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Comparison of two models of surface display of xylose reductase in the *Saccharomyces cerevisiae* cell wall

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ABSTRACT

In order to display xylose reductase at the surface of *S. cerevisiae* cells two different gene constructs have been prepared. In the first, xylose reductase gene *GRE3* was fused with two parts of the *CCW12* gene, the N-terminal one coding for the secretion signal sequence, and the C–terminal coding for the glycosylphosphatidylinositol anchoring signal. Transformed cells synthesized xylose reductase and incorporated it in the cell wall through the remnant of the glycosylphosphatidylinositol anchor. The other construct was prepared by fusing the *GRE3* with the *PIR4* gene coding for one of the proteins of the Pir-family containing the characteristic N-terminal repetitive sequence that anchors Pir proteins to β-1,3-glucan. In this way xylose reductase was covalently attached to glucan through its N-terminus. For the expression of the constructs either the *GAL1*, or the *PHO5* promoters have been used. Both strains displayed active xylose reductases and their enzyme properties were compared with the control enzyme bearing the secretion signal sequence but no anchoring signals, thus secreted into the medium. The enzyme displayed through the N-terminal fusion with *PIR4* had higher affinity for xylose than the other construct, but they both expressed somewhat lower affinity than the control enzyme. Similarly, the K_m values for NADPH of both immobilized enzymes were somewhat higher than the K_m of the control XR. Both displayed enzymes, especially the one fused with Pir4, had higher thermal and pH stability than the control, while other enzymatic properties were not significantly impaired by surface immobilization.

1. Introduction

Surface display of proteins by incorporation into the cell walls of different microorganisms is a method established in the last decade as a promising way of creation of new tools for modern biotechnology [1–5]. Both bacterial and yeast cells have been used for this purpose but yeasts have been shown to possess some comparative advantages [6]. The method essentially consists of construction of fusions between heterologous, or homologous non-cell wall proteins with signals that host cells use for localization of autochthonous proteins in the wall. In yeasts there are at least two [7,8] and perhaps even three [9,10] different ways of covalent binding of cell wall proteins to β-1,3-glucan. Most covalently attached proteins carry a C-terminal signal (a stretch of 20–30 small hydrophobic amino acids) for the addition of a glycosylphosphatidylinositol (GPI) anchor. Proteins carrying such signals are transferred to pre-synthesized GPI in the ER, they migrate through the secretory pathway and are eventually exposed at the outer surface of the plasma membrane. Subsequently, GPI anchors are cleaved and the proteins still carrying parts of GPI are transferred to β-1,6-glucan linked further to non-reducing ends of β-1,3-glucan [7]. The other class of proteins covalently attached to β-1,3-glucan comprises members of the Pir family of proteins. Cell walls of *S. cerevisiae* contain four Pir proteins, all sharing high degree of sequence identity and all having a characteristic N-terminal repetitive sequence [11]. It has been shown that these proteins are attached to glucose units of β-1,3-glucan through alkali sensitive ester linkages formed by particular glutamines of the repetitive sequences located at the proteins' N-termini [8]. The addition of GPI-anchoring domains to different heterologous proteins was shown to direct these proteins into the yeast cell wall [12–14] while the addition of the Pir repetitive sequence alone was not efficient [15]. However, the addition of a larger part of a Pir protein lead to covalent incorporation of different proteins in the wall [16]. Besides, there are proteins that have dual localization mechanisms in the wall [10]. Although there were attempts to compare the efficiency of different GPI-anchoring domains in binding heterologous proteins to the yeast cell wall [17], in none of the reports have different immobilization strategies been applied for the display of the same enzyme which would allow their direct comparison [5].

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Yeast xylose reductases (XRs) belong to the aldo-keto reductase enzyme family. They catalyse reduction of xylose to xylitol. In the last twenty years the interest for XRs has intensified, largely due to their importance in xylose bioconversions to ethanol and xylitol. *Saccharomyces cerevisiae* was most often used for industrial production of both ethanol and xylitol, however, it is unable to grow on xylose as the only carbon source due to the lack of specific transporters and insufficient activity of metabolic pathways required for xylose utilisation [18,19]. In order to construct better strains with increased xylose uptake and conversion, heterologous expression of different xylose transporters, and overexpression of pentose phosphate pathway enzymes, XR and xylitol dehydrogenase have been attempted [20–22]. In the same time, attempts to genetically modify different XRs were undertaken. An alternative approach in utilization of yeasts for the reduction of xylose would be to express XR at the yeast surface relocating the reaction from cytosol to external medium, thus avoiding problems with the transport of substrate in the cell. In the same time cells would serve as the solid matrix for genetically immobilized XR. Therefore, the aims of this paper were to create *S. cerevisiae* strains with XR displayed at the surface either through the GPI anchoring domain of the *S. cerevisiae* Ccw12 protein added to the C-terminus, or through the N-terminal fusion with the Pir4 protein, evaluate their potential for the reduction of xylose to xylitol, and compare the XRs obtained by two cell wall incorporation mechanisms.

2. Materials and methods

2.1. Strains and media

Yeast strains used in this work were derived from the wild type BY4741 (MATa; *his3-1; leu2-0; met15-0; ura3-0,* [23]), except the strain VMY4567 (SEY6210 *ccw5:: (kanMX) ccw6:: (kanMX) ccw7:: (kanMX) ccw8:: URA3*), lacking all four Pir proteins [11] that was a derivative of the wild strain SEY6210 (MATα *ura3-52 leu 2–3,112 his3-Δ200 trp1-Δ901 lys2-810 suc2-Δ9 GAL*). All strains were grown in standard yeast nitrogen base selective medium (YNB) supplemented with the required amino acids and 2% sugar (glucose, raffinose or galactose) or in the synthetic phosphate-free medium supplemented with the required amino acids and 2% glucose [24]. Cultures were incubated at 30 ^{0}C, with shaking at 200 rpm for broth cultures.

Yeast transformations were carried out according to the method of Gietz et al. [25]. The list of plasmids used in this work is presented in Table 1. All cloning and transformation experiments were made in *Escherichia coli* strain DH5α. *E. coli* cells were grown in Luria-Bertani (LB) broth or LB plates supplemented with ampicillin to a final concentration of 100 μg/ml as needed. Cultures were incubated at 37°C, with shaking at 200 rpm for broth cultures. Standard procedures were used for all DNA manipulations [26].

2.2. Construction of expression plasmids

In order to construct the genetic cassette for C-terminal immobilization of heterologous proteins, pRS425/RNYCCW12-HA [27] and pBG1805(SCW4) [10] plasmids were used as templates for PCR amplifications. pRS425/RNYCCW12-HA was used as a starting plasmid which was, at the first stage upgraded by replacing the native *CCW12* promoter with the *GAL1* promoter amplified from pBG1805(SCW4) plasmid using primers GalpromXhoF (CAACTCGAGCACCGCGGGAAC GGATTAGAAGCC) and GalCcw12R (GACAGTAGAAAATTGCATCTCCT TGACGTTAAAG). Second PCR fragment was amplified using primers GalCcw12F (CGTCAAGGAGATGCAATTTTCTACTGTC) and Ccw12 R3 (CAAGAGCTCTCCGCGAGGTTCAGGAACCTG) with pRS425/ RNYCCW12-HA as template. Fragments I and II were used in overlap extension PCR reaction to produce DNA fragment containing *GAL1* promoter followed by *RNYCCW12-HA* recombinant gene. This fragment was introduced to pRS425/RNYCCW12-HA plasmid by restriction cloning, using *Xho*I and *Sac*I sites. Resulting plasmid was named pRS425GalRnyCcw12. pRS425GalRnyCcw12 plasmid was then used as a template to construct two PCR fragments with (i) pair of primers PstHAtagF (GCTCTGCAGGGCCGCATCTTTTACCCA) and PoliLHAR (GCGGCCGCTAGATCTCCCGGGAGCGCACTGAGCAGC) for PCR fragment I and (ii) pair of primers PoliLHAF (CCCGGGAGATCTAGCGGCC GCGCTACTCACTCTGTCACC) and CCW12R3 (CAAGAGCTCTCCGCGA GGTTCAGGAACCTG) for PCR fragment II. Fragment I contained secretion signal sequence of Ccw12 and HA tag, while fragment II contained restriction sites for the insertion of the gene of interest, GPI signal and downstream elements of *CCW12* gene. Fragments I and II were used in overlap extension PCR reaction to produce final genetic cassette containing *GAL1* promoter followed by the secretion signal sequence of the Ccw12 and the HA tag. After the HA tag convenient restriction sites for the insertion of the gene of interest were created, followed by the part of the *CCW12* containing the GPI anchoring domain and the downstream genetic elements of the *CCW12*. Genetic cassette was introduced into pRS425GalRnyCcw12 plasmid by restriction cloning, using *Pst*I and *Sac*I restriction sites. In this way the final plasmid pRS425GalCcw12 was created.

Another genetic cassette promoter for C-terminal immobilization of heterologous proteins under control of *PHO5* promoter was created. In construction of this genetic cassette, the first PCR fragment containing *PHO5* promoter was amplified from pPZ plasmid [24] using primers XhoIPHOF (TGCACTCGAGCCCGTCCTGTGGATCCG) and Ccw12PHOR (GACAGTAGAAAATTGCATGGGATCCCCAAACATTGGTAATC). Second PCR fragment containing secretion signal sequence of *CCW12* followed by HA tag, GPI domain and downstream genetic elements of *CCW12* was amplified from the pRS425GalCcw12 plasmid using primers PHOCcw12 F (GATTACCAATGTTTGGGGATCCCATGCAATTTTCTACT GTC) and Ccw12R3 (CAAGAGCTCTCCGCGAGGTTCAGGAACCTG). Fragments I and II were used in overlap extension PCR reaction to

Table 1
Plasmids used in this work.

Plasmid	Description	Origin/marker	Source or reference
pBG1805 (SCW4)	Plasmid encoding *SCW4* (His6-HA-3C-ZZ tag) under control of *GAL1* promoter	AmpR, *URA3*	[10]
pRS425/RNYCCW12-HA	Plasmid encoding *RNYCCW12* (HA- tag) under control of *CCW12* promoter	AmpR, *LEU2*	[9]
pRS425GalRnyCcw12	Plasmid encoding *RNYCCW12* (HA- tag) under control of *GAL1* promoter	pRS425/RNYCCW12-HA/AmpR, *LEU2*	This work
pRS425GalCcw12	Plasmid encoding part of *CCW12* (HA- tag) under control of *GAL1* promoter	pRS425GalRnyCcw12/AmpR, *LEU2*	This work
pCCW5-HA	Plasmid encoding *CCW5* (HA- tag) under control of *CCW5* promoter	AmpR, *LEU2*	[15]
Yep351Pir4	Plasmid encoding *CCW5* (HA- tag) under control of *GAL1* promoter	pCCW5-HA/AmpR, *LEU2*	This work
pRS425GalCcw12XR	Plasmid encoding recombinant *GRE3CCW12* (HA- tag) under control of *GAL1* promoter	pRS425GalCcw12 / AmpR, *LEU2*	This work
pRS425PhoCcw12XR	Plasmid encoding recombinant *GRE3CCW12* (HA- tag) under control of *PHO5* promoter	pRS425 Ccw12XR/AmpR, *LEU2*	This work
pRS425GalCcw12XRStop	Plasmid encoding recombinant *GRE3* (HA- tag) under control of *GAL1* promoter	pRS425Ccw12XR/AmpR, *LEU2*	This work
YEp351Pir4XR	Plasmid encoding recombinant *CCW5GRE3* (HA- tag) under control of *GAL1* promoter	Yep351Pir4 / AmpR, *LEU2*	This work
pPZ	Plasmid encoding *lacZ* under control of *PHO5* promoter	AmpR, *LEU2*	[24]

produce DNA fragment containing *PHO5* promoter followed by secretion signal sequence of *CCW12,* HA tag, GPI domain and downstream genetic elements of *CCW12*. Then, pRS425GalCcw12 plasmid was cut using *Xho*I and *Sac*I restriction sites and the constructed PCR fragment was inserted. The resulting final plasmid was named pRS425PhoCcw12.

In order to construct genetic cassette for N-terminal immobilization of heterologous proteins, a pCCW5-HA [15] and pBG1805(*SCW4*) plasmids were used as templates. Pair of primers GalpromF (GCTGGA GCTCCACCGCGGGAACGGATTAGAAGCC) and PIR4GalR (GACGTTTT TGAATTGCATAGCAGCTCTCCTTGACGTTAAAGTATAG) were used to amplify PCR fragment I with pBG1805(SCW4) as a template. Second PCR fragment was amplified using pair of primers galPIR4F (CTATAC TTTAACGTCAAGGAGAGCTGCTATGCAATTCAAAAACGTC) and Pir4-poliL (CCAAGCTCTAGAAGCTTGTCGACTCGAGGATCCCGGGAATTCG CGGCCGCtAGAAGAAGAAGAAGAAGAAGAACAGTCGACCAAAGAAAC AGC) and pCCW5-HA as a template. Fragments I and II were used in overlap extension PCR reaction to produce final genetic cassette containing *GAL1* promoter followed by the *PIR4/CCW5* gene. After *PIR4/CCW5* gene the spacer region coding for a stretch of eight serine residues was introduced, followed by region consisting of several convenient insertion sites for the insertion of the gene of interest and finally by the 6xHis and HA tags. Genetic cassette was inserted into pCCW5-HA plasmid by restriction cloning using restriction sites *Sac*I and *Xba*I. In this way the final plasmid Yep351Pir4 was created.

The *S. cerevisiae* gene *GRE3* coding for xylose reductase was amplified using wild type *S. cerevisiae* genomic DNA as a template and: (i) pair of primers GRE3FXmaI (TCCCCCCGGGATGTCTTCACTGGTTACTCTTAAT AAC) and GRE3NotIR (ATAAGAATGCGGCCGCGGCAAAAGTGGGGAAT TTACCATC), and (ii) pair of primers NotIGRE3F (AATGAATGCGGCCG CATGTCTTCACTGGTTACTCTTAATAAC) and GRE3XhoCCW5R2 (CCGC TCGAGGGCCCAAAAGTGGGGAATTTACCATC), respectively. The obtained PCR fragment (i) was inserted by restriction cloning in the genetic cassette of pRS425GalCcw12 and pRS425PhoCcw12 plasmids using *Not*I and *Xma*I, while PCR fragment (ii) was inserted by restriction cloning in the genetic cassette of YEp351Pir4 using *Not*I and *Xho*I restriction sites, respectively. Resulting plasmids were pRS425GalCcw12XR, pRS425PhoCcw12XR and YEp351Pir4XR.

pRS425GalCcw12XR plasmid was additionally rearranged in order to remove the part of the recombinant gene coding for GPI signal. pRS425GalCcw12XR was cut with *Sac*I and *Not*I restriction enzymes, after that the restriction sites were blunted and re-ligated to gain the plasmid pRS425GalCcw12XRStop. Recombinant gene in this plasmid contained *GAL1* promoter followed by the secretion signal sequence of the Ccw12 and the HA tag in frame with *GRE3* and the downstream genetic elements of the *CCW12*. All plasmids were validated by DNA sequencing, and the final constructs for the expression of XR, XR-GPI, and Pir-XR are presented in Fig. 1.

2.3. Enzyme assay

The recombinant *S. cerevisiae* cells containing surface displayed XR-GPI or Pir-XR and cells secreting XR were grown overnight at 30 °C in YNB media with 2% raffinose and after that re-inoculated in the fresh media to reach the logarithmic growth phase. Once the log phase was obtained, cells were transferred to the YNB media with 2% galactose and grown overnight at 30 °C. Yeast cells with surface displayed XR-GPI or Pir-XR were harvested (3000 rpm, 5 min), washed two times with distilled water and two times with 50 mM citrate-phosphate buffer pH 5 and resuspended in the same buffer to final OD_{600} = 350. Cell suspension was used for the activity assay of XR-GPI and Pir-XR, respectively. Growth media containing secreted XR were separated from yeast cells (3000 rpm, 5 min) and concentrated ten times by ultrafiltration using Amicon Ultra-15 10 K centrifugal filter devices (10,000 MW cut-off). In the same step the media were replaced by 50 mM citrate-phosphate buffer pH 5.

The recombinant *S. cerevisiae* cells containing surface displayed XR-GPI under control of the *PHO5* promoter were grown overnight at 30 °C in YNB media with 2% glucose and after that re-inoculated in the same fresh media to reach the logarithmic growth phase. Once the log phase was obtained, cells were transferred in the phosphate free synthetic media, containing 0–5 mM K-phosphate, and harvested after 15 h.

The activity of xylose reductase was determined spectrophotometrically by monitoring the decrease in A_{340} upon oxidation of NADPH at 30 °C for 10 min using a modification of the procedure described by Webb and Lee [28]. The reaction mixture was shaken using the thermoshaker TS-100 (Biosan) at 1400 rpm. The standard assay mixture contained 50 mM citrate-phosphate buffer pH 5, 0.1 M xylose, 0.2 mM NADPH and 3.5 OD_{600} units of yeast cells with immobilized XR-GPI or Pir-XR or equivalent volume of secreted XR. Every activity measurement has been performed in triplicate, and every experiment has been repeated three times. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation of 1 μmol of NADPH per min under the above-described assay conditions. Kinetic constants K_m and V_{max} were determined by varying xylose concentration in a range from 50 to 500 mM and NADPH concentration from 0.01 to 0.25 mM. Concentrations of other substrates (galactose, arabinose, fructose, glucose) varied from 100 to 500 mM. The kinetic constants were then estimated using Lineweaver-Burk linear regression plots.

2.4. Effect of temperature on activity and stability of XR

To determine the optimum temperature of the XRs, the activity was measured at temperatures ranging from 30°C to 80°C at pH 5. XR thermal stability was measured by incubating the cells containing surface displayed forms of XR, or secreted XR at 30°C, 40°C and 50°C, respectively, for different time intervals up to 3 h at pH 5. Residual XR activity was assayed under standard assay conditions. The experiment has been repeated three times and average values of individual measurements, together with the corresponding standard deviations were presented in Results.

Fig. 1. Constructs prepared for: a) binding of xylose reductase to cell wall glucan through the fusion with the Pir4 cell wall protein (Pir-XR) expressed under regulation by the *GAL1* promoter; b) binding of xylose reductase to cell wall glucan through the Ccw12 GPI domain (XR-GPI) expressed under regulation by the *GAL1* promoter; c) secretion of xylose reductase into the medium (XR) expressed under regulation by the *GAL1* promoter; d) binding of xylose reductase to cell wall glucan through the Ccw12 GPI domain (XR-GPI) expressed under regulation by the *PHO5* promoter.

2.5. Effect of pH on activity and stability of XR

XR activity was analysed over a pH range from 3.0 to 10.0 to determine its optimum pH. The following buffers were used: 50 mM citrate-phosphate buffer (3.0–5.5), 50 mM sodium phosphate buffer (6.0–8.0) and 50 mM glycine-NaOH buffer (9.0–10.0). The stability of XR at various pH was determined by incubating cells with surface displayed forms of XR or secreted XR at pH 3 (50 mM acetate buffer); pH 4 (50 mM citrate-phosphate buffer); pH 5 (50 mM citrate-phosphate buffer) or pH 6 (50 mM Na-phosphate buffer), respectively, for different time intervals up to 20 h at 4 ^{0}C, then the residual XR activity was assayed under standard assay conditions. The experiment has been repeated three times and average values of individual measurements, together with the corresponding standard deviations were presented in Results.

2.6. Effect of different chemical agents on stability of XRs

Effects of different detergents (1% solution of Triton-X-100, Tween-20, Tween-80, or SDS), organic solvents (20% ethanol), and 1 mM PMSF, EDTA or β-mercaptoethanol, respectively, on the stability of recombinant enzymes were determined by incubating the cells containing surface displayed forms of XR or secreted XR for 1 h at 4 °C in the above solutions. Relative activities of XRs in the absence of the above substances was regarded as 100%. Residual XR activity was assayed under standard assay conditions. The experiment has been repeated three times and average values of individual measurements, together with the corresponding standard deviations were presented in Results.

2.7. Isolation of surface displayed and secreted XR

The cells with surface displayed XRs were harvested (3000 rpm, 5 min), washed twice with water and twice with 50 mM K-phosphate buffer pH 8.0. After that, cells were resuspended in the same buffer and broken with glass beads using BeadBug Microtube Homogenizer (Sigma) for 6 min at maximal speed. Cell walls were separated from intracellular material (8000 rpm, 1 min), washed 4 times with the 50 mM K-phosphate buffer pH 8.0 and boiled 2 times for 10 min in Laemmli buffer to extract non-covalently bound cell wall proteins [11]. After that, cell walls containing Pir-XR were washed 4 times in the 50 mM K-phosphate buffer pH 8.0 and twice in distilled water and then extracted by 30 mM NaOH overnight at 4 °C. Cell walls containing XR-GPI were washed 4 times in the 50 mM K-phosphate buffer pH 8.0 and twice in 50 mM K-phosphate buffer pH 6. Cell walls were resuspended in the same buffer and digested with β-glucanase (NZYTech, Portugal) in concentration of 1 U of β-glucanase per 10 OD_{600} of yeast cell walls, for 2 h at 55 °C. Secreted XR was precipitated by 40% (v/v) acetone at 4 °C for 1 h. The precipitated XR was separated from the supernatant by centrifugation for 1 h at 10,000 rpm. Supernatant was discarded and the precipitate was dried at room temperature for 30 min and solubilized in Laemmli buffer. Proteins were subjected to electrophoresis and detected by Western blot using anti-HA-peroxidase conjugate.

2.8. Electrophoresis and blotting

Electrophoresis was performed in 10% polyacrylamide gel by the method of Laemmli [29]. To visualize proteins with HA tag, the proteins were blotted to nitrocellulose which was then incubated for 1 h in 10 ml of buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 0.1% v/v Triton X-100) with 1% w/v non-fat dry milk, then 1,5 h in the same buffer with anti-HA-peroxidase mouse monoclonal antibodies (Roche) at a 1:8000 dilution. Finally, nitrocellulose was washed with 5 ml of the same buffer for 10 min. This washing step was repeated three times. Finally, blots were developed using the Clarity Western ECL Substrate solution (BioRad) and scans were taken using C-Digit (Li-Cor).

3. Results

Three gene constructs have been prepared in order to attempt and evaluate the surface display of XR at the *S. cerevisiae* cell walls as described in Materials and Methods. In the first construct XR was modified by the N-terminal addition of the secretion signal sequence and the C-terminal GPI-anchoring domain of Ccw12, one of the most abundant GPI anchored cell wall proteins [30]. Besides, the protein has been labelled by the insertion of the hemagglutinin (HA) tag between the end of the secretion signal sequence and the original XR. The obtained protein was designated as XR-GPI. The second construct was obtained by fusing Pir4/Ccw5 protein to the N-terminus of XR and the addition of the HA tag to the C-terminus of the construct. This protein was named Pir-XR. The third construct (designated simply as XR) was the control and it was constructed in the same way as the first one but omitting the GPI-anchoring domain. Thus, control XR was secreted into the medium. In all three constructs XR transcription was under the control of the *GAL1* promoter and the enzymes' synthesis was triggered by shifting the cells to the galactose medium.

Proper localizations of XR, XR-GPI, and Pir-XR were tested by extracting non-covalently linked cell wall proteins by hot SDS, GPI-proteins by β-1,3-glucanase, and Pir proteins by mild alkali. Extracted proteins were investigated by immunoblots and as shown in Fig. 2. XR was recovered from the growth medium, XR-GPI was found in the glucanase extract, while Pir-XR was found in the alkali extract of the walls. This was an expected result showing that all XRs were localized according to the signals contained in their sequences. Second, the question was addressed whether all three XR constructs retained their active conformations. XR activities were compared and it was found that the activity of XR-GPI was 1.97 U / 1 OD_{600} unit of cells, the activity of Pir-XR was 1.68 U/1 OD_{600} unit, while the activity of XR was 10.03 U per the amount of medium corresponding to 1 OD_{600} unit. The K_m values of the three constructs for xylose and NADPH were determined and the results were given in Table 2. It can be seen that XR-GPI had about 2-fold, and Pir-XR 1.5-fold lower affinity for xylose than the control XR. The K_m values of both displayed XRs for NADPH were 2–3 times higher than that of the control XR (Table 2).

Xylose reductases from different sources have quite different specificities for xylose as the preferred substrate [31]. Moreover, Gre3 is generally described as "aldose reductase". Therefore, it was of interest to find out how specific the XR used in this study was and, particularly, if the specificity was influenced by surface immobilization at one or the other side of the enzyme. The results presented in Fig. 3 showed that the enzyme was not particularly specific for xylose but had significant activity with other sugars, as well. The affinities for different sugar substrates were compared in Table 2. All monosaccharides tested could

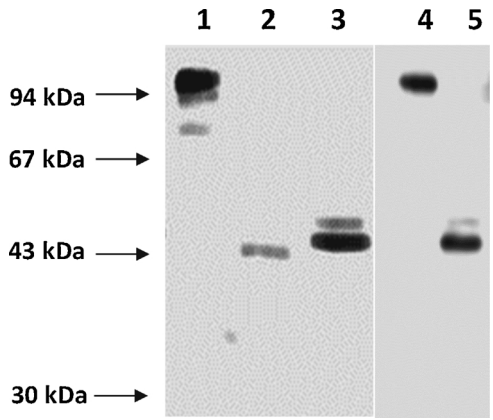

Fig. 2. Western blot of recombinant XR enzymes: 1. Pir-XR SDS extract; 2. secreted XR; 3. XR-GPI SDS extract; 4. Pir-XR NaOH extract; 5. XR-GPI glucanase extract.

Table 2
K_m values for xylose, NADPH, glucose, arabinose, fructose and galactose for XR, Pir-XR and XR-GPI recombinant enzymes.

	xylose (mM)	glucose (mM)	fructose (mM)	Arabinose (mM)	Galactose (mM)	NADPH (μM)
XR	157	790	466	1030	360	60
Pir-XR	227	1920	420	1000	350	170
XR-GPI	343	3190	530	900	410	140

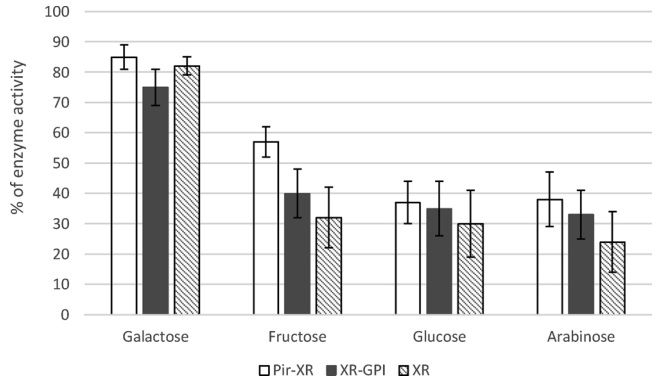

Fig. 3. Activities of recombinant XRs with different monosaccharides as substrates measured under standard assay conditions. Activity obtained with xylose was considered 100%. Every activity measurement has been performed in triplicate, the experiment has been repeated three times and the average values with standard deviations (error bars) are presented.

bind to the enzyme's active site but with lower affinities than xylose. Galactose and fructose were better substrates than arabinose and glucose. Immobilization to glucan has influenced the affinity of XR to glucose but had much less effect on the Km values for the other three sugars.

Different enzyme properties of XR-GPI, and Pir-XR have been investigated and compared to those of the control XR. All three enzymes had the same pH-optimum at pH 5, and 50% of the maximal activity at about pH 3.8. At the basic side of the pH profile free XR's activity dropped to 50% at pH 6.2, while the both immobilized XRs had 50% of the maximal activity at pH 7.5. The three enzymes had similar temperature profiles, as well, with the optimal temperature around 55 ^{0}C, and the 50% of this value at around 30 °C, and 70 °C (not shown). All three enzymes were resistant to the addition of EDTA and were not much impaired by non-ionic detergents like Tweens, or Triton X-100 but they were sensitive to SDS, PMSF and β-mercaptoethanol (Fig. 4). Immobilization of XR decreased to some extent the loss of activity in

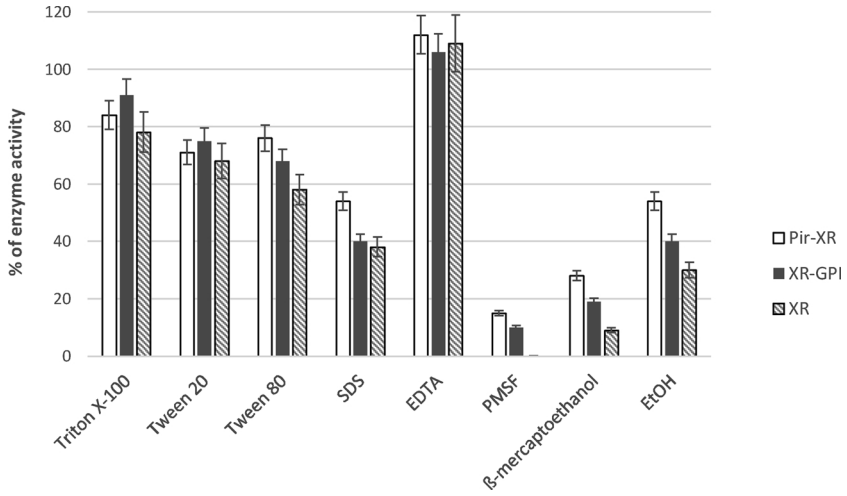

Fig. 4. Effect of 1 mM EDTA, 1% detergents, 1 mM PMSF, 1 mM β-mercaptoethanol, or 20% ethanol, respectively, on recombinant XRs' activities. Every activity measurement has been performed in triplicate, the experiment has been repeated three times and the average values with standard deviations (error bars) are presented.

Table 3
Thermal and pH stability of different forms of xylose reductase. Half-lives of XR, Pir-XR, and XR-GPI are presented in the table.

conditions	XR	Pir-XR	XR-GPI
−20 °C	36 h	84 h	72 h
30 °C	60 min	120 min	120 min
40 °C	45 min	100 min	100 min
50 °C	30 min	60 min	40 min
pH 3.5	1.2 h	1.2 h	1.2 h
pH 5.0	10 h	30 h	30 h
pH 6.0	5 h	20 h	20 h
pH 7.0	4 h	7.5 h	12.5 h

SDS (Pir-XR but not XR-GPI), PMSF, and β-mercaptoethanol.

All three XRs were rather sensitive to the addition of ethanol, as well. Immobilized XRs, particularly Pir-XR, were less sensitive and Pir-XR retained twice the activity of the control XR after a 1-hour incubation in 20% ethanol at 4 °C (Fig. 4).

An important problem in biotechnological utilization of xylose reductases is their rather low thermal stability. Therefore, it was of interest to explore if immobilization of XR at the yeast cell wall could enhance this property. Table 3 shows the stability of control and surface immobilized XRs at different temperatures. Xylose reductases are known to be sensitive enzymes and even simple storage of the enzyme at −20 °C can lead to the enzyme denaturation. It can be seen that the control enzyme lost most of its activity during 5 days of storage at −20 °C, and that both XRs displayed at the yeast surface had enhanced stability. Similar enhancement was obtained when the enzymes were incubated at 30, 40, or 50 °C (Table 3). Besides, immobilization increased the stability of XR at pH 5.0, pH 6.0, and at pH 7.0. There was no enhance of stability at more acidic pH 3.5 (Table 3).

One of the most pronounced disadvantages in the application of surface display methodology is the limited capacity of cell walls to incorporate and covalently bind proteins. A reason for this may be the competition of the heterologous protein with autochthonous cell wall proteins for available glucan binding sites. To check this, the Pir-XR has been expressed in a *S. cerevisiae* mutant VMY5678 lacking all four Pir proteins, and its parental SEY6210 wild type strain. The results showed that mutant cells were able to bind about twice more Pir-XR than the wild type cells (Fig. 5) indicating that indeed the available number of "binding sites" may be the limiting factor at least in case of the Pir-like proteins.

XRs used in the experiments described in this paper (both the immobilized and the control) were obtained by expressing corresponding gene constructs under the control of the *GAL1* promoter. As a strong and easily regulated promoter it was most often used for surface display

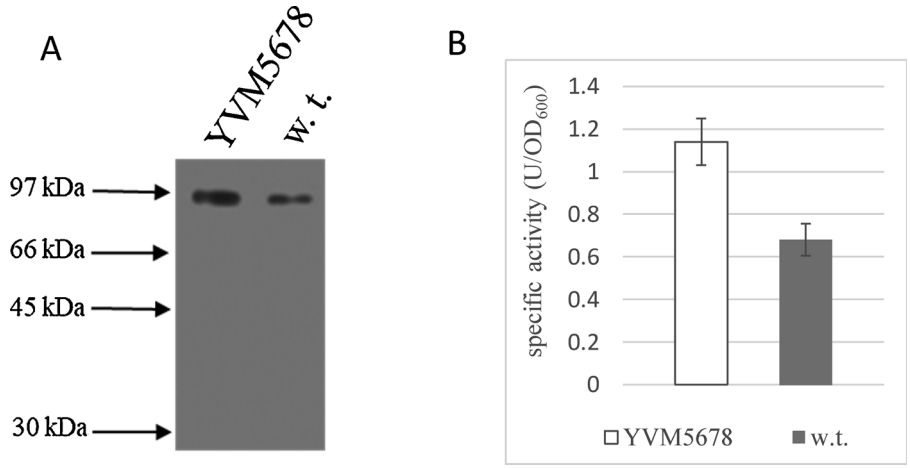

Fig. 5. A. The amount of Pir-XR extracted from the cell walls of the mutant lacking all four Pir proteins (YVM5678), and wild type cells. **B.** Specific activity of Pir-XR in the mutant lacking all four Pir proteins (YVM5678), and in the wild type cells.

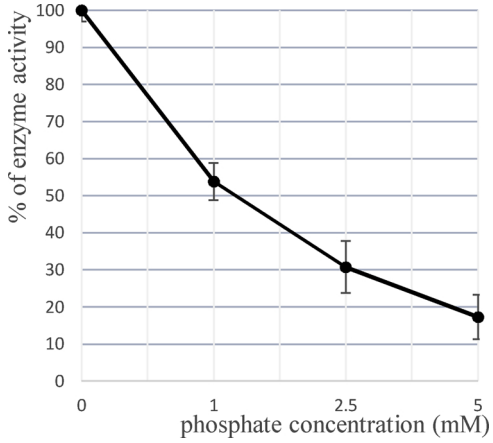

Fig. 6. Relative activity of XR-GPI under *PHO5* promoter at different concentrations of phosphate in growth media. Every activity measurement has been performed in triplicate, the experiment has been repeated three times and the average values with standard deviations (error bars) are presented.

of other proteins in *S. cerevisiae*, as well [5]. Its disadvantage, however, is a rather limited possibility of modulation which may present a problem in cases when intermediate rates of expression are required. Besides, it requires that expression strains grow on galactose. To overcome these limitations a much better controlled level of expression of recombinant proteins could be achieved using the *PHO5* promoter. XR-GPI under *PHO5* promoter in the synthetic media without phosphate showed 88% of activity achieved under *GAL1* promoter upon galactose induction. As shown in Fig. 6, the amount of enzyme displayed at the yeast surface using the *PHO5* promoter varied according to the phosphate concentration in the medium, and could be very precisely adjusted.

4. Discussion

In spite of quite a number of papers reporting surface display of different proteins in yeasts and bacteria, no systematic comparison of different immobilization strategies performed with the same enzyme has been performed. Therefore, this work was an attempt to compare properties of XR immobilized to yeast glucan either through its C-terminus using the GPI anchor, or through its N-terminus by the fusion with a Pir protein. Generally, results showed that both procedures were successful in localizing active XR at the cell surface. The activity of surface displayed enzymes were significantly lower than that of secreted XR indicating that there is a limited cell wall capacity for binding proteins and that the further research should be directed in increasing this potential in order to obtain better biotechnological tools using surface display technology. Our results also showed that better results have been obtained with the Pir-XR in terms of the apparent enzyme's affinity for xylose and its thermal stabilization than those obtained with XR-GPI. However, it is worth noting that both immobilized XRs had K_m values higher than the control for both xylose and NADPH. This could be a result of a conformation change related to the enzyme-substrate interaction, or to the hydrodynamic factors related to the substrate concentrations in the immediate surrounding of the XRs immobilized in the yeast cell wall. C-terminal immobilization of the recombinant enzyme decreased the apparent affinity of the enzyme for xylose more than the N-terminal immobilization. These data may *per se* be interesting for the XR application but without more information about the enzyme structure and the more sophisticated kinetic investigation it is difficult to assess the exact influence of immobilization on the protein conformation and kinetic properties.

Immobilization brought about thermal stabilization of XR. Although the effect was not too strong, every stabilization of XR may be of importance since this class of enzymes is known to be quite unstable [31–33]. Besides, genetic immobilization makes it easy to obtain large quantities of surface displayed XR which clearly diminishes the problem of protein instability.

In the other part of this work the question of the cell wall capacity for binding heterologous proteins has been addressed. In particular, we wanted to examine if autochthonous cell wall proteins limit the amount of surface displayed heterologous enzyme. In case of the Pir proteins, at least, it was found that their depletion from the wall enhanced the binding of Pir-XR significantly increasing the capacity of cell walls for binding Pir-XR. It was not possible to perform the same investigation with XR-GPI due to the high number of GPI anchored proteins in the wall and we can only speculate that yeasts containing less covalently bound cell wall proteins may be better hosts for surface display.

Furthermore, the results showed that a precise control of expression of the recombinant XR under *PHO5* promoter could be achieved. Accordingly, it was possible to control the level of expression of the surface displayed recombinant protein simply by adjusting the concentration of phosphate in the growth medium.

Declarations of interest

None.

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