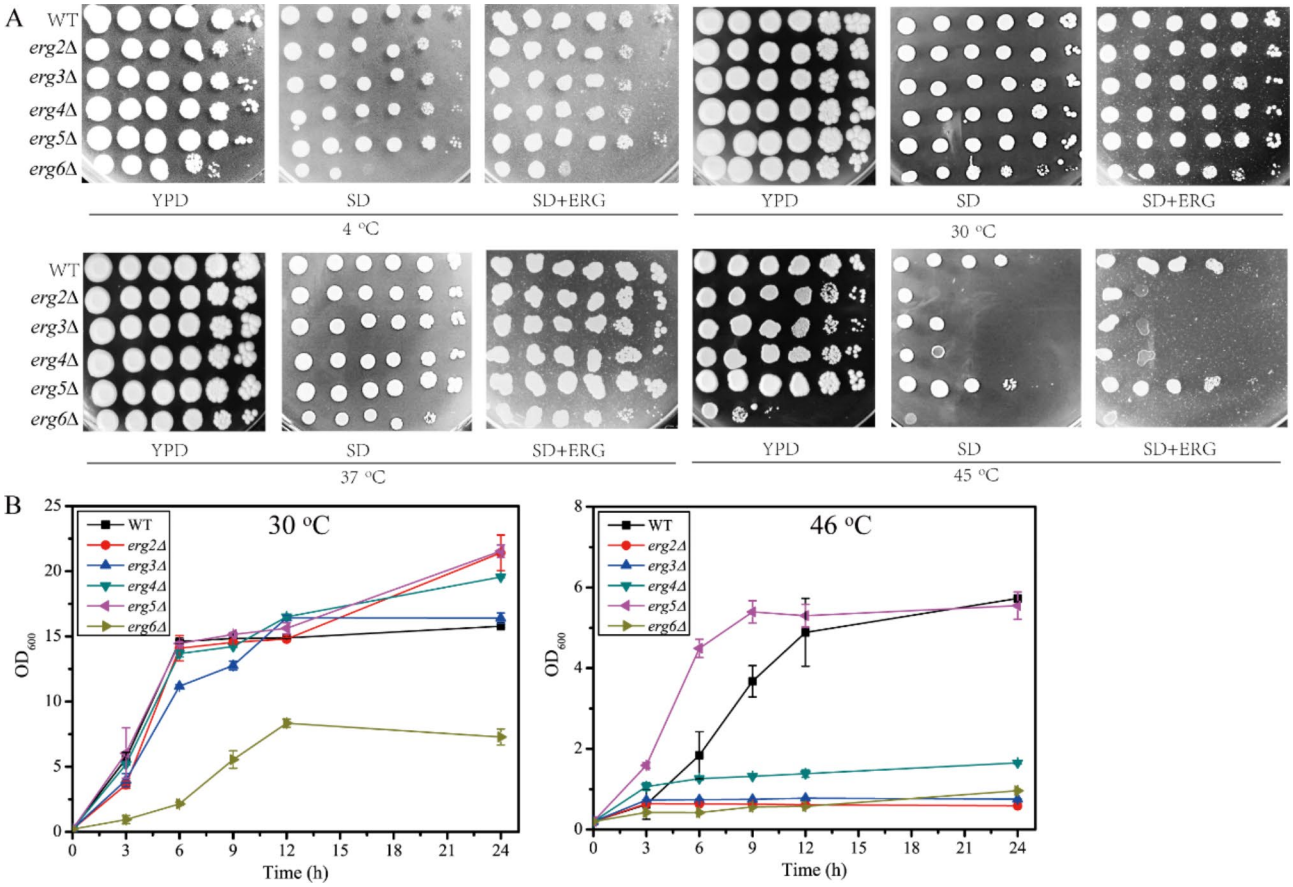

Fig. 2 (**A**) The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under different temperatures. (**B**) The growth curves of strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* cultured in YPD medium at 30°C and 46°C. Data are shown as the mean ± standard deviation from at least three experiments. The bars in the figures indicate the ranges of standard deviation

strains were completely inhibited, while the WT and *Erg5* deleted strains still grew well, and the *Erg5* deleted strain grew much better than the WT strain that reached the stationary phase 6 h earlier (Fig. 2B).

The effects of *Erg2-Erg6* overexpression on the thermotolerance of *K. marxianus* strains

The plate growth phenotypes of the WT and *Erg2-6* overexpression strains are similar at high temperatures (Fig. 3A), thus the thermotolerance of *Erg2-Erg6* overexpressed strains in liquid YPD were tested. As shown in Fig. 3B, all of the overexpressed strains grew similarly to the WT strain at 30°C, however, when the culture temperature reached to 46°C, the *Erg6* overexpressed strain grew much better than the WT strain and 52.82% more biomass was obtained than the WT strain (Fig. 3C). Besides, the *Erg5* overexpressed strain also grew much better than the WT strain, and the *Erg3* and *Erg4* overexpressed strain grew similar to the WT strain, while the *Erg2* overexpressed strain grew worse than the WT strain (Fig. 3C). Further increased the culture temperature to 47°C, all the growth of the strains was inhibited except *Erg6* and *Erg5* overexpressed strains, and *Erg6* overexpressed stain grew much better than the *Erg5* overexpressed strain with about 116.31% more biomass than the *Erg5* overexpressed strain (Fig. 3D).

The effects *Erg2-Erg6* on multiple industrial inhibitors of *K. marxianus* strains

In addition to high temperature, strains in industrial fermentation processes also face multiple stress challenges, such as hypertonic, organic acids, ethanol, oxidation, and inhibitors generated from biomass hydrolysis (furfural, 5-HMF, and phenols), etc. To understand the role of *Erg2-Erg6* in *K. marxianus* resistance to these inhibitors, the phenotypes of *Erg2-Erg6* knockout strains on SD plates containing NaCl, acetic acid, ethanol, furfural, 5-HMF, phenol, SDS, H_2O_2, CR at concentrations of 0.75 M, 1 g/L, 1 g/L, 12% (v/v), 1 g/L, 0.5 mg/L, 3 mM, 150 μg/mL respectively, were studied. As shown in Fig. 4A, the *erg6Δ* strain exhibited stronger tolerance to hypertonic stress than the WT strain, while the *erg3Δ* strain was more sensitive. Other strains were similar to WT. The *erg2Δ*, *erg3Δ*, and *erg5Δ* strains were more sensitive to

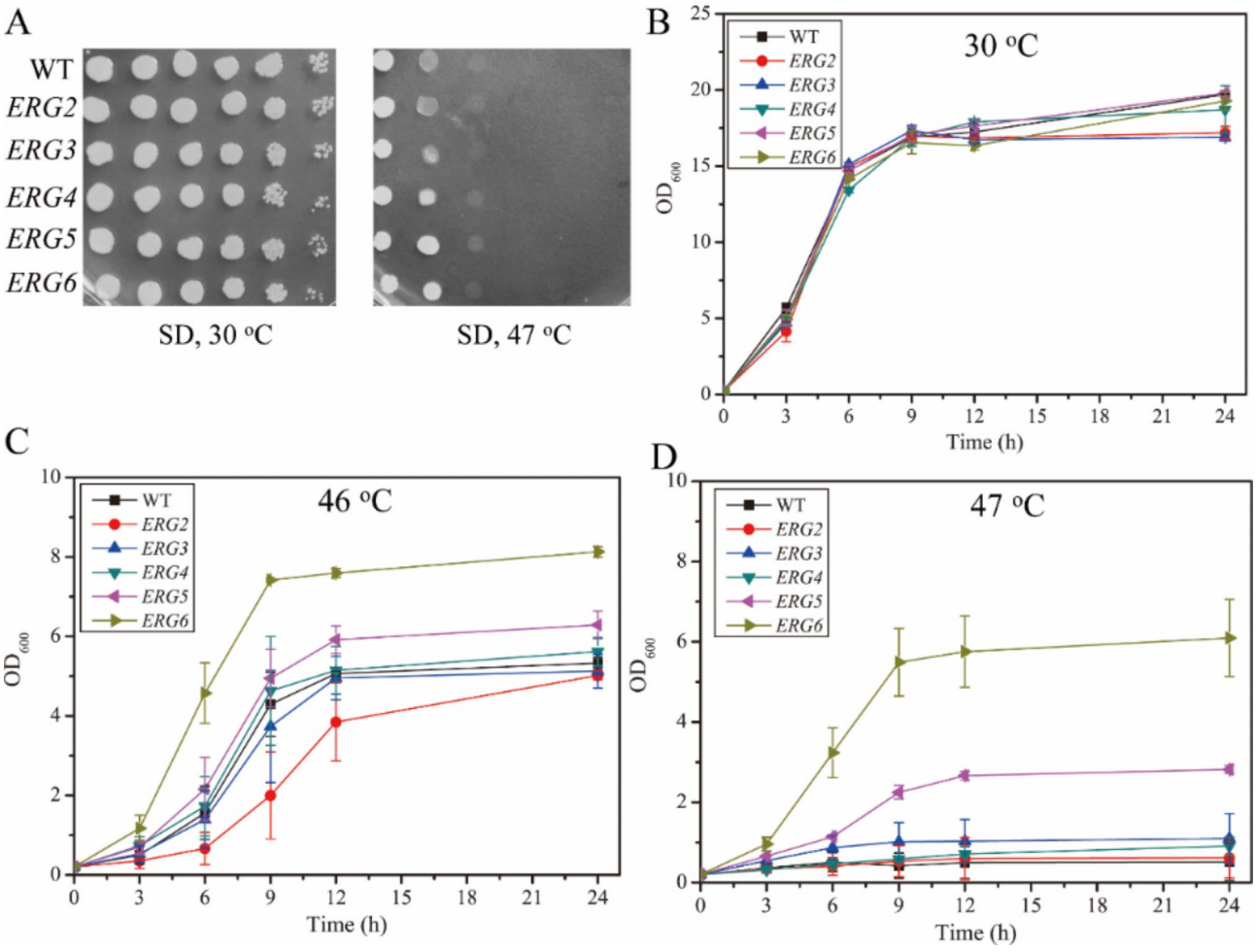

Fig. 3 The performance of *K. marxianus* strains overexpressed *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* with the WT strain under 30°C and 47°C (**A**). The growth curves of strains overexpressed *Erg2*, *Erg3*, *Erg4*, *Erg5*, and *Erg6* with the WT strain cultured in YPD medium at 30°C (**B**), 46°C (**C**), and 47°C (**D**). Data are shown as the mean ± standard deviation from at least three experiments. The bars in the figures indicate the ranges of standard deviation

acetic acid than the WT strain, while the growth of *erg4Δ* and *erg6Δ* strains was completely inhibited by acetic acid. Compared to the WT strain, *erg2Δ-erg5Δ* strains were more sensitive to ethanol, while the *erg6Δ* strain was significantly inhibited by 12% ethanol. The knockout of *Erg* genes had little effect on the response of *K. marxianus* to oxidative stress, except for *Erg4* and *Erg6*. For the stress of inhibitors furfural and 5-HMF, *Erg2-Erg6* did not show a significant effect. For phenol stress, compared to the WT strain, *erg2Δ-erg5Δ* strains all showed reduced tolerance to varying degrees. *Erg2*, *Erg3*, and *Erg6* were important for the response of *K. marxianus* to SDS stress, while the deletion of *Erg4* and *Erg5* had no effect. The deletion of *Erg2*, *Erg3*, and *Erg5* had no effect *K. marxianus* on CR stress, and the deletion of *Erg4* increased its sensitivity, while the deletion of *Erg6* increased the tolerance of *K. marxianus* to CR.

Since the deletion of *Erg4* and *Erg6* completely inhibits the growth of *K. marxianus* on the SD plate containing 1 g/L acetic acid, whether the overexpressing of *Erg2* and *Erg6* enhance the acid tolerance of *K. marxianus*? The growth curves of *Erg2* and *Erg6* overexpressing strains in YPD medium containing 2 g/L and 4 g/L acetic acid were tested. As shown in Fig. 4B, both strains overexpressed *Erg2* or *Erg4* enhanced the acid tolerance of *K. marxianus*, with *Erg6* showing the best effect. For ethanol stress, the *Erg6* knockout strain showed higher sensitivity, but the *Erg6* overexpressing strain exhibited similar growth to the WT strain at 5% (v/v) ethanol concentrations. However, when the ethanol concentration increased to 7% (v/v), the tolerance of the *Erg6* overexpressing strain decreased, indicating that the *Erg6* overexpressing strain is not suitable for bioethanol production (Fig. 4C). For hyperosmotic stress, the *Erg6* knockout strain showed stronger hyperosmotic tolerance on plates, but liquid culture showed that the *Erg6* overexpressing strain had stronger tolerance to hyperosmotic stress, while the *Erg6*

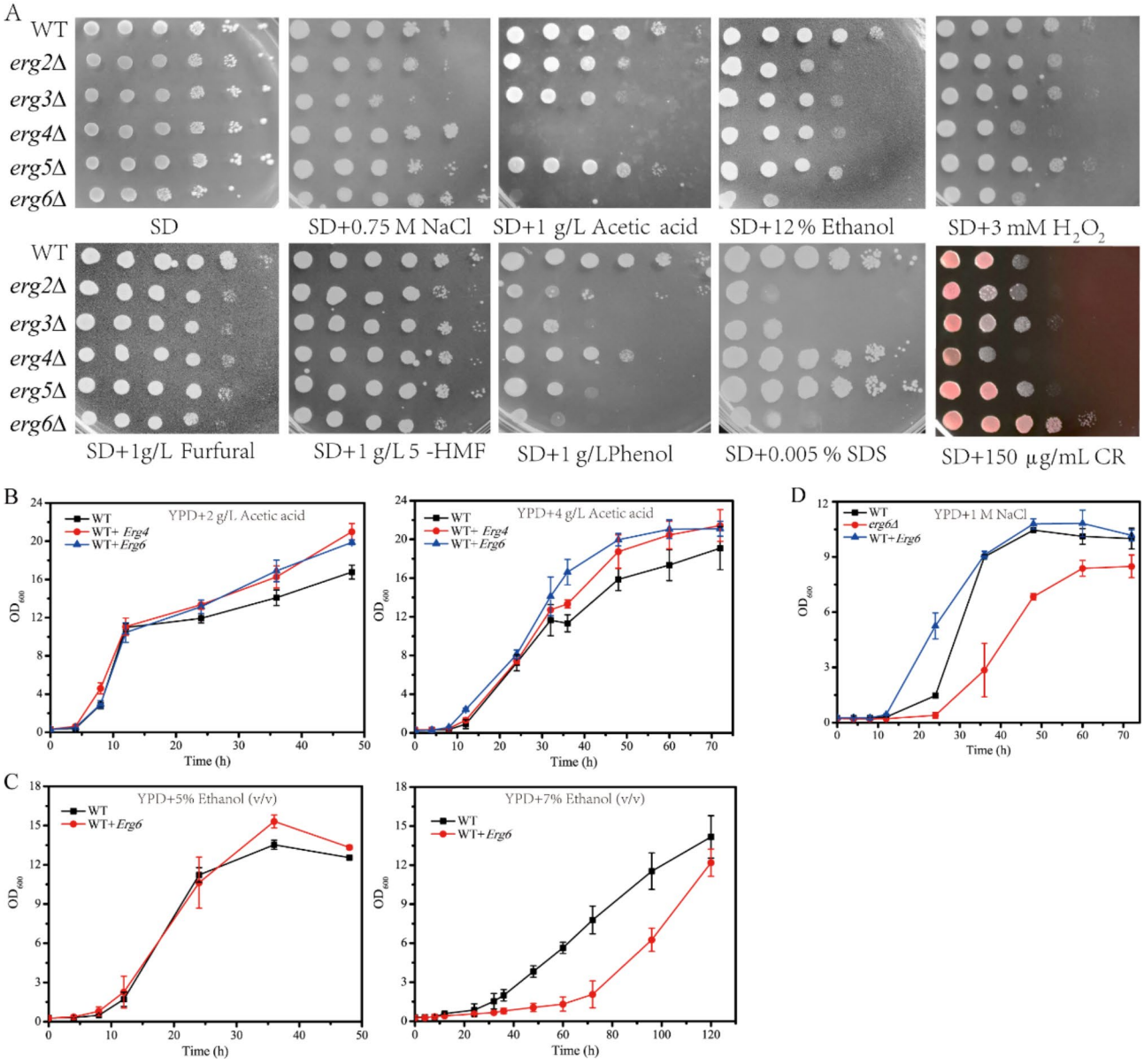

Fig. 4 (**A**) The performance of *K. marxianus* strains *erg2Δ*, *erg3Δ*, *erg4Δ*, *erg5Δ*, and *erg6Δ* under multiple chemical stress. (**B**) The growth of *K. marxianus* strains WT, WT + *Erg4*, and WT + *Erg6*under acetic acid stress. (**C**) The growth of *K. marxianus* strains WT and WT + *Erg6* under ethanol stress. (**D**) The growth of *K. marxianus* strains WT, *erg6Δ*, and WT + *Erg6* under hyperosmotic stress. Data are shown as the mean ± standard deviation from at least three experiments. The bars in the figures indicate the ranges of standard deviation

deletion strain was more sensitive, which seemed to contradict the results in Fig. 4A.

Xylitol production at elevated temperature by sterol engineering with *K. marxianus*

The strain overexpressing *Erg6* not only enhances the thermotolerance of *K. marxianus* but also enhances its hyperosmotic tolerance and acid tolerance, all of which are desirable characteristics for xylitol industrial fermentation. The deletion of the *Erg5* gene, on the other hand, mainly leads to a reduction in phenol tolerance and an increase in high-temperature tolerance of *K. marxianus*, with no significant changes in tolerance to other factors (Fig. 4A). Therefore, the overexpression of *Erg6* or deletion of *Erg5* for xylitol synthesis at higher temperatures were studied. Based on strain YZJ074, an effective xylitol production with glycerol as co-substrate at 42°C reported in the previous study [20], the *Ku70* was deleted for increasing gene integration efficiency to obtain YZB152 [19]. After that, the *Erg5* deletion strain YZB414, *Erg6* overexpression strain YZB453, and both deleting *Erg5* and overexpression *Erg6* strain YZB452

were constructed. When fermented at 46ºC in 250-mL flasks containing 50 mL medium for xylitol production, the growth conditions of YZB152, YZB414, and YZB452 are similar, with OD values of 1.91, 1.91, and 2.07, respectively. Among them, the YZB453 has the strongest growth ability, with an OD value of 2.6 (Fig. S3A). Compared with the YZB152, the xylose consumption abilities of YZB414 and YZB452 reduced by 9.62% and 16.95%, respectively, while, YZB453 increased by 16.53%. Similarly, the xylitol production abilities of YZB414 andYZB452 were weaker than YZB152, with xylitol yields of 4.32 g/L and 3.97 g/L, respectively, while YZB453 produced the highest xylitol titer of 5.57 g/L, which is 1.17 times that of the YZB152 (Fig. S3C). The parent strain YZB074 overexpressed xylose reductase (XR) from *Neurospora crassa*, which is one of the most active XRs characterized thus far [13]. Furthermore, the optimal catalytic temperature of *Nc*XR is between 45 and 55 °C [28], which is not achievable by mesophilic yeast. So, after enhancing the high-temperature stability of the host, the catalytic efficiency of *Nc*XR was also improved at elevated temperatures, and enabling the engineered strain to synthesize more xylitol than the parent strain above 45 °C.

Fermentation with YZB453 using a fermenter at elevated temperature for xylitol production

As shown in Fig. 5; Table 1, using 100 g/L xylose and 40 g/L glycerol in a fermenter at 45 °C, YZB152 produced 62.83 g/L xylitol with 42.17 g/L xylose residual, while YZB453 utilized almost all the xylose and produced 101.22 g/L xylitol, which was 61.10% higher than YZB152 produced (Fig. 5A and B; Table 1). When the xylose and glycerol concentrations were improved to 150 and 60 g/L, although both fermentation of YZB152 and YZB453 were inhibited, and produced 57.41 and 110.99 g/L xylitol, respectively (Fig. S4A and S4B, Table 1) the xylitol production of YZB453 was still 93.33% higher than YZB152 produced. In another experiment using 2.5 g/L xylose and 1 g/L glycerol in a fermenter at 46 °C, the fermentation of YZB152 was almost completely inhibited and produced 1.36 g/L xylitol with 2.28 g/L xylose residual, while YZB453 utilized almost all the xylose and produced 2.36 g/L xylitol (Fig. 5C and D; Table 1). Further improving the xylose to 50 g/L and 75 g/L and fermented at 46 °C, YZB453 still could use all the xylose out and produced 47.29 g/L and 75.11 g/L xylitol with productivity of 1.24 and 1.56 g/L/h (Fig. 5E and F; Table 1). When the xylose and glycerol concentrations were improved to 100 and 40 g/L, YZB453 was inhibited (Fig. S4C, Table 1).

Higher temperatures during SSF promoted saccharification and enhanced the synthesis of xylitol

Using the YZB453 strain to perform SSF on corncob residue and hydrolysate at 42 °C and 46 °C, as shown in Fig. 6, when the temperature is lower (42 °C), the rate of saccharification is slower than the rate of xylose utilization by yeast, leading to insufficient substrate utilization and reduced xylitol production (21.28 g/L) (Fig. 6A). However, when the temperature is increased to 46 °C, the rate of saccharification is significantly improved, allowing the substrate to be fully utilized and converted into xylitol (45.98 g/L) (Fig. 6B).

Discussion

The different resistances of various yeast strains to the three antifungal drugs, amphotericin B, pimaricin, and nystatin, are attributed to changes in the composition of sterols in the yeast cell membrane, as gene knockouts lead to varying degrees of changes in the sterols on the cell membrane. As shown in Fig. 1A and D, knocking out *Erg4* increased the resistance of yeast to amphotericin, because yeast accumulates epista-5,7,22,24(28)-trienol instead of ergosterol, the target of amphotericin. Similarly, knocking out *Erg5* causes the yeast to accumulate epista-5,7,24(28)-trienol instead of epista-5,7,22,24(28)-trienol and ergosterol, resulting in significantly enhanced resistance to amphotericin B in the *erg5Δ* strain. After knocking out *Erg3*, the yeast can still synthesize ergosterol through a compensatory pathway, despite the small quantity (Fig. 1D), so the *erg3Δ* strain remains sensitive to amphotericin but cannot synthesize the target of pimaricin, epista-5,7,24(28)-trienol, resulting in significantly enhanced resistance to pimaricin in the *erg3Δ* strain. Strains with the *Erg2* and *Erg6* genes knocked out cannot synthesize the target of amphotericin, ergosterol, the target of pimaricin, epista-5,7,24(28)-trienol, or the target of nystatin, episterol. Therefore, these two strains exhibit extremely strong resistance to the three antifungal drugs (Fig.s 1A and 1D). The strong resistance to azole drugs exhibited by all strains was attributed to *K. marxianus* has strong sterol synthesis ability, and the resistance to azole drugs is positively correlated with the yeast's sterol synthesis ability [29]. Additionally, antifungal drugs such as ketoconazole and fluconazole primarily target key enzymes on the fungal cell membrane, such as CYP51A1 (Erg11) and the cytochrome P450 enzyme system. They inhibit the activity of these enzymes to block the biosynthesis of ergosterol, thereby disrupting the fungal cell membrane and inhibiting the growth and reproduction of fungi, which suggesting that *K. marxianus* can bypass the inhibited pathway to synthesize ergosterol through paralogous proteins [30]. Currently, there are also many studies on drug tolerance in different yeast strains. Previous study reported that the *Erg6* knockout mutant of *Kluyveromyces lactis* exhibited enhanced resistance to amphotericin B, nystatin, and pimaricin, consistent with the results of this study. However, the *Erg6* knockout mutant of *K. lactis* is more sensitive to azole

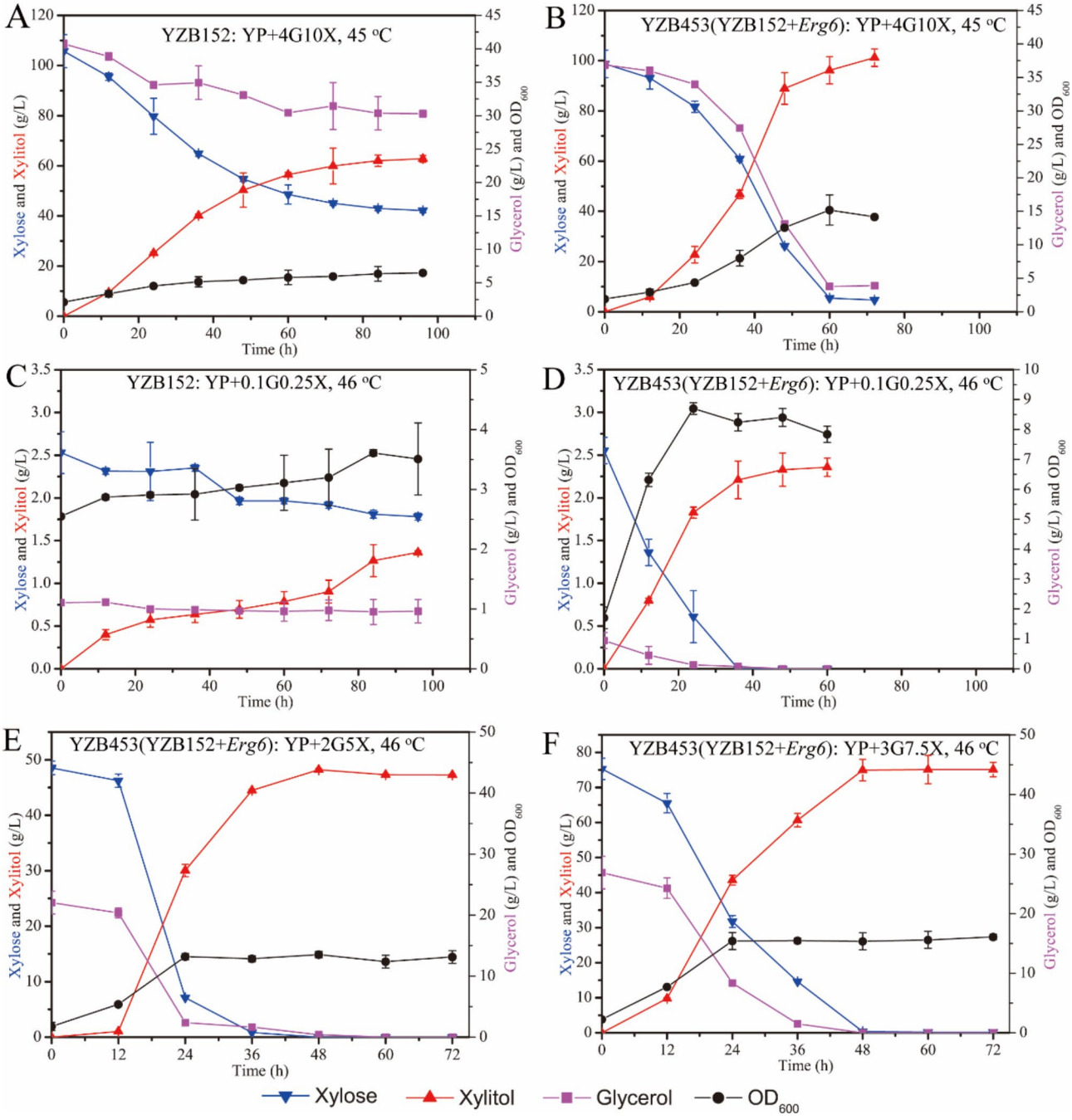

Fig. 5 (**A**) YZB152 was fermented with a fermenter at 45°C using the YP medium containing 40 g/L glycerol and 100 g/L xylose. (**B**) YZB453 was fermented with a fermenter at 45°C using YP medium containing 40 g/L glycerol and 100 g/L xylose. (**C**) YZB152 was fermented with a fermenter at 46°C using the YP medium containing 1 g/L glycerol and 2.5 g/L xylose. (**D**) YZB453 was fermented with a fermenter at 46°C using the YP medium containing 1 g/L glycerol and 2.5 g/L xylose. (**E**) YZB453 was fermented with a fermenter at 46°C using the YP medium containing 20 g/L glycerol and 50 g/L xylose. (**F**) YZB453 was fermented with a fermenter at 46°C using the YP medium containing 30 g/L glycerol and 75 g/L xylose. Data are shown as the mean ± standard deviation from at least three experiments. The bars in the figures indicate the ranges of standard deviation

antifungal drugs (fluconazole, itraconazole, ketoconazole, and miconazole), indicating differences in sterol synthesis ability between these two closely related yeasts [31]. In *Schizosaccharomyces pombe*, although no significant defects in endocytosis were observed in ergosterol knockout mutants, they also exhibit strong resistance to polyene drugs.

Similarly, after knocking *Erg5* out, the sterol synthesized by yeast probably changed from ergosterol to epista-5,7,24(28)-trienol, which improved the

Table 1 Performance of *K. marxianus* strains YZB152 and YZB453 in the conversion of xylose to xylitol at elevated temperatures

Strains	Initial substrate concentrations		Temperatures (°C)	Xylitol production (g/L)	Xylitol productivity (g/L/h)	Xylitol yield (g/g)
	Glycerol (g/L)	Xylose (g/L)				
YZB152	40.73	105.74	45	62.83	0.87	0.59
YZB453	36.88	99.72	45	101.22	1.41	1.01
YZB152	57.78	167.36	45	57.41	0.80	0.34
YZB453	57.06	173.68	45	110.99	1.54	0.64
YZB152	1.10	2.53	46	1.36	0.06	0.54
YZB453	0.94	2.38	46	2.36	0.98	0.99
YZB453	22.05	48.54	46	47.29	0.99	0.98
YZB453	26.88	75.29	46	75.11	1.56	0.99
YZB453	37.03	101.39	46	66.14	1.10	0.65

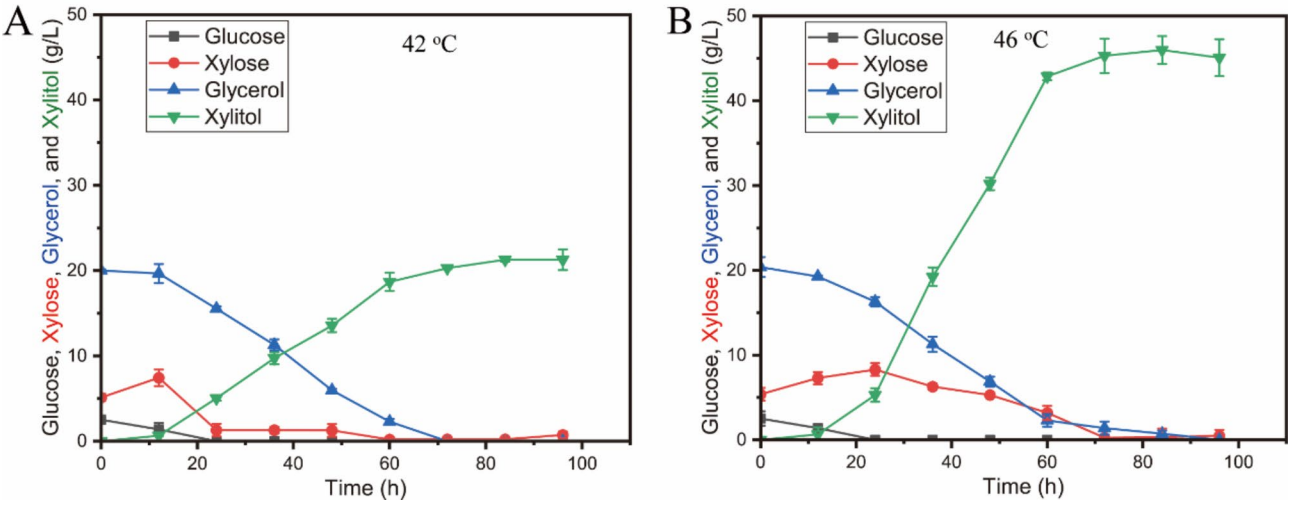

Fig. 6 Xylitol producing with YZB453 at elevated temperatures through SSF at 42°C (**A**) and 46°C (**B**). Data are shown as the mean ± standard deviation from at least three experiments. The bars in the figures indicate the ranges of standard deviation

thermotolerance of *K. marxianus*, consistent with previous reports in *S. cerevisiae* and *P. oxalicum* (Fig. 1) [21].A comparison of Figs. 2B and 3D reveals that knocking out *Erg5* and overexpressing *Erg5* both enhanced the thermotolerance of *K. marxianus*, which seems contradictory. It is speculated that this is achieved through different mechanisms. When *Erg5* was knocked out, the yeast cell membrane accumulated more epista-5,7,24(28)-trienol, which contains more unsaturated bonds than ergosterol (Fig. 1), neutralizing the effect of increased membrane fluidity under high temperature [1]. Moreover, knocking out *Erg5* reduces yeast's consumption of NADPH, leaving more NADPH to eliminate the damage caused by the rise of oxygen (ROS) radicals due to high-temperature stress [1]. On the other hand, overexpressing *Erg5*, while maintaining the original sterol homeostasis of *K. marxianus*, increases the content of epista-5,7,22,24(28)-trienol, the sterol with the most unsaturated bonds (Fig. 1A), which can more effectively neutralize the effect of increased membrane fluidity under high temperature.

For hyperosmotic stress, the *Erg6* knockout strain showed stronger hyperosmotic tolerance on plates, but liquid culture showed that the *Erg6* overexpressing strain had stronger tolerance to hyperosmotic stress, while the *Erg6* deletion strain was more sensitive, which seemed to contradict the results in Fig. 4A. It is speculated that this phenomenon occurs because the deletion of *Erg6* has a significant impact on the normal growth of *K. marxianus* (Fig. 2B). Although the deletion of *Erg6* enhances the hyperosmotic tolerance of *K. marxianus* on SD plates, this advantage is overshadowed by the rapid growth of the WT strain during liquid culture. The reason for the enhanced hyperosmotic tolerance of the *Erg6* overexpressing strain is different from that of the *Erg6* deletion strain. It is due to the generation of smaller perturbations in normal cellular membrane sterol levels, rather than the complete alteration of cellular membrane sterol species caused by *Erg6* deletion (Fig. 1D).

Compared with the YZB152, the xylose consumption abilities of YZB414 and YZB452 was reduced by 9.62% and 16.95%, respectively, and the xylitol production

abilities of YZB414 andYZB452 were weaker than YZB152, with xylitol yields of 4.32 g/L and 3.97 g/L, respectively (Fig. S3C). This result seems to be inconsistent with the growth curve in Figs. 2 and 3, and the possible reasons are speculated to be that the overexpression of *Erg6* increased the thermotolerance of *K. marxianus* by increasing the sterol synthesis based on maintaining the steady state of sterols, thus minimizing the negative effects of other stresses on the strain faced, and the deletion of *Erg5* increased the thermotolerance of *K. marxianus* by destroying the steady state of sterols and thus decreased the robustness of the engineering strain in fermentation (Fig. S3). Thus, yeast overexpressed *Erg6* enhanced thermotolerance was reported for the first time in this study and YZB453 was chosen for further study.

K. marxianus was proved to be an ideal host for xylitol production at high temperatures [13, 19, 26]. However, the effective temperature for xylitol production was 42ºC in these studies, when the temperature reached 45ºC, the xylitol yields sharply declined, although *K. marxianus* was reported to grow at elevated temperatures above 45ºC. That is because industrial fermentation faced multiple stresses such as hyperosmotic, oxygen supply, or acid et al., which decreased the thermotolerance of microorganisms. On the other hand, the fermentation temperature reached to 45ºC is very important for SSF, because the optimal temperature of cellulase and hemicellulase for the hydrolysis of cellulose and hemicellulose is usually above 45ºC [22]. Thus, xylitol production at a temperature above 45ºC was important for the biological synthesis of xylitol instead of the chemical method [32]. There have been some reports about xylitol production at elevated temperatures, such as *K. marxianus* KCTC17555 produced 63.76 g/L xylitol at 40ºC [32], *Kluyveromyces sp.* IIPE453 produced 11.5 g/L xylitol at 50ºC [33], *Debaryomyces hansenii* produced 68.6 g/L xylitol at 40ºC [34], *K. marxianus* YZJ015 produced 60.03 g/L xylitol at 45ºC, and *K. marxianus* CICC 1727-5 produced 24.2 xylitol at 40ºC [35]. However, the results in these studies were unable to maintain the xylitol yield while increasing temperature, and the highest xylitol productions were obtained in this study with engineering *K. marxianus* strain YZB453 for temperature above 45ºC (101.22 g/L xylitol at 45ºC and 75.11 g/L xylitol at 46ºC). Therefore, SSF at higher temperatures was performed, which can significantly reduce the cost of biomass enzymatic degradation. Corncob hydrolysate typically generates inhibitors such as furfural, 5-HMF, and acetic acid. The biosynthetic strains of xylitol also face high osmotic stress from substrates and products. Additionally, SSF often requires operation at higher temperatures. However, in this study, overexpressing the *Erg6* gene alone in *K. marxianus* can comprehensively enhance its thermal, hypertonic, and acid tolerance, although reduces its ethanol tolerance. Strains constructed in this study promoted green biosynthesis as a substitute for chemical synthesis.

Conclusions

Robust industrial strains of *K. marxianus*, constructed through sterol engineering, were reported in this study. The deletion of *Erg2* and *Erg6* increased *K. marxianus*'s resistance to amphotericin B, pimaricin, and nystatin, while the deletion of *Erg4* had almost no impact. The deletion of *Erg3* increased the sensitivity of *K. marxianus* to amphotericin B and nystatin but significantly enhanced its resistance to pimaricin. In contrast, the deletion of *Erg5* significantly enhanced its resistance to amphotericin B and slightly increased resistance to nystatin. Additionally, all strains demonstrated strong resistance to azole drugs. Unlike in *S. cerevisiae*, where the disruption of *Erg3* improved its thermotolerance, the deletion of *Erg2-Erg6* increased the sensitivity of *K. marxianus* to high temperatures, except for *Erg5*, which increased its thermotolerance. The overexpression of *Erg6* increased the acid, thermal, and hypertonic tolerance of *K. marxianus*, while the overexpression of *Erg4* increased only acid tolerance. The overexpressing *Erg6* strain YZB453 produced 101.22 g/L xylitol at 45 °C and 75.11 g/L xylitol at 46 °C, which is the highest xylitol titer obtained by biological fermentation above 45 °C. YZB453 produced 45.98 g/L xylitol by SSF with corncob residue and hydrolysate at 46 °C. This approach could be applied to enhance the robustness of *K. marxianus* for the production of more high-value-added products using biomass through SSF.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02546-3.

Supplementary Material 1

Acknowledgements

This research was supported by the Huaibei Normal University.

Author contributions

LR, BZ and FL developed the initial concept. LR and BZ wrote the manuscript and conducted experiments. LR and HZ conducted the experiments with the supervision of QZ, YX, JL, ZH and XT. RL and BZ supervised the study, revised the manuscript, and coordinated the project. DX and FL revised/edited the manuscript.

Funding

This work was supported by a Grant-in-Aid from the National Natural Science Foundation of China (22378156, 32102136), the Outstanding Youth Fund Project of Natural Science Foundation of Colleges and Universities in Anhui Province (2023AH020039), the Outstanding Young Talents Support Program in Colleges and Universities of Anhui Province (gxyqZD2022043), the Anhui Province University Innovation Team Project (2023AH010045), and the Innovation and Entrepreneurship Fund for the College Students (202310373029).

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Anhui Province Key Laboratory of Pollutant Sensitive Materials and Environmental Remediation, School of Life Sciences, Huaibei Normal University, Huaibei 235000, Anhui, P. R. China

Received: 23 July 2024 / Accepted: 29 September 2024
Published online: 10 October 2024

References

1. Caspeta L, Chen Y, Ghiaci P, Feizi A, Buskov S, Hallstrom BM, Petranovic D, Nielsen J. Altered sterol composition renders yeast thermotolerant. Science. 2014;346:75–8. https://doi.org/10.1126/science.1258137.
2. Guan NZ, Li JH, Shin HD, Du GC, Chen J, Liu L. Microbial response to environmental stresses: from fundamental mechanisms to practical applications. Appl Microbiol Biotechnol. 2017;101:3991–4008. https://doi.org/10.1007/s00253-017-8264-y.
3. Yu JX, Zhang Y, Liu H, Liu YX, Mohsin A, Liu ZB, Zheng YN, Xing JM, Han J, Zhuang YP et al. Temporal dynamics of stress response in *Halomonas elongata* to NaCl shock: physiological, metabolomic, and transcriptomic insights. Microbial Cell Factories. 2024, 23:88. https://doi.org/8810.1186/s12934-024-02358-5.
4. Asefi S, Nouri H, Pourmohammadi G, Moghimi H. Comprehensive network of stress-induced responses in *Zymomonas mobilis* during bioethanol production: from physiological and molecular responses to the effects of system metabolic engineering. Microbial Cell Factories. 2024, 23:180. https://doi.org/18010.1186/s12934-024-02459-1.
5. Huber G, Iborra S, Corma A. Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering. Chem Rev. 2006;106:4044–98. https://doi.org/10.1021/cr068360d.
6. Baptista M, Cunha JT, Domingues L. Establishment of *Kluyveromyces marxianus* as a microbial cell factory for lignocellulosic processes: production of high value furan derivatives. J Fungi. 2021, 7:1047. https://doi.org/104710.3390/jof7121047.
7. Singh AK, Deeba F, Kumar M, Kumari S, Wani SA, Paul T, Gaur NA. Development of engineered *Candida tropicalis* strain for efficient corncob-based xylitol-ethanol biorefinery. Microbial Cell Factories. 2023, 22:201. https://doi.org/20110.1186/s12934-023-02190-3.
8. Salli K, Lehtinen MJ, Tiihonen K, Ouwehand AC. Xylitol's health benefits beyond dental health: A comprehensive review. Nutrients. 2019, 11:1813. https://doi.org/181310.3390/nu11081813.
9. Nadimi H, Wesamaa H, Janket SJ, Bollu P, Meurman JH. Are sugar-free confections really beneficial for dental health? Br Dent J. 2011;211:E15. https://doi.org/10.1038/sj.bdj.2011.823.
10. Benahmed AG, Gasmi A, Arshad M, Shanaida M, Lysiuk R, Peana M, Pshyk-Titko I, Adamiv S, Shanaida Y, Bjorklund G. Health benefits of xylitol. Appl Microbiol Biotechnol. 2020;104:7225–37. https://doi.org/10.1007/s00253-020-10708-7.
11. Ahuja V, Macho M, Ewe D, Singh M, Saha S, Saurav K. Biological and pharmacological potential of xylitol: A molecular insight of unique metabolism. Foods 2020, 9:1592. https://doi.org/159210.3390/foods9111592.
12. IMARC. Xylitol market report by form (solid, liquid), application (chewing gum, confectionery, pharmaceutical and personal care, and others), and region 2024–2032. The International Market Analysis Research and Consulting Group 2024:1450918. https://www.imarcgroup.com/xylitol-market
13. Zhang J, Zhang B, Wang DM, Gao XL, Hong J. Xylitol production at high temperature by engineered *Kluyveromyces marxianus*. Bioresour Technol. 2014;152:192–201. https://doi.org/10.1016/j.biortech.2013.10.109.
14. Carneiro C, de Paula ESFC, Almeida JRM. Xylitol production: identification and comparison of new producing yeasts. Microorganisms. 2019;7:7110484. https://doi.org/10.3390/microorganisms7110484.
15. Baptista SL, Cunha JT, Romani A, Domingues L. Xylitol production from lignocellulosic whole slurry corn cob by engineered industrial *Saccharomyces cerevisiae* PE-2. Bioresour Technol. 2018;267:481–91. https://doi.org/10.1016/j.biortech.2018.07.068.
16. Lane M, Morrissey J. *Kluyveromyces marxianus*: A yeast emerging from its sister's shadow. Fungal Biol Rev. 2010;24:17–26. https://doi.org/10.1016/j.fbr.2010.01.001.
17. Karim A, Gerliani N, Aïder M. *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology. Int J Food Microbiol 2020, 333:108818. https://doi.org/10881810.1016/j.ijfoodmicro.2020.108818.
18. Baptista M, Domingues L. *Kluyveromyces marxianus* as a microbial cell factory for lignocellulosic biomass valorisation. Biotechnol Adv 2022, 60:108027. https://doi.org/10802710.1016/j.biotechadv.2022.108027.
19. Ren LL, Liu YY, Xia YT, Huang Y, Liu Y, Wang YM, Li PF, Chang KC, Xu DY, Li F, Zhang B. Improving glycerol utilization during high-temperature xylitol production with *Kluyveromyces marxianus* using a transient clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 system. Bioresour Technol. 2022. https://doi.org/10.1016/j.biortech.2022.128179.
20. Zhang J, Zhang B, Wang DM, Gao XL, Hong J. Improving xylitol production at elevated temperature with engineered *Kluyveromyces marxianus* through over-expressing transporters. Bioresour Technol. 2015;175:642–5. https://doi.org/10.1016/j.biortech.2014.10.150.
21. Liu G, Chen Y, Faergeman NJ, Nielsen J. Elimination of the last reactions in ergosterol biosynthesis alters the resistance of *Saccharomyces cerevisiae* to multiple stresses. FEMS Yeast Res. 2017;17:fox063. https://doi.org/10.1093/femsyr/fox063.
22. Zhang B, Ren L, Wang Y, Xu D, Zhang S, Wang HN, Wang H, Zeng X, Xin B, Li F. Glycerol production through *TPI1* defective *Kluyveromyces marxianus* at high temperature with glucose, fructose, and xylose as feedstock. Biochem Eng J. 2020;161:107689. https://doi.org/10.1016/j.bej.2020.107689.
23. Zhang B, Ren L, Zeng S, Zhang S, Xu D, Zeng X, Li F. Functional analysis of *PGI1* and *ZWF1* in thermotolerant yeast *Kluyveromyces Marxianus*. Appl Microbiol Biotechnol. 2020;104:7991–8006. https://doi.org/10.1007/s00253-020-10808-4.
24. Liu YY, Ren LL, Zhao JY, Xia YT, Zhang ZY, Guan XY, Huang SR, Wang Q, Wu J, Yu ZJ, et al. Ergosterol production at elevated temperatures by Upc2-overexpressing *Kluyveromyces marxianus* using Jerusalem artichoke tubers as feedstock. Bioresour Technol. 2022;362:127878. https://doi.org/10.1016/j.biortech.2022.127878.
25. Zhang B, Li LL, Zhang J, Gao XL, Wang DM, Hong J. Improving ethanol and xylitol fermentation at elevated temperature through substitution of xylose reductase in *Kluyveromyces marxianus*. J Ind Microbiol Biotechnol. 2013;40:305–16. https://doi.org/10.1007/s10295-013-1230-5.
26. Zhang B, Zhang J, Wang DM, Han RX, Ding R, Gao XL, Sun LH, Hong J. Simultaneous fermentation of glucose and xylose at elevated temperatures co-produces ethanol and xylitol through overexpression of a xylose-specific transporter in engineered *Kluyveromyces marxianus*. Bioresour Technol. 2016;216:227–37. https://doi.org/10.1016/j.biortech.2016.05.068.
27. Zhang B, Zhu YL, Zhang J, Wang DM, Sun LH, Hong J. Engineered *Kluyveromyces marxianus* for pyruvate production at elevated temperature with simultaneous consumption of xylose and glucose. Bioresour Technol. 2017;224:553–62. https://doi.org/10.1016/j.biortech.2016.11.110.
28. Woodyer R, Simurdiak M, van der Donk WA, Zhao H. Heterologous expression, purification, and characterization of a highly active xylose reductase from *Neurospora crassa*. Appl Environ Microbiol. 2005, 71:1642–1647. https://doi.org/71/3/1642 [pii]. 1128/AEM. 71.3.1642-1647.2005.
29. Yang H, Tong J, Lee CW, Ha S, Eom SH, Im YJ. Structural mechanism of ergosterol regulation by fungal sterol transcription factor Upc2. Nat Commun. 2015;6:6129. https://doi.org/10.1038/Ncomms7129.
30. Johnston EJ, Moses T, Rosser SJ. The wide-ranging phenotypes of ergosterol biosynthesis mutants, and implications for microbial cell factories. Yeast. 2020;37:27–44. https://doi.org/10.1002/yea.3452.
31. Konecna A, Hervay NT, Bencova A, Morvova M, Sikurova L, Jancikova I, Gaskova D, Gbelska Y. *Erg6* gene is essential for stress adaptation in *Kluyveromyces lactis*. Fems Microbiol Lett 2018, 365. https://doi.org/10.1093/femsle/fny265

32. Park JB, Kim JS, Kweon DH, Kweon DH, Seo JH, Ha SJ. Overexpression of endogenous xylose reductase enhanced xylitol productivity at 40°C by thermotolerant yeast. Appl Microbiol Biotechnol. 2019;189:459–70. https://doi.org/10.1007/s12010-019-03019-9.
33. Kumar S, Singh SP, Mishra IM, Adhikari DK. Ethanol and xylitol production from glucose and xylose at high temperature by *Kluyveromyces* Sp IIPE453. J Ind Microbiol Biotechnol. 2009;36:1483–9. https://doi.org/10.1007/s10295-009-0636-6.
34. Prakash G, Varma AJ, Prabhune A, Shouche Y, Rao M. Microbial production of xylitol from D-xylose and sugarcane bagasse hemicellulose using newly isolated thermotolerant yeast *Debaryomyces hansenii*. Bioresour Technol. 2011;102:3304–8. https://doi.org/10.1016/j.biortech.2010.10.074.
35. Du C, Li Y, Zong H, Yuan T, Yuan W, Jiang Y. Production of bioethanol and xylitol from non-detoxified corn cob via a two-stage fermentation strategy. Bioresour Technol. 2020;310:123427. https://doi.org/10.1016/j.biortech.2020.123427.

Publisher's note