Effect of Evolutionary Adaptation on Metabolic Enzyme Activities of Thermotolerant Strain *Kluyveromyces Marxianus* NIRE-K1 and NIRE-K3 for Bioethanol Production

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Abstract

Evolutionary adaptation provides stability to the strains in the challenging environment. As extension of earlier study, the evolved strains *Kluyveromyces marxianus* NIRE-K1.1 and *K. marxianus* NIRE-K3.1 were subjected for secondary adaptation on minimal salt (MS) medium with the aim to enhance xylose utilization for ethanol production together with salt tolerance. Both the strains were adapted till saturated improvement in xylose uptake i.e., 54 generations on MS medium containing xylose. Xylose utilization increased from 14.21 to 45.80% and 10.55 to 45.31%, in evolved strains KmNIRE-K1.2 and KmNIRE-K3.2, respectively. Specific xylose reductase activity has also increased 2.04 and 3.36-folds in KmNIRE-K1.2 and KmNIRE-K3.2, respectively. Xylitol dehydrogenase activity was also increased by 2.82 and 1.35-folds in KmNIRE-K1.2 and KmNIRE-K3.2, respectively. Decrease in redox imbalance was observed in evolved strains, and hence there was a reduction in xylitol production during growth and fermentation. Xylose uptake rate increased by 2.53 and 1.5-folds in KmNIRE-K1.2 and KmNIRE-K3.2, respectively with 2.20 and 6.46-folds higher ethanol concentration, and 2.25 and 5.86-folds higher volumetric productivity, respectively. This study has demonstrated the role of evolutionary adaptation for developing robust yeast strains. KmNIRE-K1.2 and KmNIRE-K3.2 have shown enhanced ethanol production, enzyme activities and less by-product formation like xylitol during xylose metabolism.

Background

Efficient conversion of lignocellulosic biomass to transportation fuels such as bioethanol is a major concern of biofuel researchers since last three decades for economic and environmental sustainability (Arora et. al., 2019; Sharma et. al., 2019). Although, many process have been developed for cellulosic ethanol production but their economic viability is still unknown due to incomplete conversion of biomass to valuable products (Achinas et. al., 2019). Lignocellulosic biomass contains C6 and C5 sugars including glucose and xylose as major sugar i.e. 90% of total sugar present and simultaneous conversion of both sugars into ethanol is still a loophole (Rodrussamee et. al. 2018).

Simultaneous saccharification and fermentation (SSF) is one of the integrated approaches in lignocellulosic ethanol production has various advantages i.e., high productivity, avoids cellulases inhibition due to glucose accumulation, etc. However, the optimal temperatures of maximum cellulases activity and fermentation by conventional yeast *Saccharomyces cerevisiae* and other mesophilic yeasts are about 50°C and 30°C, respectively, and have wide difference to perform in a single step (Zhang et. al., 2015).

Nowadays thermotolerant ethanologenic yeast *K. marxianus* has attracted increasing attention due to its thermotolerance and broad range of substrate specificity including xylose (Sharma et. al., 2016; 2017). It can grow up to 50°C and produce ethanol while most of the yeasts cannot survive at this temperature (Zhang et. al., 2015). Various genetic engineering efforts have been applied on *K. marxianus* including heterologously expression of *XYL1* and *XYL2* genes in xylose utilization pathways from *S. stipitis* or *XYL1* from *Orpinomyces sp.* (Zhang et. al., 2015; Wang et. al., 2013). However, the ethanol yield and

productivity of these strains at higher temperature are observed to be very low on xylose as carbon source.

In our previous studies, we have reported the evolutionary adaptation of *K. marxianus* NIRE-K1 and *K. marxianus* NIRE-K3 in rich medium with xylose as carbon source (YEPX) (Sharma et. al., 2016; 2017). However, the xylose assimilation of adapted strains was not significant on minimal salt medium during fermentation which is necessary for economical process. In the present study, we further adapted both the primary adapted cultures *K. marxianus* NIRE-K1.1 and *K. marxianus* NIRE-K3.1 in minimal salt medium containing xylose as carbon source and developed *K. marxianus* NIRE-K1.2 and *K. marxianus* NIRE-K3.2 with enhanced xylose utilization and ethanol fermentation capability. The effect of secondary evolutionary adaptation on the activity of metabolic enzymes involved in xylose assimilation including, XR, and XDH is explored. The changes in gene expression level of *XYL1* and *XYL2* genes and co-enzyme quantification are also examined. Further, the growth and fermentation kinetics of primary and secondary adapted strains are compared.

Materials And Methods

Microorganisms and media

Primary adapted strains thermotolerant *K. marxianus* NIRE-K1.1 (KmNIRE-K1.1) (MTCC 5933) and *K. marxianus* NIRE-K3.1 (KmNIRE-K3.1) were used for secondary evolutionary adaptation (Sharma et al. 2016; 2017). *Candida tropicalis* (MTCC 230) was procured from Microbial Type Culture Collection, CSIR-Institute of Microbial Technology, Chandigarh, India. Secondary adaptation of KmNIRE-K1.1 and KmNIRE-K3.1 was performed on minimal salt medium containing xylose as carbon source MSX1 [(g l⁻¹): yeast extract, 5; ammonium sulfate, 1.23; di-potassium hydrogen phosphate, 1.35; magnesium sulfate 1; sodium di-hydrogen phosphate, 1.05; xylose, 20; pH, 5.5] and MSX2 [(g l⁻¹): yeast extract, 4.81; ammonium sulfate, 1.99; di-potassium hydrogen phosphate, 1.10; magnesium sulfate, 0.95; sodium di-hydrogen phosphate, 1.05; xylose, 20; pH, 5.5], respectively (Arora et. al., 2017).

Evolutionary adaptation

Evolutionary adaptation in repetitive batch culture was carried out through sequential transfer in MSX medium in 100 ml of cotton plugged Erlenmeyer flask with 20 ml of medium. The initial batch was performed by culturing loopful cells from phytigel plate in Erlenmeyer flask and incubated for 24 h at 45°C in an Innova 43/43R Shaker (orbital shaker incubator) (New Brunswick, USA) at 150 rpm. Further, 1% inoculum was used for consecutive batch for subsequent growth. One ml sample was withdrawn after every 24h in each cycle for analysis of residual xylose, dry cell weight (DCW) and metabolites (ethanol and xylitol) produced during the growth. The adaptation was continued until no further improvement was observed in terms of xylose utilization and DCW formation.

Growth and fermentation condition

Growth study was carried out in a bioreactor of 3 L volume (NBS BioFlo-CelliGen 115) under aerobic condition with working volume of 2 L. Growth was performed for 24 h at 45°C for native and adapted culture. The samples were collected at an interval of 2 h and analyzed for DCW, xylose concentration and extracellular metabolites. Fermentation was carried out in NBS BioFlo-CelliGen 115 (New Brunswick, USA) bioreactor of 3 L volume under controlled temperature of 45°C and pH 5.5. Inoculum was prepared by growing the strains overnight in MSX medium at 45°C. Cells were harvested by centrifugation at 5000 × g for 10 min and used as inoculum for fermentation in bioreactor with similar composition as the growth medium except xylose concentration of 30 g xylose l⁻¹. Bioreactor was loaded with 2 L salt medium and sterilized at 121°C for 15 min. The initial cell mass concentration was kept at ~2.5 g cells l⁻¹.

Enzyme assay

The strains were grown in YEP medium containing 20 g xylose l⁻¹ and/or 20 g glucose l⁻¹ overnight at 45°C to gain high cell mass. Cells were harvested at 3000 × g for 10 min at 4°C in a centrifuge. Protein extraction was done by using Yeast Buster protein extraction reagent (Novagen, Germany) and procedure was followed according to manufacturer's instructions. Enzyme activity of xylose reductase (XR) and xylitol dehydrogenase (XDH) was determined according to Ikeuchi et al. (2000) using spectrophotometer at A₃₄₀ for KmNIRE-K1.1, KmNIRE-K1.2, KmNIRE-K3.1 and KmNIRE-K3.2 at variable temperatures ranging 30 to 50°C. One unit (U) of XR activity is defined as the amount of enzyme required to oxidize 1.0 μmole NADPH to NADP⁺ for reduction of D-xylose to xylitol in one min; and one unit (U) of XDH activity are defined as the amount of enzyme required to reduce 1.0 μmole NAD⁺ to NADH for oxidation of xylitol to xylulose in one min. Enzyme activities were calculated as per the formulae given by Ikeuchi et al. (2000):

$$\text{Volumetric activity (U l}^{-1}\text{)} = \frac{1000 \times TV \times dA/dt}{\epsilon \times V \times CF} \quad (1)$$

Where, *TV* is total volume; *V* is volume of cell extract, ϵ is molar extinction coefficient (6.22 l mmol⁻¹ for a path length of 1.0 cm); *CF* is dilution of cell extract.

$$\text{Specific activity (U mg}^{-1}\text{)} = \frac{\text{Volumetric activity (U l}^{-1}\text{)}}{\text{Protein concentration (mg l}^{-1}\text{)}} \quad (2)$$

Co-enzyme quantification

Intracellular co-enzyme concentrations were determined using the cells harvested from YEPX medium containing 2% xylose as carbon source in log phase. Two milliliter sample was withdrawn and cell pellets were washed with phosphate buffer (pH 7.0) followed by extraction of NADH/NAD⁺ and NADPH/NADP⁺ by using NADH/NAD⁺ and NADPH/NADP⁺ extraction buffers (Sigma Aldrich, USA), respectively, as manufacturer's instructions. The co-enzyme quantification was done calorimetrically at

A₄₅₀ in a UV-Vis spectrophotometer by using NADH/NAD⁺ and NADPH/NADP⁺ Kit (Sigma Aldrich, USA) by following the manufacturer's instructions.

Real Time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to determine relative expression of *XYL 1* and *XYL2* genes in KmNIRE-K1.1, KmNIRE-K1.2, KmNIRE-K3.1 and KmNIRE-K3.2, respectively. These strains were cultivated in YEP medium containing 20 g l⁻¹xylose under aerobic condition at 45°C. Total RNA was isolated by using Aurum total RNA kit (Biorad, USA) and purified according to manufacturer's instructions, followed by cDNA synthesised using iScript cDNA synthesis kit (Biorad, Canada). RT-PCR was performed in CFX connect real time system (Biorad, India) with iTaq universal syber green supermix (Biorad, Canada). *KmAtc1* encoding for actin was used as control. List of primers used in the gene expression analysis were reported in Table 1.

Analytical methods and kinetics

The concentration of xylose, ethanol, xylitol, glycerol and acetic acid were estimated using high-pressure liquid chromatography (HPLC) system (Agilent Technologies, USA) with HiPlex H column with 1mM H₂SO₄ as the mobile phase and flow rate of 0.7 ml min⁻¹ at 57°C and detected by refractive index detector (RID) at 50°C. Yeast cell concentration was measured through dry cell weight (DCW). Protein concentrations in cell extracts were estimated using Lowry's method. Growth and fermentation kinetic parameters were calculated using the formulae by Bailey and Ollis (Bailey et. al., 1986; Sharma et. al., 2016; 2017).

Table 1. List of primers used in the gene expression analysis

Gene	Orientation	Sequence (5'→3')	References
<i>KmXYI1</i>	Fwd-1	CAGAGCCCTTGAGAAGTTGG	Singori et al. 2014
	Rev-1	AAGGATAGTGGGCCGAAACT	
<i>KmXYI1</i>	Fwd-2	GAGAAATACCCTCCAGGTTTCTA	Zhang et al. 2011
	Rev-2	CCATTCCCAAGGGTCATTGAATC	
<i>KmXYI1</i>	Fwd-3	ATGACATACCTCGCACCAACA	Zhang et al. 2011
	Rev-3	TTAGATAAAGGTTGGGAATTCG	
<i>KmXYI2</i>	Fwd-1	GCTGTAGAGCCAGGTGTTCC	Singori et al. 2014
	Rev-1	CAAATCCAGTACCGGCAAGT	
<i>KmXYI2</i>	Fwd-2	ACCAACACTCAAAAAGCCG	Wang et al. 2013
	Rev-2	TGGACCATCAATGATAGTCT	
<i>KmXYI2</i>	Fwd-3	CCAATGGTTTTGGGTCACGAATC	Lulu et al. 2013
	Rev-3	CCTACAGGACCGGCACCAAAGAC	
<i>KmATC1</i>	Fwd-1	AGCACCCAGTTTTGTTGACC	Singori et al. 2014

Results And Discussion

As reported in our previous studies, KmNIRE-K1.1 and KmNIRE-K3.1 were developed through primary adaptation of wild type strains (Sharma et. al., 2016; 2017). The significant improvement was observed in adapted cultures, but still it was not significant in terms of xylose utilization and ethanol fermentation when grown in MSX. Hence, the primary adapted strains were further adapted in MSX medium for further improvement.

Evolutionary adaptation of KmNIRE-K1.1 and KmNIRE-K3.1

KmNIRE-K1.1 and KmNIRE-K3.1 were undergone secondary adaptation in MSX medium containing xylose as carbon source and improvement were monitored in the form of DCW and sugar concentrations in the broth after completion of each batch as shown in Fig. 1. There was almost 2-fold increase in xylose utilization in first 10 batches in both the cultures. KmNIRE-K1.1 and KmNIRE-K3.1 utilized approximately 50% and 45% of total sugar after 50 batches. After 50 batches, no significant improvement was observed in cell growth for both the cultures and hence, the sub-culturing was stopped after 54 cycles. There was 3.5-fold increase in xylose utilization by KmNIRE-K1.1 (Fig. 1A) and renamed the adapted culture as KmNIRE-K1.2, while, there was 4.5-fold increase in xylose utilization by KmNIRE-K3.1 (Fig. 1B) and renamed the adapted culture as KmNIRE-K3.2. DCW of KmNIRE-K1.2 increased exponentially and was about 4-fold increase, while, no significant improvement (1.17-fold) was observed in DCW in KmNIRE-K3.2. Martín et al. (2007) developed an adapted strain of genetically engineered *S. cerevisiae* through

cultivation on medium containing the inhibitors including aliphatic acids, furaldehydes and phenolic compounds for 353 h. Apart from the adaptation of culture on inhibitors, the xylose consumption also increased significantly through adaption. In another study, Liu et al. (2009) developed a strain *S. cerevisiae* NRRL Y-50049 by using an evolutionary adaption, which could detoxify furfural and HMF *in-situ* while producing ethanol. Similarly, Dhaliwal et al. (2011) adapted thermotolerant yeast *Pichia kudriavzevii* on galactose medium and observed 30% enhanced ethanol production from sugarcane juice. Similarly, the present study on the secondary adaptation of both strains in MSX medium proved to be a possible approach for enhanced xylose utilization.

Enzyme assays of adapted strains

The enzyme assays were conducted to determine the effect of adaptation on the activities of XR and XDH in both the secondary adapted cultures KmNIRE-K1.2 and KmNIRE-K3.2. The enzyme activities of XR and XDH were determined in the crude enzyme extracts obtained from cells grown in glucose, xylose, and glucose/xylose mixture separately. The enzyme activity was determined at different temperatures varying from 30 to 50°C to validate the thermostability of XR and XDH enzymes.

XR assay

XR activity for xylose grown KmNIRE-K1.2 was observed to be 1.14, 1.03, 1.24, 1.27 and 1.20-folds higher as compared to xylose grown KmNIRE-K1.1 at 30, 35, 40, 45 and 50°C, respectively, while, these activities were found to be 2.26, 1.61, 1.50, 1.58 and 1.28-folds higher in glucose/xylose mixture grown KmNIRE-K1.2 than that of glucose/xylose mixture grown KmNIRE-K1.1 at 30, 35, 40, 45 and 50°C, respectively (Fig. 2A). On comparing XR activities in KmNIRE-K1.2 grown in xylose and glucose/xylose mixture, it was found higher in glucose/xylose mixture at all the temperatures between 30-50°C with maximum activity at 50°C. XR activity in KmNIRE-K1.2 was about 1.34-fold higher in glucose/xylose mixture than xylose at 45°C. The higher XR activity in cells grown in glucose/xylose mixture might be due to up regulation of *XYL1* gene in the presence of glucose, which enhances xylose transport (Hamacher et. al., 2002).

Similarly, XR activities in KmNIRE-K3.2 were found to be 14.11, 2.39, 3.95, 3.37 and 1.04-folds higher than that of KmNIRE-K3.1 at 30, 35, 40, 45 and 50°C, respectively in xylose, while, these activities were 1.32, 1.26, 1.09 and 1.08-folds higher in KmNIRE-K3.2 than that of KmNIRE-K3.1 at 30, 40, 45 and 50°C, respectively when grown in glucose/xylose mixture (Fig. 2B). However, similar XR activity was observed in glucose/xylose mixture grown KmNIRE-K3.2 and KmNIRE-K3.1 at 35°C. Although, the maximum activity was were found at 50°C in glucose/xylose mixture grown KmNIRE-K3.2 (Fig. 2B). Unlike KmNIRE-K1.2, XR activity decreased 0.48-fold in KmNIRE-K3.2 when grown in glucose/xylose mixture as compared to xylose at 45°C.

Jeppsson et al. (2003) have obtained the activity 6.07 U mg^{-1} for XR in *S. cerevisiae* TMB2360 grown in xylose as sole carbon source. Similarly, Khattab et al. (2013) also estimated the activity XR in genetically modified *S. cerevisiae* strains SK-N2, SK-N3 and SK-N4 and found as 1.50, 2.52 and 1.11 U mg^{-1} ,

respectively when grown with xylose as carbon source. Further, activity of XR in thermotolerant *K. marxianus* YZJ088 has been reported as 0.35 U mg^{-1} (Zhang et al., 2015). Similarly, Dasgupta et al. (2016) have calculated an activity of XR in *Kluyveromyces sp.* IIPE453 as $1.036 \pm 0.006 \text{ U mg}^{-1}$, when grown in xylose.

Further, on comparing XR activities in both the xylose grown evolved strains, KmNIRE-K3.2 was found to be 1.53-fold higher than that of KmNIRE-K1.2 at 45°C. However, XR activity was found to be 1.79-fold higher in glucose/xylose mixture grown KmNIRE-K1.2 than that of in glucose/xylose mixture grown KmNIRE-K3.2 at 45°C.

XDH assay

XDH activities in KmNIRE-K1.2 were found to be 2.09, 2.75, 2.25, 2.83, 1.36-folds higher than that of KmNIRE-K1.1 at 30, 35, 40, 45 and 50°C, respectively in xylose (Fig. 2C). The maximum XDH activity KmNIRE-K1.2 was observed 0.65 U mg^{-1} at 45°C in xylose, whereas it was observed maximum 0.33 U mg^{-1} for KmNIRE-K1.1 at 50°C in xylose. On the other hand, XDH activities in KmNIRE-K1.2 were found as 1.28, 1.18, and 1.04-folds higher as compared to KmNIRE-K1.1 at 30, 45 and 50°C, respectively in glucose/xylose mixture. However, it was observed that XDH activities in KmNIRE-K1.2 were lower at 35 and 40°C than that of in KmNIRE-K1.1 in glucose/xylose mixture. The maximum XDH activity $1.34 \pm 0.04 \text{ U mg}^{-1}$ in was found at 45°C in KmNIRE-K1.2, while it was maximum $1.34 \pm 0.06 \text{ U mg}^{-1}$ at 40°C in KmNIRE-K1.1 in glucose/xylose mixture. On comparing XDH activities in xylose and glucose/xylose mixture grown KmNIRE-K1.2, the activity was higher in glucose/xylose mixture at all the temperatures between 30-50°C. XDH activity in KmNIRE-K1.2 was found to be 2.06-fold higher in glucose/xylose mixture than xylose at 45°C.

Similarly, XDH activities in KmNIRE-K3.2 was observed $1.04 \pm 0.01 \text{ U mg}^{-1}$, which was 1.35-fold higher than KmNIRE-K3.1 at 45°C in xylose (Fig. 2D). However, lower XDH activities were found in KmNIRE-K3.2 as compared KmNIRE-K3.1 at rest of temperatures. On the contrary, XDH activities in KmNIRE-K3.2 were found to be 2.45, 5.09, 3.83, 3.57 and 2.35-folds higher than that of KmNIRE-K3.1 at 30, 35, 40, 45 and 50°C, respectively in glucose/xylose mixture. However, the adapted strain showed maximum XDH activity $1.78 \pm 0.06 \text{ U mg}^{-1}$ at 35°C, whereas KmNIRE-K3.1 showed maximum activity at 30°C i.e. $0.64 \pm 0.04 \text{ U mg}^{-1}$ in. It was also observed that XDH activity KmNIRE-K3.2 was found to be 1.20-fold higher in glucose/xylose mixture than xylose at 45°C.

Yablochkova et al. (2003) have compared the activity of XDH in different native xylose utilizing yeast strains *Candida silvanoru* VGI-II, *C. tropicalis* Y-456, *K. marxianus* Y-488, *C. intermedia* TabII and *C. shehatae* Y-1632 and reported as 4.32, 1.16, 0.02, 3.83 and 13.95 U mg^{-1} , respectively. Goshima et al. (2013) have reported XDH activity as 1.11 U mg^{-1} for *K. marxianus* DMB3-7, grown in xylose as carbon source. Zhang et al. (2015) have obtained 0.45 U mg^{-1} XDH activity in genetically modified *K. marxianus*

YZJ088. Similarly, Zhang et al. (2016) has estimated $0.60 \pm 0.10 \text{ U mg}^{-1}$ XDH activity in modified yeast *S. cerevisiae* W32N55.

The increased XDH activities in the secondary adapted strains might be due to the combined effect of enhanced XR activity and decreased efflux of xylitol from cells. On comparing the activities of XDH in both the evolved strains, the activity of XDH in KmNIRE-K3.2 was found to be 1.6-fold higher than that of KmNIRE-K1.2 at 45°C in xylose. However, XDH activity in KmNIRE-K1.2 was found 1.1-fold higher than that of KmNIRE-K3.2 at 45°C in glucose/xylose mixture.

Co-enzyme quantification

Co-enzyme concentration was determined during the growth of KmNIRE-K1.1, KmNIRE-K1.2, KmNIRE-K3.1 and KmNIRE-K3.2 in xylose to ensure the improvement in redox balance. NADPH/NADP⁺ and NAD⁺/NADH ratio play an important role in xylose assimilation as the redox imbalance results xylitol efflux (Zhang et. al., 2015). XR oxidizes NADPH to NADP⁺ for conversion of xylose to xylitol and XDH reduces NAD⁺ to NADH to convert xylitol to xylulose, which is further phosphorylated to xylulose-5-phosphate by XK. KmNIRE-K1.2 showed improved concentrations of NAD⁺ and NADH i.e. 1814.09 ± 6.98 and $754.95 \pm 3.72 \text{ nmol g}^{-1} \text{ DCW}$, respectively, whereas, in KmNIRE-K1.1, these concentrations were 1052.64 ± 5.69 and $237.57 \pm 5.69 \text{ nmol g}^{-1} \text{ DCW}$, respectively (Table 2). Similarly, the concentrations of NAD⁺ and NADH were also increased in KmNIRE-K3.2 i.e., 1142.94 ± 5.92 and $1838.7 \pm 4.65 \text{ nmol g}^{-1} \text{ DCW}$, respectively, as compared to KmNIRE-K3.1, which were 714.77 ± 4.96 and $958.4 \pm 4.34 \text{ nmol g}^{-1} \text{ DCW}$, respectively (Table 2). Furthermore, the ratio of NAD⁺/NADH in KmNIRE-K1.2 was 2.4, whereas in KmNIRE-K1.1, it was 4.43. Similarly, there was a value of 0.62 of NAD⁺/NADH ratio in KmNIRE-K3.2, whereas, in KmNIRE-K3.1 it was 0.75. The decreased values of ratio of NAD⁺/NADH showed the better activity of XDH which converts more NAD⁺ to NADH for production of xylulose from xylitol. Hence, the secondary adapted strains had the better XDH activity in terms of co-factor ratio.

Table 2

NAD⁺/NADH and NADP⁺/NADPH quantifications of primary and secondary adapted strains.

Strain	NAD ⁺ (nmol g ⁻¹ DCW)	NADH (nmol g ⁻¹ DCW)	NAD ⁺ / NADH	NAD ⁺ + NADH (nmol g ⁻¹ DCW)	NADP ⁺ (nmol g ⁻¹ DCW)	NADPH (nmol g ⁻¹ DCW)	NADPH/ NADP ⁺	NADP ⁺ + NADPH (nmol g ⁻¹ DCW)
<i>K. marxianus</i> NIRE-K1.1	1052.64 ± 5.69	237.57 ± 5.69	4.43	1290.21	165.9 ± 5.29	94.26 ± 4.21	0.57	260.16
<i>K. marxianus</i> NIRE-K1.2	1814.09 ± 6.98	754.95 ± 3.72	2.4	2569.04	138.33 ± 2.69	62.71 ± 1.9	0.45	201.04
<i>K. marxianus</i> NIRE-K3.1	714.77 ± 4.96	958.4 ± 4.34	0.75	1673.17	82.99 ± 5.17	153.12 ± 2.67	1.85	236.11
<i>K. marxianus</i> NIRE-K3.2	1142.94 ± 5.92	1838.7 ± 4.65	0.62	2981.64	165.85 ± 4.14	105.71 ± 4.95	0.64	271.56

Concentrations of NADPH and NADP⁺ in KmNIRE-K1.2 were found to be 62.71 ± 1.9 and 138.33 ± 2.69 nmol g⁻¹ DCW, respectively, whereas, KmNIRE-K1.1 showed the concentrations of 62.71 ± 1.9 and 138.33 ± 2.69 nmol g⁻¹ DCW, respectively. Similarly, the concentrations of NADPH and NADP⁺ in KmNIRE-K3.2 were found to be 105.71 ± 4.95 and 165.85 ± 4.14 nmol g⁻¹ DCW, respectively, which were found to be 153.12 ± 2.67 and 82.99 ± 5.17 nmol g⁻¹ DCW, respectively in KmNIRE-K1.1. Moreover, the ratios of NADPH/NADP⁺ were 0.57 and 0.45 in KmNIRE-K1.1 and KmNIRE-K1.2, respectively and 1.85 and 0.64 in KmNIRE-K3.1 and KmNIRE-K3.2, respectively. The lower values of NADPH/NADP⁺ ratio and higher concentration of NADP⁺ showed the higher activities of XR in KmNIRE-K1.2 and KmNIRE-K3.2. Aforesaid results suggested that redox imbalance reduced together with decreased xylitol efflux in evolved strains KmNIRE-K1.2 and NIRE-K3.2. In the similar way, Zhang et al. (2016) engineered *K. marxianus* YZJ088 with NADPH preferring XR, NADP⁺ preferring XDH and NADH dependent GLN1 and reported the increased co-enzyme concentration by 6.72-fold as compared to parent strain *K. marxianus* NBRC1777. Moreover, NADH/NAD⁺ and NADPH/NADP⁺ ratios were also higher i.e. 0.89 and 0.80 than *K. marxianus* NBRC1777 i.e. 0.28 and 0.69, respectively Zhang et al., 2016. In another study, Wei et al. (2013), developed *FPS1* deleted strain *S. cerevisiae* SR8-fps1Δ to enhance ethanol production. SR8-fps1Δ showed 10 to 30 % enhanced ethanol yield together with increased NADP⁺/NADPH ratio as compared to parent strain *S. cerevisiae* SR8. However, NAD⁺/NADH ratio was similar as parent strain (Wei et al., 2013).

Comparative gene expression analysis of primary and secondary adapted strains

The expression of endogenous genes *XYL1* and *XYL2*, plays key role in xylose metabolism, were analysed using RT-PCR. The relative level of gene expression was determined between KmNIRE-K1.1 and KmNIRE-K1.2, and KmNIRE-K3.1 and KmNIRE-K3.2. The expression level of *XYL1* and *XYL2* in KmNIRE-K1.2 was 2.23 and 7.73-folds higher, respectively, than that of KmNIRE-K1.1 (Fig. 3A). Similarly, the expression level of *XYL1* and *XYL2* in KmNIRE-K3.2 were 2.53 and 6.99-folds higher, respectively, than that of KmNIRE-K3.1 (Fig. 3B). Increased expression of *XYL1* and *XYL2* in secondary adapted cultures KmNIRE-K1.2 and KmNIRE-K3.2 may be due to enhanced xylose uptake, which is supposed to trigger the mRNA synthesis.

The researchers have reported various studies of relative expression of *XYL1*, *XYL2* and other xylose metabolic genes. In another study, Qi et al. (2015), analysed gene expression of *XYLA* and *XKS1* of evolutionary adapted recombinant *S. cerevisiae* SyBE003 and reported 0.53 and 3-folds relative expression, respectively, than that of SyBE002. Biswas et al. (2013) investigated the effect of different carbon sources on the expression level of *XYL2* gene of *Debaryomyces hansenii* and reported 7.5-fold higher expression of *XYL2* gene in xylose grown cells as compared glucose grown cells. Similarly, Yamasaki-Yashiki et al. (2014) also reported higher expression of *XYL2* in *Rhizomucor pusillus* grown in xylose as compared to glucose. In another study, expression of *XYL1* in *R. pusillus* NBRC 4578 was found to be higher in the cells grown in xylose and arabinose than that of grown in glucose (Komeda et al., 2015).

Comparative growth profiling of primary and secondary adapted strains

Growth profiling of primary and secondary adapted cells of KmNIRE-K1 and KmNIRE-K3 were performed in 3L Bioflow Bioreactor containing 2L MSX at 45°C, 5.5 pH and 250 rpm. Growth pattern was drawn as shown in Fig. 4A and 4B. KmNIRE-K1.2 utilized 22.08 g xylose l⁻¹ in 22 h, whereas KmNIRE-K1.1 utilized the same amount of sugar in 28 h. A significant difference was observed in maximum specific growth rate between the KmNIRE-K1.2 ($0.22 \pm 0.02 \text{ h}^{-1}$) and KmNIRE-K1.1 ($0.04 \pm 0.02 \text{ h}^{-1}$), which was about 5.5-fold higher in KmNIRE-K1.2 as compared to KmNIRE-K1.1. Similarly, KmNIRE-K3.2 and KmNIRE-K3.1 utilized about 20 g xylose l⁻¹ in 16 and 22 h, respectively. However, KmNIRE-K3.2 and KmNIRE-K3.1 showed almost similar maximum specific growth rate, i.e. 0.18 ± 0.02 and $0.20 \pm 0.02 \text{ h}^{-1}$ (Table 3). On comparing the maximum specific growth rate of KmNIRE-K1.2 and KmNIRE-K3.2, it was found to be 1.22-fold higher in KmNIRE-K1.2 than that of KmNIRE-K3.2.

Table 3

Growth and fermentation kinetics of KmNIRE-K1.1, KmNIRE-K1.2, KmNIRE-K3.1 and KmNIRE-K3.2

Kinetic parameters	KmNIRE-K1.1	KmNIRE-K1.2	KmNIRE-K3.1	KmNIRE-K3.2
Growth				
Maximum specific growth rate ($\mu_{\max}$, h^{-1})	0.04 ± 0.02	0.22 ± 0.02	0.20 ± 0.02	0.18 ± 0.02
Specific sugar consumption rate (q_s , $\text{g g}^{-1} \text{h}^{-1}$)	0.19 ± 0.02	0.48 ± 0.038	0.32 ± 0.02	0.48 ± 0.01
Cell mass yield ($Y_{x/s}$, g g^{-1})	0.36 ± 0.005	0.26 ± 0.006	0.35 ± 0.002	0.30 ± 0.01
Fermentation				
Ethanol ($P_{ethanol}$, g l^{-1})	0.9 ± 0.02	1.98 ± 0.12	0.46 ± 0.02	2.97 ± 0.12
Xylitol ($P_{xylitol}$, g l^{-1})	nd	nd	2.35 ± 0.014	3.89 ± 0.06
Cell mass yield ($Y_{x/s}$, g g^{-1})	0.19 ± 0.03	0.072 ± 0.006	0.19 ± 0.02	0.08 ± 0.003
Ethanol yield ($Y_{p/s}$, g g^{-1})	0.14 ± 0.02	0.1 ± 0.006	0.06 ± 0.005	0.14 ± 0.006
Xylitol yield ($Y_{p/s}$, g g^{-1})	–	–	0.28 ± 0.02	0.19 ± 0.002
Volumetric substrate uptake (Q_s , $\text{g l}^{-1} \text{h}^{-1}$)	0.088 ± 0.01	0.28 ± 0.006	0.12 ± 0.007	0.28 ± 0.006
Volumetric product productivity (Ethanol) (Q_p , $\text{g l}^{-1} \text{h}^{-1}$)	0.012 ± 0.002	0.027 ± 0.002	0.007 ± 0.0003	0.041 ± 0.001
Volumetric product productivity (Xylitol) (Q_p , $\text{g l}^{-1} \text{h}^{-1}$)	–	–	0.03 ± 0.002	0.054 ± 0.001
Specific sugar consumption rate (q_s , $\text{g g}^{-1} \text{h}^{-1}$)	0.03 ± 0.003	0.085 ± 0.004	0.05 ± 0.01	0.085 ± 0.003
Specific product formation rate (Ethanol) (q_p , $\text{g g}^{-1} \text{h}^{-1}$)	0.004 ± 0.0001	0.008 ± 0.0001	0.003 ± 0.001	0.013 ± 0.001
Specific product formation rate (Xylitol) (q_p , $\text{g g}^{-1} \text{h}^{-1}$)	–	–	0.016 ± 0.01	0.015 ± 0.002

Not detected

Kinetic parameters	KmNIRE-K1.1	KmNIRE-K1.2	KmNIRE-K3.1	KmNIRE-K3.2
Conversion rate (%) into ethanol	28.84 ± 4.1	19.51 ± 1.8	11.56 ± 0.43	29.39 ± 0.5
Conversion rate (%) into xylitol	–	–	28.68 ± 0.28	19.19 ± 0.03
Not detected				

A shorter lag phase was observed in KmNIRE-K1.2 (6 h) as compared to KmNIRE-K1.1 (10 h). Similarly, KmNIRE-K3.2 also showed shorter lag phase i.e., 6 h than KmNIRE-K3.1 (8 h). The lag phase during microbial growth usually increases due to change in environment, which could be reduced through adaptation in the same media. Hence, reduction in lag phase after adaptation may be due to stabilization of regulatory mechanisms (Sharma et al., 2016; New et al., 2014). Variation in growth environment results slow metabolic activity, because transcription is not adapted for new changes (New et al., 2014). However, after adaptation in new environment microorganisms show improved growth i.e. shorter lag phase (Sharma et al., 2016; New et al., 2014). Shorter lag phase was also found after adaptation with various inhibitors including furfural, HMF and acetic acid (Koppram et. al., 2012; Landaeta et. al., 2013; Slininger et. al., 2015). Engineered strains also showed shorter lag phase after evolutionary adaptation (Martín et. al., 2007, Koppram et. 2012).

During growth in MSX medium xylose uptake rate increased by 2.53-fold in KmNIRE-K1.2 ($0.48 \pm 0.038 \text{ g g}^{-1} \text{ h}^{-1}$) as compared to KmNIRE-K1.1. Similarly, xylose uptake rate of KmNIRE-K3.2 was found to be 1.5-fold higher than that of KmNIRE-K3.1 (Table 3). However, the cell mass yield was 1.38-fold lower in KmNIRE-K1.2 as compared to KmNIRE-K1.1. Similarly, the cell mass yield of KmNIRE-K3.2 was also slightly lower i.e., $0.30 \pm 0.01 \text{ g g}^{-1}$ as compared to KmNIRE-K3.1. The lower cell mass yield may be due to the formation of other metabolite i.e. ethanol under aerobic condition (Arora et. al., 2015). However, the ethanol was not detected in the broth, which is supposed to escape of ethanol from vent due to air sparging. Sonderegger et al. (2003) developed *S. cerevisiae* TMB3001C1 and TMB3001C5 through adaptation with 460 generations and reported improved maximum growth rate as 0.64 ± 0.001 and $0.119 \pm 0.001 \text{ h}^{-1}$, with xylose uptake rate of 0.13 ± 0.00 and $0.27 \pm 0.02 \text{ g g}^{-1} \text{ h}^{-1}$, respectively on 5 g xylose l⁻¹ initial concentration. Similarly, Zhou et al. (2012) applied 3-stage evolutionary adaptation on engineered *S. cerevisiae* H131-A3 and reported improved specific growth rate of 0.12 h^{-1} on xylose as sole source of carbon. Several improved features like enhanced fermentation rates, improved ethanol tolerance and yield have also been reported through adaptation. The current study shows that the evolutionary adaption in salt medium has proved to be a better approach for improvement of yeast strain in context to utilize xylose present in lignocellulosic biomass.

Fermentation profiling of adapted strain

Fermentation batches using primary and secondary adapted cells of KmNIRE-K1 and KmNIRE-K3 were carried out under anaerobic condition in 3L Bioflow Bioreactor containing 2L salt medium MSX at 45°C, 5.5 pH and 200 rpm. After 72 h of fermentation, KmNIRE-K1.2 utilized 20.26 ± 0.48 g xylose l⁻¹ of and produced 4.1 ± 0.09 g DCW l⁻¹ from 30.92 ± 0.32 g xylose l⁻¹ and $2.63 \pm$ g DCW l⁻¹ of initial, respectively (Fig. 4C). However, KmNIRE-K1.1 utilized only 6.38 ± 0.72 g xylose l⁻¹ of and produced 3.37 ± 0.06 g DCW l⁻¹ of from 30.38 ± 0.77 g xylose l⁻¹ and 2.13 ± 0.15 g DCW l⁻¹ of initial, respectively (Fig. 4C).

Simultaneously, the maximum concentration of 1.98 ± 0.2 g ethanol l⁻¹ was obtained by KmNIRE-K1.2 which was 2.11-fold higher than that of KmNIRE-K1.1 (0.91 ± 0.02 g ethanol l⁻¹) (Fig. 4E). Moreover, KmNIRE-K1.2, also produced 3.61 ± 0.09 g glycerol and 2.47 ± 0.2 g acetic acid l⁻¹, whereas, KmNIRE-K1.1 produced only 0.14 ± 0.02 g glycerol l⁻¹ and 0.68 ± 0.02 g acetic acid l⁻¹, respectively.

KmNIRE-K3.2 utilized 20.18 ± 0.48 g xylose l⁻¹ and produced 4.2 ± 0.1 g DCW l⁻¹ using 28.95 ± 0.61 g xylose l⁻¹ and 2.53 ± 0.06 g DCW l⁻¹ of initial, respectively in 72 h, whereas, KmNIRE-K3.1 utilized only 8.31 ± 0.52 g xylose l⁻¹ and produced 3.89 ± 0.22 g DCW l⁻¹ of 29.59 ± 0.64 g xylose l⁻¹ and 2.33 ± 0.15 g DCW l⁻¹ of initial (Fig. 4D). The maximum ethanol concentration produced by KmNIRE-K3.2 was found to be 2.97 ± 0.12 g ethanol l⁻¹ in 72 h, which was 6.45-fold higher than that of KmNIRE-K3.1 (0.46 ± 0.02 g ethanol l⁻¹) (Fig. 4F). Furthermore, KmNIRE-K3.2 also produced 3.89 ± 0.06 g xylitol l⁻¹, 2.2 ± 0.21 g glycerol l⁻¹ and 2.94 ± 0.02 g acetic acid l⁻¹, respectively, as by-product together with ethanol production, which were 1.65, 5.51, 5.44-folds higher concentration of xylitol (2.35 ± 0.15 g l⁻¹), glycerol (0.4 ± 0.02 g l⁻¹) and acetic acid (0.54 ± 0.04 g l⁻¹), respectively, than KmNIRE-K3.1. Similarly, solid agar adapted *S. stipitis* CBS 6054 cells showed increased ethanol production (19.4 g ethanol l⁻¹) than that of native strain (16.3 g l⁻¹) (Agbogbo et. al., 2007).

The comparative fermentation kinetics of all the evolved strains has presented in Table 3. The specific sugar consumption rate (q_s) in KmNIRE-K1.2 (0.085 ± 0.004 g g⁻¹ h⁻¹) was 2.83-fold higher than that of KmNIRE-K1.1, whereas, the specific product (ethanol) formation rate (q_p) was found to be 2-fold higher in KmNIRE-K1.2 (0.008 ± 0.0001 g g⁻¹ h⁻¹) than that of KmNIRE-K1.1. Similarly, specific sugar consumption rate (q_s) in KmNIRE-K3.2 (0.085 ± 0.003 g g⁻¹ h⁻¹) was 1.7-fold higher than that of KmNIRE-K3.1, whereas, the specific product (ethanol) formation rate (q_p) was found to be 4.34-fold higher in KmNIRE-K3.2 (0.013 ± 0.001 g g⁻¹ h⁻¹) than that of KmNIRE-K3.1. On the other hand, ethanol yield ($Y_{p/s}$) of KmNIRE-K1.2 (0.1 ± 0.006 g g⁻¹) was nearly similar as KmNIRE-K1.1 (0.14 ± 0.02 g g⁻¹). However, ethanol yield ($Y_{p/s}$) was 2.43-fold higher in KmNIRE-K3.2 (0.14 ± 0.02 g g⁻¹) as compared to KmNIRE-K3.1, whereas, xylitol yield ($Y_{p/s}$) in KmNIRE-K3.2 (0.19 ± 0.002 g g⁻¹) was 1.47-fold lower than that of KmNIRE-K3.1. Adapted cells of *Candida guilliermondii* showed 3-fold higher xylitol yield after adaptation in rice straw hydrolysate (Silva et. al., 2001). The volumetric substrate uptake (Q_s) and volumetric productivity (Ethanol) (Q_p , g l⁻¹ h⁻¹) were 3.18 and 2.25-folds higher in KmNIRE-K1.2 ($Q_s = 0.28 \pm 0.006$, $Q_p = 0.027 \pm 0.002$) than that of KmNIRE-K1.1, respectively. Similarly, the volumetric substrate uptake (Q_s) in KmNIRE-

K3.2 (0.28 ± 0.006) was 2.34-fold higher than that of KmNIRE-K3.1. Moreover, volumetric productivities (Q_p) for ethanol and xylitol were 5.86 and 1.8-folds higher in KmNIRE-K3.2 ($Q_{p(ethanol)} = 0.041 \pm 0.001$; $Q_{p(xylitol)} = 0.0540 \pm 0.001$) than that of KmNIRE-K3.1, respectively.

Furthermore, final xylose to ethanol conversion (%) in KmNIRE-K1.2 was 19.51 ± 1.8 %, whereas it was 28.84 ± 4.1 % in KmNIRE-K1.1. Similarly, xylose to ethanol and xylitol conversions (%) in KmNIRE-K3.2 were 29.39 ± 0.5 and 19.19 ± 0.03 %, respectively, whereas it was 11.56 ± 0.43 and 28.68 ± 0.28 %, respectively in KmNIRE-K3.1. Moreover, the cell mass yields ($Y_{x/s}$) in KmNIRE-K1.2 (0.072 ± 0.006 g g⁻¹) and KmNIRE-K3.2 (0.08 ± 0.003 g g⁻¹) were 2.63 and 2.38-fold lower than that of KmNIRE-K1.1 and KmNIRE-K3.1, respectively. Comparable results of fermentation have also been reported by few researchers in support of evolutionary adaptation strategy. Variants of *S. stipitis* were obtained with tolerance to hardwood spent sulfite liquor (HSSL), when evolutionary engineering was performed (Pereira et. al., 2015). HSSL tolerance of evolved strain increased from 20 to 60 % with increased substrate uptake rate (0.22 g l⁻¹ h⁻¹) and ethanol yield (0.16 g g⁻¹). Several researchers have adopted this strategy to stabilize engineered strains. Xylose utilizing *S. cerevisiae* showed 6 % higher relative flux in pentose phosphate pathway after application of long term adaptation (Cadière et. al., 2011). Developed strain produced 53 g l⁻¹ of ethanol by utilizing 40 and 80 g l⁻¹ of xylose and glucose, respectively, in 24 h. Nielsen et al. (2015) applied short term adaptation on xylose utilizing *S. cerevisiae* during propagation. Adapted culture showed enhanced xylose utilization along with 30 % improved ethanol yield. Moreover, cell vitality and viability also increased more than 10 and 20 %, respectively, in adapted culture.

Aforesaid results indicate the role of secondary evolutionary engineering for enhancement of the xylose uptake, cell mass growth, and ethanol production and yield in MSX medium. Moreover, it has also enhanced the gene expression of metabolic gene required for xylose metabolism. Both the strains KmNIRE-K1.2 and KmNIRE-K3.2 showed better ratio of NAD⁺/NADP and NADPH/NADP⁺, which show the redox balance. However, further improvement is also suggested to reduce acetic acid and glycerol efflux during fermentation, which could further increase the ethanol production.

Conclusion

Two thermotolerant strains KmNIRE-K1.2 and KmNIRE-K3.2 were developed through evolutionary adaptation with faster xylose utilization capacity. Both the strains showed significant growth on xylose under aerobic condition along with better xylose uptake rate. Specific activities of metabolic enzymes including XR and XDH drastically improved in both the strains. Further, co-enzyme quantification study also revealed the reduction in redox imbalance after the evolutionary adaptation. Gene expression of *XYL1* and *XYL2* also increased many folds during xylose metabolism. These improvements occurred due to some evolutionary mutation inside the genome. Higher activity of XR and XDH in adapted strain at 45°C, will be fruitful for the SSF process and may increase the productivity of bioethanol. Therefore, further adaptation, optimization and modification at the genetic level will surely enhance bioethanol production and reduce by-product formation including acetic acid and glycerol which will be acceptable

at the industrial point of view. However, the genomic characterization of the strains will be done in future together with possible intergeneric hybridization. Moreover, the method of strain development using evolutionary adaptation has been proven to be useful for bioprocessing using robust non-conventional strains according to this study and opens the scope for integration of targeted and non-targeted genetic engineering methods.

Declarations

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Author Contribution: NKS conceived, planned, executed experiments and wrote the original draft; SB and RA edited the draft; and SK conceived, planned and reviewed the draft.

References

Agbogbo FK, Haagensen FD, Milam D, Wenger KS (2007) Fermentation of acid-pretreated corn stover to ethanol without detoxification using *Pichia stipitis*. In: Biotechnology for Fuels and Chemicals. Springer; p53-58.

Arora R, Behera S, Kumar S (2015) Bioprospecting thermophilic/thermotolerant microbes for production of lignocellulosic ethanol: a future perspective. *Renew Sust Energ Rev* 51: 699-717.

Arora R, Behera S, Sharma NK, Kumar S (2015) A new search for thermotolerant yeasts, its characterization and optimization using response surface methodology for ethanol production. *Front Microbiol* 6:889.

Arora R, Behera S, Sharma NK, Kumar S (2017) Augmentation of ethanol production through statistically designed growth and fermentation medium using novel thermotolerant yeast isolates. *Renew Ener* 109:406-421.

- Arora R, Behera S, Sharma, NK, Kumar S (2019) Evaluating the pathway for co-fermentation of glucose and xylose for enhanced bioethanol production using flux balance analysis. *Biotechnol Bioproc Eng* 24:924-933.
- Bailey JE, Ollis DF (1986) *Biochemical engineering fundamentals*. New York, NY: McGraw-Hill.
- Biswas D, Datt M, Aggarwal M, Mondal AK (2013) Molecular cloning, characterization, and engineering of xylitol dehydrogenase from *Debaryomyces hansenii*. *Appl Microbiol Biotechnol* 97:1613-1623.
- Cadière A, Ortiz-Julien A, Camarasa C, Dequin S (2011) Evolutionary engineered *Saccharomyces cerevisiae* wine yeast strains with increased in vivo flux through the pentose phosphate pathway. *Metab Eng* 13:263-271.
- Dasgupta D, Ghosh D, Bandhu S, Agrawal D, Suman SK, Adhikari DK (2016) Purification, characterization and molecular docking study of NADPH dependent xylose reductase from thermotolerant *Kluyveromyces sp.* IPE453. *Process Biochem* 51:124-133.
- Dhaliwal SS, Oberoi HS, Sandhu SK, Nanda D, Kumar D, Uppal SK (2011) Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. *Bioresour Technol* 102:5968-5975.
- Goshima T, Negi K, Tsuji M, Inoue H, Yano S, Hoshino T, Matsushika A (2013) Ethanol fermentation from xylose by metabolically engineered strains of *Kluyveromyces marxianus*. *J Biosci Bioeng* 116:551-554.
- Hamacher T, Becker J, Gárdonyi M, Hahn-Hägerdal B, Boles E (2002) Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization. *Microbiol* 148:2783-2788.
- Ikeuchi T, Kiritani R, Azuma M, Ooshima H (2000) Effect of D-glucose on induction of xylose reductase and xylitol dehydrogenase in *Candida tropicalis* in the presence of NaCl. *J Basic Microbiol* 40:167-175.
- Jeppsson M, Träff K, Johansson B, Hahn-Hägerdal B, Gorwa-Grauslund MF (2003) Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant *Saccharomyces cerevisiae*. *FEMS Yeast Res* 3:167-175.
- Khattab SMR, Saimura M, Kodaki T (2013) Boost in bioethanol production using recombinant *Saccharomyces cerevisiae* with mutated strictly NADPH-dependent xylose reductase and NADP⁺-dependent xylitol dehydrogenase. *J Biotechnol* 65:153-156.
- Komeda H, Yamasaki-Yashiki S, Hoshino K, Asano Y (2015) Identification and characterization of d-xylose reductase involved in pentose catabolism of the zygomycetous fungus *Rhizomucor pusillus*. *J Biosci Bioeng* 119:57-64.

- Koppram R, Albers E, Olsson L (2012) Evolutionary engineering strategies to enhance tolerance of xylose utilizing recombinant yeast to inhibitors derived from spruce biomass. *Biotechnol Biofuels* 5:32.
- Landaeta R, Aroca G, Acevedo F, Teixeira JA (2013) Mussatto SI. Adaptation of a flocculent *Saccharomyces cerevisiae* strain to lignocellulosic inhibitors by cell recycle batch fermentation. *Appl Ener* 102:124-130.
- Martín C, Marcet M, Almazán O, Jönsson L (2007) Adaptation of a recombinant xylose-utilizing *Saccharomyces cerevisiae* strain to a sugarcane bagasse hydrolysate with high content of fermentation inhibitors. *Bioresour Technol* 98:1767-1773.
- New AM, Cerulus B, Govers SK, Perez-Samper G, Zhu B, Boogmans S, Xavier JB, Verstrepen K (2014) Different levels of catabolite repression optimize growth in stable and variable environments. *Plos Biol.* 12:e1001764.
- Nielsen F, Tomás-Pejó E, Olsson L, Wallberg O (2015) Short-term adaptation during propagation improves the performance of xylose-fermenting *Saccharomyces cerevisiae* in simultaneous saccharification and co-fermentation. *Biotechnol Biofuels* 8:219.
- Pereira SR, Nogué VS, Frazão CJ, Serafim LS, Gorwa-Grauslund MF, Xavier, AMRB (2015) Adaptation of *Scheffersomyces stipitis* to hardwood spent sulfite liquor by evolutionary engineering. *Biotechnol Biofuels* 8:50.
- Qi X, Zha J, Liu GG, Zhang W, Li BZ, Yuan YJ (2015) Heterologous xylose isomerase pathway and evolutionary engineering improve xylose utilization in *Saccharomyces cerevisiae*. *Front. Microbiol* 6:1165.
- Rodrussamee N, Sattayawat P, Yamada M (2018) Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2. *BMC Microbiol* 18:73.
- Sharma NK, Behera S, Arora R, Kumar S (2016) Enhancement in xylose utilization using *Kluyveromyces marxianus* NIRE-K1 through evolutionary adaptation approach. *Bioproc Biosyst Eng* 39:835-843.
- Sharma NK, Behera S, Arora R, Kumar S (2017) Evolutionary adaptation of *Kluyveromyces marxianus* NIRE-K3 for enhanced xylose utilization. *Front Ener Res* 5:32.
- Sharma NK, Bibra M, Kumar S (2019) Genetic and metabolic engineering of microbes for efficient lignocellulosic ethanol. In: *Advances in bio-fuel production*, Kumar A, Garg S (Eds.) 1:79-136. Nova Science Publishers Inc, New York.
- Signori L, Passolunghi S, Ruohonen L, Porro D, Branduardi P (2014) Effect of oxygenation and temperature on glucose-xylose fermentation in *Kluyveromyces marxianus* CBS712 strain. *Microb Cell Fact* 13:51.

- Silva C, Roberto I (2001) Improvement of xylitol production by *Candida guilliermondii* FTI 20037 previously adapted to rice straw hemicellulosic hydrolysate. *Lett Appl Microbiol* 32:248-252.
- Slininger PJ, Shea-Andersh MA, Thompson SR, Dien BS, Kurtzman CP, Balan V, da Costa Sousa L, Uppugundla N, Dale BE, Cotta MA (2015) Evolved strains of *Scheffersomyces stipitis* achieving high ethanol productivity on acid-and base-pretreated biomass hydrolyzate at high solids loading. *Biotechnol Biofuels* 8:60.
- Sonderegger M, Sauer U (2003) Evolutionary engineering of *Saccharomyces cerevisiae* for anaerobic growth on xylose. *Appl Environ Microbiol* 69:1990-1998.
- Wang R, Li L, Zhang B, Gao X, Wang D, Hong J (2013) Improved xylose fermentation of *Kluyveromyces marxianus* at elevated temperature through construction of a xylose isomerase pathway. *J Ind Microbiol Biotechnol* 40:841-854.
- Wei N, Xu H, Kim SR, Jin YS (2013) Deletion of FPS1 coding for aquaglyceroporin Fps1p improves xylose fermentation by engineered *Saccharomyces cerevisiae*. *Appl Environ Microbio*. 79:3193-3201.
- Yablochkova E, Bolotnikova O, Mikhailova N, Nemova N, Ginak A (2003) The activity of xylose reductase and xylitol dehydrogenase in yeasts. *Microbiol* 72:414-417.
- Yamasaki-Yashiki S, Komeda H, Hoshino K, Asano Y (2014) Molecular analysis of NAD⁺-dependent xylitol dehydrogenase from the zygomycetous fungus *Rhizomucor pusillus* and reversal of the coenzyme preference. *Biosci Biotechnol Biochem* 78:1943-1953.
- Zhang B, Sun H, Li J, Wan Y, Li Y, Zhang Y (2016) High-titer-ethanol production from cellulosic hydrolysate by an engineered strain of *Saccharomyces cerevisiae* during an in situ removal process reducing the inhibition of ethanol on xylose metabolism. *Process Biochem* 51:967-972.
- Zhang J, Zhang B, Wang D, Gao X, Sun L, Hong J (2015) Rapid ethanol production at elevated temperatures by engineered thermotolerant *Kluyveromyces marxianus* via the NADP (H)-preferring xylose reductase-xylitol dehydrogenase pathway. *Metab Eng* 31:140-152.
- Zhou H, Cheng JS, Wang BL, Fink GR, Stephanopoulos G (2012) Xylose isomerase overexpression along with engineering of the pentose phosphate pathway and evolutionary engineering enable rapid xylose utilization and ethanol production by *Saccharomyces cerevisiae*. *Metab Eng* 14:611-622.

Figures

Figure 1

Secondary adaptation of (A) *KmNIRE-K1.1* and (B) *KmNIRE-K3.1* in MSX medium containing 20 g l⁻¹ xylose (♦) Xylose utilization; (■) Dry cell weight; (—) Trend line of xylose utilization; (---) Trend line of dry cell weight

Figure 2

Specific enzyme activities of xylose reductase (XR) and xylitol dehydrogenase (XDH) in KmNIRE-K1.1, KmNIRE-K3.1, KmNIRE-K1.2, and KmNIRE-K3.2 at different temperatures, grown in xylose and glucose/xylose mixture. A) XR of KmNIRE-K1.1 and KmNIRE-K1.2; B) XR of KmNIRE-K3.1 and KmNIRE-K3.2; C) XDH of KmNIRE-K1.1 and KmNIRE-K1.2; D) XDH of KmNIRE-K3.1 and KmNIRE-K3.2

Figure 3

Comparative gene expression analysis of XYL1 and XYL2 genes between (A) KmNIRE-K1.1 (■) and KmNIRE-K1.2 (■), and (B) KmNIRE-K3.1 (■) and KmNIRE-K3.2 (■)

Figure 4

Growth and fermentation pattern of *KmNIRE*-K1.1 (---) and *KmNIRE*-K1.2 (—), and *KmNIRE*-K3.1 (---) and *KmNIRE*-K3.2 (—) using xylose as a carbon source, (◆) Xylose; (■) Dry cell weight (DCW); (▲) Glycerol; (×) Acetic acid; (●) Ethanol; (+) Xylitol. (A) Growth profile of *KmNIRE*-K1.1 and *KmNIRE*-K1.2; (B) Growth profile of *KmNIRE*-K3.1 and *KmNIRE*-K3.2; (C) Fermentation profile of *KmNIRE*-K1.1 and *KmNIRE*-K1.2; (D) Fermentation profile of *KmNIRE*-K3.1 and *KmNIRE*-K3.2; (E) Product profile during fermentation of *KmNIRE*-K1.1 and *KmNIRE*-K1.2; (F) Product profile during fermentation of *KmNIRE*-K3.1 and *KmNIRE*-K3.2.

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