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Overview of Catalytic Properties of Fungal Xylose Reductases and Molecular Engineering Approaches for Improved Xylose Utilisation in Yeast

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Abstract

Background and Objective: Xylose reductases belong to the aldo-keto reductase family of enzymes, which catalyse the conversion of xylose to xylitol. Yeast xylose reductases have been intensively studied in the last two decades due to their significance in biotechnological production of ethanol and xylitol from xylose. Due to its GRAS status and pronounced tolerance to harsh conditions, *Saccharomyces cerevisiae* is the ideal organism for industrial production of both xylitol and ethanol. However, *Saccharomyces cerevisiae* is unable to use xylose as the sole carbon source due to the lack of xylose specific transporters and insufficient activity of metabolic pathways for xylose utilisation. The aim of this paper is to give an overview of attempts in increasing biotechnological potential of xylose reductases and to highlight the prospective of this application.

Results and Conclusion: In order to create strains with improved xylose utilization, different approaches were attempted including simultaneous overexpression of xylitol dehydrogenase, xylose reductase and pentose phosphate pathway enzymes, heterologous expression of putative xylose transporters or heterologous expression of genes coding for enzymes included in the xylose metabolism, respectively. Furthermore, number of attempts to genetically modify different xylose reductases is increasing. This review presents current knowledge about yeast xylose reductases and the different approaches applied in order to improve xylose metabolism in yeast.

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1. Introduction

In the last two decades, interest in xylose reductases (XRs) has increased due to their potential use primarily in the fermentation of plant biomass (wheat straw, sugar cane bagasse, non-recyclable paper etc.) to ethanol and in the microbial industrial production of xylitol. High oil prices and supply instability increased the interest in the production of biofuel from renewable plant material [1,2]. Xylose is the second monosaccharide in natural abundance and represents the predominant pentose sugar in plant hemicelluloses [3]. Efficient utilization of xylose is essential for the development of feasible conversion processes of biomass to energy. Many microorganisms including fungi could grow on xylose aerobically, but only few of them are capable to produce ethanol from it. Due to its ability to withstand low pH and high concentration of ethanol and ferment under strictly anaerobic conditions *Saccharomyces* (*S.*) *cerevisiae* is the ideal organism for industrial production of ethanol. However, in the presence of high glucose concentrations, *S. cerevisiae* cannot simply uptake xylose because both sugars are transported into the cell by the same transport system with a higher affinity for glucose. Numerous genetic modifications of *S. cerevisiae* have been taken in the last two decades in order to increase the efficiency of xylose utilization, including expression of heterologous proteins predicted to be xylose transporters [4,5], overexpression of native pentose phosphate pathway (PPP) enzymes [6,7], XR and xylitol dehydrogenase (XDH) [8]. Heterologous expression, including surface display [9], of different genes from native pentose catabolising organisms coding for enzymes included in the xylose metabolism was also attempted [10-12]. Some XRs were genetically modified in order to change cofactor

preferences and resolve cofactor regeneration deficiency which represents as one of the biggest bottlenecks in the xylose utilisation [13-15]. Furthermore, the method of directed evolution in medium containing xylose was carried out under aerobic [16,17], or anaerobic conditions [7,18] in order to improve xylose metabolism and ethanol production. The main goal of this attempt was to redirect metabolism toward increased ethanol and decreased xylitol production. On the other hand, to improve production of xylitol, different approaches are needed, including disruption of the XDH gene and overexpression of XR [19,20] which prevents further metabolism of xylitol to ethanol. Furthermore, attempts to optimize composition of growth media (i.e. type and ratios of different sugars and nitrogen sources) and conditions of fermentation in order to increase xylitol yield using artificial neural network strategy [21] were carried out. On an industrial scale, xylitol is currently produced by high-cost and low-yield chemical process using Ni/Al_2O_3 as a catalyst under high temperature and pressure [22]. Economically effective biological process for xylitol production has not been developed yet, mainly due to the lack of efficient XR cofactor regeneration system.

In *S. cerevisiae* cells xylitol is produced by reduction of xylose in the reaction catalysed by XR. Xylitol is then oxidized by the XDH to xylulose, which in the next step is phosphorylated by xylulose kinase (XK) to xylulose-5-phosphate. Finally, xylulose-5-phosphate enters the PPP (Fig. 1). Xylose reductase, the first enzyme in the pathway, is essential for utilization of xylose and has been shown to control the rate of xylose utilization [23]. Accordingly, XR is of great importance for both ethanol, and xylitol production. XRs from different yeasts (*Pichia* (*P.*) *stipitis*, *Candida* (*C.*) *tropicalis*, *C. tenuis*, *C. intermedia*, *C. parapsilosis*, *Debaryomyces (D.) nepalensis*, *D. hansenii*, *S. cerevisiae*) and from fungi *Neurospora* (*N.*) *crasa*, *Rhizopus* (*R.*) *oryzae* and *Talaromyces* (*T.*) *emersonii* were isolated, purified and characterised so far [24-33]. *P. stipitis*, *C. tenius and S. cerevisiae* XRs are the most thoroughly investigated and engineered yeast XRs, and *S. cerevisiae* is the most frequently used for the heterologous expression of native or modified genes from other yeasts.

In this paper, the overview of the current knowledge on yeast XRs and bottlenecks in xylose utilisation in yeasts are presented, as well as the outline of different approaches applied in order to improve the xylose metabolism for the production of biofuels and xylitol.

1. Fungal xylose reductases, catalytic properties and mode of action

Yeast XRs are members of 2b subfamily of the aldo/keto reductase (AKR) family of enzymes [34]. The reaction favours alcohol formation, whereas the reverse reaction occurs only to a limited extent. XRs are commonly found in fungi and yeasts, frequently with several isozymes in single species [26,35]. Many genes coding for XRs have been sequenced and expressed in different hosts [26,27,30,35-37], but many XR genes are still unidentified or poorly characterized.

Fungal XRs are usually dimers of small monomeric units with molecular weights between 30 and 40 kDa. Most of them are found to be strictly NADPH-dependent, but some can utilise both NADPH and NADH [26,34,38]. However, unlike all other XRs characterised so far, *C. parapsilosis* XR prefers NADH over NADPH [27]. Yeast XRs exhibit Michaelis–Menten kinetics [39] and their catalytic and coenzyme binding sites have characteristics of both the short-chain dehydrogenases/reductases (SDR) and the AKR families. Namely, they possess the coenzyme-binding site typical for AKRs, containing Ile-Pro-Lys-Ser motif and conserved Arg situated five residues downstream, as well as the GlyXXXGlyXGly region for the binding of coenzyme that is characteristic for SDR enzymes [40] in which the last glycine residue is substituted by Glu or Asp in yeast XRs [41].

Kinetic characteristics of so far characterised XRs are displayed in Table 1, showing that the k_{cat} for different XRs varies from 860 min^{-1} (*S. cerevisiae*) to 3600 min^{-1} (*N. crassa*), K_m for xylose from 13,6 mM (*S. cerevisiae*) to 72 mM (*C. tenius*) and K_m for NADPH from 1,8 μM (*N. crassa*) to 56 μM (*C. intermedia*). Such pronounced differences in the kinetic characteristics among different yeast XRs could indicate that the yeast XRs were not particularly conserved. However, the amino acid sequence alignment of the six yeast XRs (XR from *S. cerevisiae*, *Kluyveromyces* (*K.*) *lactis*, *P. stipitis*, *C. tropicalis* and *Pachysolen tannophilus*) and two human aldo-keto reductases showed a significant homology and 24% identity of aligned sequences [41]. Moreover, this alignment showed that all the residues with direct roles in binding of cofactor or catalysis were strictly conserved. However, a number of non-polar residues placed in the human aldose reductase catalytic site, which were supposed to be in contact with the substrate, were replaced by different polar amino acids in the yeast. The differences in substrate specificity and kinetic constants between yeast XRs may be caused by these substitutions. Furthermore, the K_m values for xylose are about 1000 fold higher than that for NADPH for all yeast XRs characterised so far (Table 1). High affinity for coenzyme binding insures that the coenzyme will be tightly bound to the enzyme and that the reaction will proceed even with small concentration of the coenzyme in the cell.

Table 1. Kinetic constants of fungal xylose reductases

Yeast	k_{cat} (min^{-1})	K_m for xylose (mM)	K_m for NADPH (μM)	k_{cat}/K_m for xylose ($mM^{-1}min^{-1}$)	k_{cat}/K_m for NADPH ($\mu M^{-1}min^{-1}$)	K_m for NADH (μM)	Reference
P. stipitis	1500	42	9	36	167	21	38
C. tropicalis	ND	5.9	12.0	-	-	ND	24
C. intermedia	900	50	56	18	16	28	35
C. parapsilosis	3100[a]	32	36.5	97.9[a]	83.8	3.3	27
C. tenius	1300	72	4.8	18	271	25	36
S. cerevisiae	860	13.6	7.6	63	113	ND	30
N. crasa	3600	34	1.8	106	2000	16	31
R. oryzae	1800	42.7	19.2	42.15	93.75	972	32

Optimal pH for most XRs is around 6, and optimal temperature is around 40°C. Although XR with higher optimal temperature than 40-50°C was not found so far, such results might be the outcome of high thermal instability of NADPH that prevents detection of enzyme activity at higher temperatures. This presumption is additionally supported by the fact that even XR that originates from thermophilic fungus *T. emersonii* [33], from which some thermostable enzymes have been previously characterized, showed optimum activity at 37°C.

The most efficient xylose-fermenting yeast, *P. stipitis*, has NAD(P)H-dependent XR. The enzyme can utilise various aldoses as substrates (arabinose, glyceraldehyde, galactose, ribose, xylose and glucose). Kostrzynska *et al.* [42] have shown that Lys_{270} in the coenzyme-binding site of *P. stipitis* XR was involved in the binding of both NADPH and xylose. Site-directed mutagenesis of Lys_{270} reduced about five-fold the affinity for binding of NADPH, without changes in the affinity for NADH [42]. Optimal pH for this enzyme is 6.0 and it shows approx. 30% lower activity with NADH than with NADPH [38]. Most of the yeast XRs characterised so far showed activity with different aldose, and even ketose substrates. Some XRs, like XR from *C. tenuis* and *S. cerevisiae*, show higher affinity for other substrates than xylose, opening the question if they should actually be named aldose reductases instead of XRs. Advantage of such a broad substrate specificity is clearly that the yeast could use the same enzyme to metabolise different sugars available in natural surroundings. However, in industrial applications it could result in the formation of different side products during fermentation in complex media. Furthermore, it is not yet completely understood what might be the biological advantage of XRs that utilise both NADPH and NADH as coenzymes. Possible reason for developing such a dual coenzyme utilisation might be the ability to resolve redox imbalance in the cell that occurs under anaerobic conditions. Namely, during anaerobic fermentation of xylose in yeasts using NADPH-linked XR and NAD^+-linked XDH (as shown in Fig. 1), an overproduction of NADH occurs. Accumulated NADH cannot be oxidized without oxygen, so the metabolism is blocked. However, if there is an NADH-specific XR activity present in the cell, it could oxidase accumulated NADH. In this way the imbalance of the redox system under anaerobic conditions is bypassed, permitting fermentation of xylose to ethanol and growth on xylose. Bruinenberg *et al*. [43] investigated capability of various xylose-assimilating yeasts under anaerobic conditions to grow and produce ethanol. Results of this study showed that substantial fermentation of xylose to ethanol occurred only in yeasts which contained NADH-specific XR.

Cocotle-Ronzon *et al.* [24] partially purified and characterized XR from *C. tropicalis* IEC5-ITV. The molecular weight of this enzyme was 32.42 kD. This XR exhibited the highest activity at pH 6.0 and 40°C, with K_m for xylose 5.9 mM, and K_m for NADPH 12.0 mM. No activity was detected with NADH as a cofactor.

Mayr *et al.* [26] isolated and characterised two structurally similar forms of XR (Alr1 and Alr2) from *C. intermedia* with different coenzyme specificities. Alr1 showed to be strictly specific for NADPH, whereas Alr2 utilised both NADH and NADPH. Both enzymes are composed of two subunits, but they differ in pI. Nidetzky *et al.* [35] showed that aldehyde-binding modes were identical for both XR forms, thus the specificity for NADH and for NAD(P)H were the main features that distinguished Alr1 from Alr2.

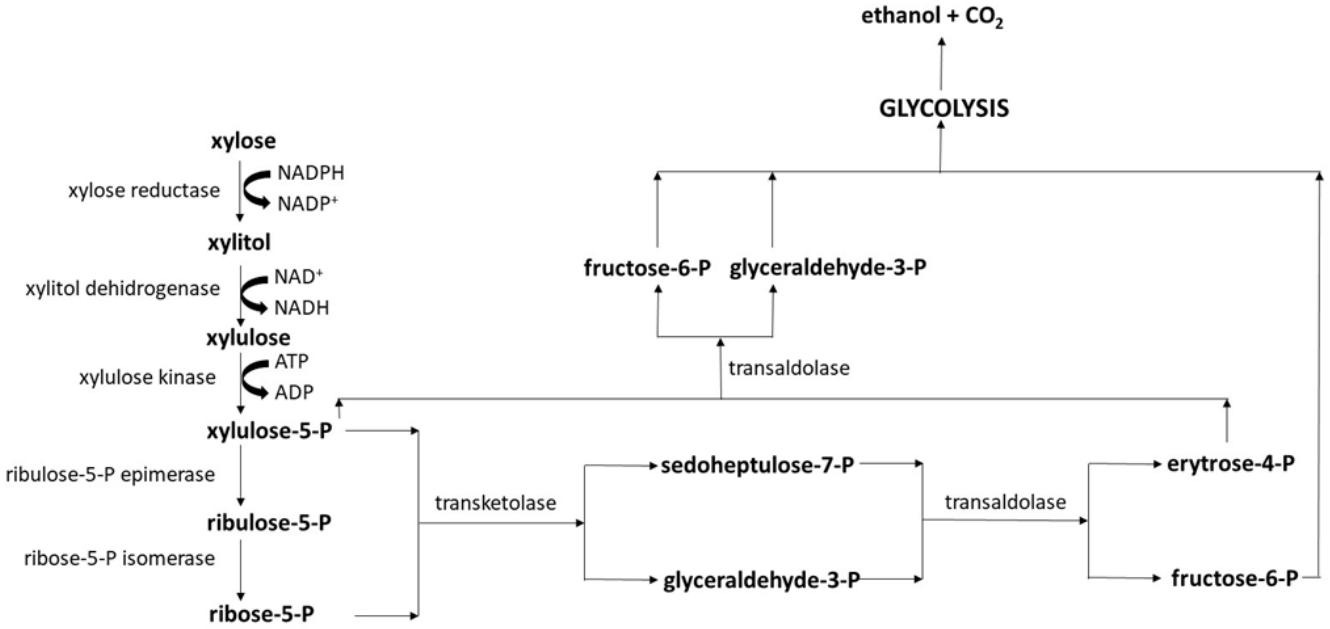

Fig. 1. Metabolic scheme for conversion of xylose to xylitol or ethanol in yeast cells.

An NADH-preferring XR with a molecular mass of 36.62 kDa was purified from *C. parapsilosis* KFCC-10875 [27]. This XR, unlike all other XRs characterized so far, showed a lower efficiency with NADPH (k_{cat}/K_m = $1.27x10^2$ s^{-1} mM^{-1}) than with NADH (k_{cat}/K_m = $1.39x\ 10^4$ s^{-1} mM^{-1}) [27]. The K_m for xylose was similar to those from other *Candida* (Table 1), but this XR showed a high catalytic efficiency with xylose as well as a high specificity for xylose in comparison to other yeast XRs [27]. The high K_m values for NADPH for *C. parapsilosis* XR compared with K_m for NADPH of XRs from other sources (Table 1) could be caused by the differences in the amino acid sequence in the region of binding of NADPH. Namely, the Lys residue in the IPKS motif of *C. parapsilosis* XR is replaced by an Arg. However, this assumption has not been confirmed by site-directed mutagenesis of specific Arg residue in IPKS motif so far.

The yeast *C. tenuis* produces single aldose reductase that is active with both NADPH and NADH. However, the apparent binding of NADPH is 5.3- fold stronger than that of NADH and the k_{cat} observed with NADH is approx. 20% lower than that with NADPH [36]. Crystallographic studies and site-directed mutagenesis of the *C. tenuis* XR confirmed that the coenzyme binding and selectivity depends on Lys residue in the IPKS region [25,44]. This enzyme consists of a single 43 kDa polypeptide with an isoelectric point of 4.7, with optimum temperature of 50°C and optimum pH of 6.0. The enzyme is active with three-carbon to seven-carbon aldose sugars and aliphatic and aromatic aldehydes, but the preferred substrate is erythrose [36].

S. cerevisiae nonspecific xylose reductase encoded by *GRE3* (YHR104w) catalyses the reduction of a variety of aldehydes using NADPH exclusively as a cofactor [30]. The enzyme is a monomer with the molecular mass between 35 and 36.8 kDa which shows the coenzyme-binding and the catalytic features of both SDR and AKR families [30,41,45]. In general, a crucial Lys residue forms H-bonds with a Tyr residue, and a Tyr residue donates its proton to the substrate during catalysis by enzymes of both AKR and SDR families [45]. However, the *S. cerevisiae* XR comprises two Lys residues, one matching the AKR (Lys_{78}) and the other matching the SDR family of enzymes (Lys_{53}), capable to form H-bond with Tyr_{49}. Jeong *et al.* [46] determined by site-directed mutagenesis that the essential residue that forms H-bonds to Tyr_{49} is Lys_{78}. According to this finding it is clear that the active site of *S. cerevisiae* XR correlates with those of the AKR family. Site-directed mutagenesis of the individual residues in the SDR-like ($G_{128}A$, $G_{132}A$, $D_{134}G$, and $D_{134}A$) coenzyme-binding motif did not show any significant change of kinetic characteristics in any of the mutants. However, it was found that this motif contributes significantly to the overall conformation of the enzyme [47].

XR from *D. hansenii* UFV-170 was partly characterised by Sampaio *et al.* [29]. This XR showed to be NADPH-dependent, with optimal pH of 5.3 and optimal temperature of 39°C. The enzyme retained almost 100% activity at 4°C and about 50% activity at 39°C for 360 min, while at temperatures ≥50°C the activity was almost completely lost in 20 min.

Kumar and Gummadi [28] purified XR from the *D. nepalensis* NCYC 3413. The enzyme favoured pentoses over hexoses and showed an absolute specificity for NADPH over NADH. It had a molecular mass of 36.4 kDa, pI of 6.0 and the optimal activity at pH 7.0 and 45°C. The enzyme showed high activity in 100 mM NaCl or KCl (90% of optimal activity) and in 1 M KCl (40% of optimal activity).

Woodyer *et al.* [31] purified and characterized a XR from *Neurospora crassa*. XR from *N. crassa* is a dimer with the molecular mass of 53 kDa. Purified *N. crassa* XR had a 100-fold better catalytic efficiency with NADPH as the cofactor, having about 10 times higher k_{cat} and about 10 times lower K_m with NADPH than with NADH. The enzyme can utilise ribose, glucose, arabinose and galactose, while there was no activity with fructose and sucrose. Compared to yeast XRs, its k_{cat} was two-fold higher than the best performing yeast NADPH-dependent XR (*P. stipitis*) [38] while its catalytic efficiency with NADPH was higher than that of the *C. tenuis* XR about 7-fold [36]. The optimal temperature for this enzyme was between 45 and 55°C, and the optimal pH around pH 5.5. The *N. crassa* XR retained activity at 4°C for several months and over 1 month at room temperature.

Superior characteristics of *N. crassa* XR, relative to yeast XRs, raise the question whether characteristics of XRs from filamentous fungi in general are better than those of yeast XRs. However, there is a lack of published data on this topic. Two more filamentous fungi XRs, apart from *N. crassa* XR, were characterised. Zhang *et al*. [32] cloned and expressed XR from *Rhizopus oryzae* in the *P. pastoris* cells. Recombinant enzyme had a molecular mass about 37 kDa and showed the highest activity at pH 5.8 and 50°C. However, the 50% of enzyme activity was lost after 83 min at 50°C, and after 10 min at 58°C, thus showing instability at temperatures close to the optimal temperature. Similar to the XR from *N. crassa*, purified enzyme was stable at 4°C for over 3 weeks. *R. oryzae* XR displayed activity towards xylose, arabinose, ribose, fructose and galactose. The enzyme exhibited a lower catalytic efficiency (k_{cat}/K_m) for xylose (0.596 mM^{-1} s^{-1}) than for arabinose (0.336 mM^{-1} s^{-1}). According to the values of the K_m constants, *R. oryzae* XR is NADPH dependent (K_m= 19.2 µM), with catalytic efficiency of 1562.50 mM^{-1} s^{-1}, and shows very low affinity and catalytic efficiency for NADH (K_m= 972 µM; k_{cat}/K_m= 4.14 mM^{-1} s^{-1}).

Fernandes *et al.* [33] characterised XR from the fungus *T. emersonii* as a monomeric enzyme with a molecular mass of 36 kDa. The XR of this filamentous thermophilic fungus exhibits high sequence identity with yeast XRs. It showed the highest activity at pH 6.5, and it was active in ranges between pH 5 and pH 8. Although it originates from a thermophilic organism, from which thermostable endo-betaglucanases, cellobiohydrolases and xylan degrading enzymes have previously been characterized [48,49], this XR showed optimum activity at 37°C. The enzyme showed dual coenzyme specificity, with just a slightly higher affinity for NADPH (K_m = 244 µM) than for NADH (K_m=263 µM). However, its catalytic efficiency was 24.5-fold lower with NADH as coenzyme. Unfortunately, stability of this enzyme was not investigated.

According to these results, it seems that XRs from filamentous fungi in general have higher stability than yeast XRs. However, catalytic characteristics of filamentous fungi XRs in general are not better than those of yeast XRs, and the results obtained for XR from *N. crassa* are rather an exception.

2. Bottlenecks in xylose utilisation by yeasts

Inability of wild type *S. cerevisiae* strains to utilise xylose as the only source of carbon for growth and fermentation is partly caused by the lack of efficient xylose transporters [50,51]. Since an overexpression of native XR and XDH genes resulted only in slow growth on xylose [8], numerous attempts have been taken to construct recombinant *S. cerevisiae* strains with efficient xylose intake.

2.1. Hexose transporters in xylose utilisation

There are 18 transporters of hexoses (Gal2, Hxt1-17) in *S. cerevisiae*, and seven of them (Hxt1-7) are glucose transporters. Glucose transporters are classified into three groups based on their K_m for glucose: high-affinity transporters with K_m 1-2 mM (Gal2, Hxt6 and Hxt7); intermediate-affinity transporters with K_m~10 mM (Hxt5, Hxt4 and Hxt2); and low-affinity transporters with K_m~50–100 mM (Hxt3 and Hxt1) [52]. The transporters Gal2, Hxt1, Hxt4, Hxt5 and Hxt7 are xylose-permissive, but the uptake of xylose by these transporters depends on the xylose to glucose concentration ratio [53]. When the only available source of carbon is xylose, or if xylose is in the mixture with low concentration of glucose, the main transporters for the xylose uptake are Hxt7 and Hxt5 [53]. *HXT7* and *HXT5* are expressed when the glucose is absent, , while their transcription is severely repressed in high glucose concentration (>200 mM). Glucose repress *GAL2* transcription as well. However, hexose transporters Hxt7 and Gal2 are the most efficient xylose transporters when expressed constitutively [53]. In order to generate yeast strains with efficient xylose transport, *S. cerevisiae* has been genetically engineered to express different heterologous hexose transporters. More than 80 potential heterologous xylose-specific transporters have been expressed in *S. cerevisiae* so far. However, many of them were not functional, most probably because they were not properly folded or due to miss-localization. Proteins coded

by *STL12* (coding for the protein named Xyp29), *XUT1*, *XUT3*, *XUT4*, *SUT1*, *SUT2* and *SUT3*, from *P. stipitis*, and proteins from *C. intermedia* coded by *GXF1* and *GXS1*, proteins An25 and An29-2 from *N. crassa*, proteins xtrD from *Aspergillus nidulans*, At5g59250 from *Arabidopsis thaliana*, Xylh from *Debaryomyces hansenii* and MgT05196 from *Meyerozyma guilliermondii* have accomplished to transport xylose into the cells of the null hexose-transporter *S. cerevisiae* strain [54-57]. Three of them (An25, Xyp29 and Xut1) were characterized as xylose-specific transporters. Xyp29 and An25 uptake xylose only, showing no affinity towards glucose, while Xut1 transported xylose and glucose, but with a lower affinity for glucose [54]. Expression of *C. intermedia* Gxs1 and Gxf1 significantly improved growth rate of recombinant *S. cerevisiae* when using mixture of glucose and xylose as source of carbon [55]. Co-expression of these two transporters resulted in efficient xylose transport, although they showed a high affinity for glucose as well [56,57].

2.2. Redox imbalance in xylose utilisation

Another problem of xylose utilization in yeast is a cellular redox imbalance due to the XR and XDH preferences toward NADPH and NAD^+ respectively. Possible strategies to solve this problem consist of changing the cofactor preference of XR towards NADH [4,13,14,44,58,59] or XDH towards $NADP^+$ [60]. The redox imbalance in the cell appears under anaerobic conditions when NADH, produced in the reaction catalysed by XDH, accumulates because it cannot be oxidized without oxygen. At the same time, $NADP^+$ will accumulate as a product of the reaction catalysed by XR. XR engineered to utilize NADH instead of NADPH can oxidase the accumulated NADH and solve the redox imbalance. Similarly, if XDH is engineered to utilize $NADP^+$ instead of NAD^+, such imbalance will not appear. In order to increase the efficiency of xylose utilization additionally, the regeneration of NADPH in the cell and the rate of non-oxidative part of PPP should be enhanced (Fig. 1). Regeneration of NADPH could be improved by overexpression of homologous $NADP^+$ coenzyme dependent enzymes of PPP [61,62], or by expression of heterologous $NADP^+$ coenzyme dependent enzymes. Bera *et al*. [6] used this approach and expressed *K. lactis* $NADP^+$-dependent D-glyceraldehyde-3-phosphate dehydrogenase. However, this leads towards an increased xylitol production. If the final goal is to increase ethanol production, the rate of the non-oxidative part of PPP should be increased by overexpression of the enzymes catalysing the reactions of that part of the pathway [7,62].

In the Metzger and Hollenberg [63] study the complete replacement of *P. stipitis* XDH binding motif for NAD^+ with the *Thermoanaerobium brockii* alcohol dehydrogenase binding motif for $NADP^+$ was conducted. Although the apparent K_m for NAD^+ was nine-fold higher in comparison to native enzyme, this modification resulted in approximately the same affinity towards NAD^+ and $NADP^+$.

Watanabe *et al.* [60] engineered the triple (Asp207Ala/Ile208Arg/ Phe209Ser) and the quadruple (Asp207Ala/Ile208Arg/ Phe209Ser/Asn211Arg) mutant of XDH form *P. stipitis* reaching values of k_{cat}/K_m with $NADP^+$ comparable to those with NAD^+, i.e. about 4500-fold higher enzyme efficiency with $NADP^+$ than the native enzyme.

2.3. Simultaneous expression of enzymes of other metabolic pathways

Several authors tried to express xylose isomerase (XI) in the yeast [64-66]. XI is bacterial enzyme that converts xylose to xylulose and requires no coenzyme, so in this way the redox imbalance would be eluded. However, small number of XIs have been successfully expressed in yeast, probably due to the problems in posttranslational modifications and protein miss-folding [64, 66]. Ota *et al.* [65] expressed XI from *Clostridium cellulovorans* on the *S. cerevisiae* cell surface, enabling xylulose production outside the cell. In this way both redox imbalance and xylose transport into the cell could be solved, because xylulose could easily be transported into the cell. In last two decades different heterologous proteins have been expressed and immobilised on the surface of yeast cell [9,67-69]. This system might also be used to display XRs in combination with a system for the coenzyme regeneration. This approach might efficiently address problems of both xylose transport into the cell and the redox imbalance caused by intracellular conversion of xylose to xylulose.

Jeppsson *et al.* [61] reported decreased xylitol and increased ethanol production by lowering the rate of the NADPH-producing part of the PPP by the disruption of either 6-phosphogluconate dehydrogenase (*GND1*), or glucose 6-phosphate dehydrogenase (*ZWF1*) gene. The PPP flux was additionally lowered by decreasing the phosphoglucose isomerase activity. Such approach, however, apart from lowered xylitol production, resulted in a slower consumption of xylose as well. Such results indicated that xylose utilisation and xylitol yield depend on the rate of the oxidative part of the PPP.

Johansson and Hahn-Hagerdal [62] overexpressed the *S. cerevisiae* genes coding for enzymes of the non-oxidative part of the PPP (transketolase, transaldolase, ribose 5-phosphate isomerase and ribulose 5-phosphate epimerase). They showed that the growth on xylulose and the rate of xylulose fermentation were only partly affected at the level of the non-oxidative PPP, while the xylose fermentation rate depended on the efficiency of xylose

transport and the activity of XR and XDH. Demeke *et al.* [7] constructed and integrated into the industrial strain of *S. cerevisiae* an expression cassette containing genes coding for the enzymes of non-oxidative PPP, *XYLA* (encoding xylose isomerase) from *Clostridium phytofermentans*, and the gene *HXT7* coding for the hexose transporter. The strain obtained in this way was additionally submitted to ethyl methanesulfonate mutagenesis, selection in hydrolysate of lignocellulose with high content of xylose, and directed evolution in the xylose containing complex medium. This resulted in the selection of a strain with enhanced tolerance to inhibitors and increased efficiency of xylose utilization.

To facilitate NADPH regeneration, Verho *et al*. [70] overexpressed the fungal *GDP1* from *K. lactis* and deleted native *ZWF1* gene. Deletion of *ZWF1* resulted in decreased CO_2 production because the oxidative part of the PPP was blocked. Since there is no CO_2 production during the regeneration through an $NADP^+$-GAPDH, the resulting strain produced ethanol from xylose with a higher yield and without excess CO_2 production. Overexpression of *GDP1* in combination with overexpression of genes coding for the non-oxidative part of the PPP did not yield any additional enhancement in xylose fermentation [6].

3. Genetic engeneering of xylose reductase

Numerous attempts to change the cofactor specificity of XR have been conducted. Amino acid sequence alignment in combination with three dimensional structures showed that proteins of the AKR superfamily shared a common $(\alpha/\beta)_8$-barrel three-dimensional fold and had a highly conserved $NAD(P)^+$-cofactor-binding pocket, suggesting a similar mode of cofactor binding. Even among AKRs with less than 30% amino acid sequence identity, one third of the residues of the cofactor pocket are strictly identical, and more are highly conserved [71]. The connections between enzyme and cofactor are nearly identical in all known AKRs, and mutagenesis studies showed the importance of amino acids at positions between 260 and 280 in discriminating between the binding of NAD(H) and NADP(H) [71]. In order to enhance utilization of NADH, mutagenesis of XRs from *C. tenius* [44,72], *P. stipitis* [13,15,42,58,59], *H. polymorpha* [14], *R. oryzae* [32] and *T. emersonii* [33] were attempted.

Petschacher *et al.* [44] changed the coenzyme selectivity of *C. tenuis* XR by site-directed mutagenesis. The double mutant (Lys274Arg, Asn276Asp) showed 6-fold higher affinity for NADH over NADPH, while the native enzyme had 34-fold higher affinity for NADPH. The apparent dissociation constant K_{iA} (NADPH) for the Lys274Arg, Asn276Asp mutant was 64-fold higher compared to the value for the wild type XR, reflecting the mutant's high discrimination against the binding of NADPH. Furthermore, the Lys274Arg, Asn276Asp mutant retained the catalytic efficiency of the wild-type XR.

Xylose reductase of *P. stipitis* was mutated several times during the last two decades by different investigators, with different techniques, and at different sites. Most investigators used site-directed mutagenesis to introduce single or double mutations at specific positions in the primary sequence of the enzyme, but random mutagenesis approach was used as well in the form of error-prone PCR and combinatorial active site saturation mutagenesis. All these methods emphasized Lys270, Asn272 and Arg276 residues as responsible for the coenzyme selectivity.

First site-directed mutagenesis on *P. stipitis* XR was done by Kostrzynska *et al.* [42] in the course of their investigation in the role of Lys_{270} in the coenzyme and substrate binding. The Lys270Met variant showed 5-16-fold lower affinity for NADPH, without changes in the affinity for NADH, while the K_m value for xylose decreased by 14-fold.

Liang *et al.* [58] changed the coenzyme specificity of *P. stipitis* XR by applying CASTing (combinatorial active site saturation) mutagenesis method to the NAD(P)H binding site of the enzyme. By this method they targeted residues predicted to bind the NAD(P)H, and carried out three cycles of site-saturation mutagenesis to randomize these residues. The best mutant active site, carrying four mutations (Lys270Ser, Asn272Pro, Ser271Gly, Arg276Phe), showed a 13-fold preference for NADH over NADPH, and 42-fold higher catalytic efficiency. The mutant k_{cat} with NADPH lowered about 10 times, while the k_{cat} with NADH was only somewhat lower in comparison with the native enzyme.

A single site-directed mutagenesis of Arg276His and the double site-directed mutagenesis of Lys270Arg, Asn272Asp [59] resulted in 52- and 146-fold improvement in catalytic efficiency compared with the native enzyme. The Lys270Arg, Asn272Asp mutant showed an increase of K_m for NADPH, while Arg276His mutant showed decrease of k_{cat} with NADPH.

Bengtsson *et al.* [13] introduced the Lys270Arg mutation to *P. stipitis* XR and overexpressed it in the genetically modified *S. cerevisiae* strain with deleted *GRE3* and overexpressed native *P. stipitis* non-oxidative PPP enzymes, xylulokinase and XDH. Metabolic flux analysis of the mutant obtained showed that, although the K_m value for NADPH seemed to be about twice lower than the K_m value for NADH, the Lys270Arg XR *in vivo* used more NADH than NADPH.

Runquist *et al*. [15] mutated the cofactor binding region of *P. stipitis* XR by error-prone PCR, and expressed the resulting library in *S. cerevisiae*. After sequential anaerobic batch cultivation, used as the selection process, a strain

harbouring the double XR mutations Asn272Asp, Pro275Gln was enriched from the library. The NADPH/NADH utilization ratio of this mutant was significantly decreased, while V_{max} was about 10-fold higher compared to that of the native enzyme. However, the single Pro275Gln mutation showed negligible effect on metabolic efficiency, while the Asn272Asp mutation was responsible for increased selectivity of the enzyme towards NADH and for increased V_{max}.

As it was mentioned earlier, the XR of *H. polymorpha* can use both NADH and NADPH as cofactors. However, its affinity for NADPH is about 10-fold higher than affinity forNADH. Based on the gene sequence of *C. tenuis* XR Dmytruk *et al*. [14] mutated the cofactor binding site of *H. polymorpha* XR using the site-specific mutagenesis at the positions Lys341 and Asn343. This resulted in a substantial increase of the K_m for NADPH in the Lys341Arg, Asn343Asp mutant, while K_m for NADH remained practically unaffected. Although the mutated XR resulted in lowered specific activity with NADPH and in an increased ethanol productivity, additional overexpression of XDH was needed to gain 2.4-fold improvement in the ethanol production, with concomitant 2.6-fold decrease in the production of xylitol.

Zhang *et al.* [32] performed site-directed mutagenesis of XR from *R. oryzae*. Sequence alignments with different yeast XRs proposed two potential sites for binding of coenzyme - Thr226 and Val274. Replacing of Val274 with Asn, or Thr226 with Gln, respectively, resulted in the 1.4-fold lower affinity for NADH in the Val226Asn mutant, and 11.9-fold higher affinity for NADH in the Thr226Gln mutant.

Fernandes *et al*. [33] constructed Lys271Arg, Asn273Asp double mutant of the *T. emersonii* XR in order to change coenzyme binding preference. The mutated form of the enzyme showed 16-fold higher preference for NADH, compared to the native XR. However, this change in overall preference toward NADH is due to decreased affinity for NADPH and decreased k_{cat} with NADPH, rather than the increased affinity for NADH. Namely, affinity of mutated XR for NADPH became 3.1-fold lower and the affinity for NADH remained unchanged. However, double mutant's k_{cat} was 1.6-fold higher with NADH and 3.2-fold lower with NADPH. Consequently, the mutant catalytic efficiency (k_{cat}/K_m) was 1.4-fold higher with NADH and 10.8-fold lower with NADPH compared to the native XR.

Almost all attempts to change XRs specificity for the coenzyme resulted in decreased affinity towards NADPH without significant increase in affinity for NADH. In some cases, decrease of k_{cat} with NADPH was also achieved, resulting in higher catalytic efficiency of mutated forms of enzyme with NADH as coenzyme. However, all these results showed that interventions in characteristics of individual enzymes were not sufficient to improve the metabolism of xylose to the level needed for industrial application. Thus, a combination of approaches should be utilized in the future in order to construct strains with characteristics that would fulfil demands of industrial production of xylitol or ethanol from lignocellulose materials.

4. Conclusion

Increased global demands for sustainable energy supplies by conversion of lignocellulose biomass into fuels, and for the development of economically effective biological xylitol production, resulted in an increased interest in yeast XRs and the capability of *S. cerevisiae* to metabolize xylose in general. Although significant improvements of xylose utilisation have been made in the last two decades, there are still bottlenecks that need to be addressed in the future. According to the results published so far, it is becoming clear that individual interventions in the *S. cerevisiae* genome are not sufficient to enhance the xylose utilisation to the level necessary for industrial application. In order to increase industrial ethanol production, overexpression of the enzymes catalysing the reactions of non-oxidative part of the PPP and, at the same time, deletion of genes coding for the enzymes catalysing the reactions of oxidative part of the pathway should be attempted. In this way, increased flux through the non-oxidative part of PPP could be obtained resulting in increased production of ethanol and decreased production of CO_2 during fermentation. Simultaneously, alternative way for regeneration of NADPH should be established, possibly by heterologous expression of some $NADP^+$ - dependent enzyme(s). Furthermore, in order to increase xylose-fermenting capabilities of engineered *S. cerevisiae*, the expression of heterologous xylose transporters and the enzymes included in the xylose metabolism, as well as the overexpression (or deletion) of genes coding for XR and XDH, should be combined with adaptive evolution and site directed mutagenesis of targeted enzymes. Furthermore, when combined with genome sequencing, adaptive evolution could be used for identification of possible novel gene targets which could be mutated/ deleted/ overexpressed, to obtain phenotypes of interest.

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6. Conflict of Interest

The authors declare no conflict of interest.

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مروری بر خواص کاتالیزوری ردوکتاز زایلوز قارچی و رویکردهای مهندسی مولکولی به‌منظور بهبود مصرف زایلوز در مخمر

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چکیده

سابقه و هدف: ردوکتاز زایلوزهای مخمری به آنزیم‌های خانواده آلدوکتوردوکتاز تعلق دارد، که تبدیل زایلوز به زایلیتول را کاتالیست می‌کنند. در دو دهه اخیر، ردوکتاز زایلوزهای مخمری به علت اهمیت در تولید اتانول و زایلیتول از زایلوز به روش زیست‌فناوری مورد مطالعات دقیق قرار گرفته است. *ساکارومیسس سرویزیه* به‌علت ایمن (Generally recognized as safe-GRAS) و مقاوم بودن در برابر شرایط سخت، اندامگان (Organism) مناسبی برای تولید صنعتی اتانول و زایلیتول می باشد. به‌علت نبود انتقال‌دهنده‌های اختصاصی زایلوز و فعالیت ناکافی مسیرهای سوخت و ساز برای استفاده از زایلوز، *ساکارومیسس سرویزیه* قادر نیست از زایلوز به‌عنوان تنها منبع کربن استفاده کند. هدف این مقاله مروری است بر تلاش‌های انجام شده برای افزایش پتانسیل ردوکتازهای زایلوز در زیست فناوری و نشان دادن نکات برجسته کاربرد آن می‌باشد.

یافته‌ها و نتیجه‌گیری: به‌منظور ایجاد گونه‌هایی با بهبود توانایی مصرف زایلوز، روش‌های گوناگون به‌کار گرفته شده است که عبارتند از: به‌ترتیب بیش‌بیان (Overexpression) همزمان ژن زایلیتول دهیدروژناز، زایلوز ردوکتاز و آنزیم-های مسیر پنتوز فسفات، بیان نامتجانس ژن انتقال دهنده‌های احتمالی زایلوز یا بیان نامتجانس ژن‌های کد کننده آنزیم‌های درگیر در سوخت و ساز زایلوز. علاوه بر این، تلاش‌ها به‌منظور اصلاح ژنتیکی ردوکتازهای گوناگون زایلوز رو به‌افزایش است. مقاله مروری حاضر دانش امروز را درباره ردوکتاز زایلوز مخمری و راهکارهای گوناگون به‌کار گرفته شده برای بهبود سوخت و ساز زایلوز در مخمر نشان می‌دهد.

تعارض منافع: نویسندگان اعلام می‌کنندکه هیچ تعارض منافعی وجود ندارد.

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- سوخت و ساز زایلوز

***نویسنده مسئول**

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