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(54) Title: ENGINEERING OF XYLOSE REDUCTASE AND OVEREXPRESSION OF XYLITOL DEHYDROGENASE AND XYLULO Kinase IMPROVES XYLOSE ALCOHOLIC FERMENTATION IN THE THERMOTOLERANT YEAST HANSENULA POLYMORPHA

(57) Abstract: Recombinant genetic constructs and strains of *H. polymorpha* having significantly increased ethanol productivity with a simultaneous decreased production of xylitol during high-temperature xylose fermentation are disclosed. The constructs include a *H. polymorpha* *XYL1* gene encoding xylose reductase mutated to decrease affinity of the enzyme toward NADPH. The modified version of *XYL1* gene under control of a strong constitutive *HpGAP* promoter was overexpressed in a $\Delta xyl1$ background. A recombinant *H. polymorpha* strain overexpressing the mutated enzyme together with native xylitol dehydrogenase and xylulokinase in the $\Delta xyl1$ background was also constructed. Xylose consumption, ethanol and xylitol production by the constructed strain were evaluated during high-temperature xylose fermentation (48°C). A significant increase in ethanol productivity (up to 7.4 times) was shown in the recombinant strain as compared with the wild type strain. Moreover, the xylitol production by the recombinant strain was reduced considerably: 0.9 mgx(Lxh)⁻¹ versus 4.2 mgx(Lxh)⁻¹ by the wild type strain.



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**Title: ENGINEERING OF XYLOSE REDUCTASE AND OVEREXPRESSION OF
XYLITOL DEHYDROGENASE AND XYLULOKINASE IMPROVES XYLOSE
ALCOHOLIC FERMENTATION IN THE THERMOTOLERANT YEAST
HANSENULA POLYMORPHA**

Priority and Incorporation by Reference

- [0001] This application claims priority to U.S. provisional application No. 61/057,515 filed May 30, 2008, which is incorporated by reference in its entirety, including all references cited therein (repeated herein) to the extent such references aid in understanding the invention or in obtaining the materials and methods that would facilitate the practice of the invention. If the content of a cited reference conflicts with teaching of the present application, the present application shall be deemed the controlling understanding.

Technical Field

- [0002] This application relates to the field of cellulosic ethanol production by fermentation, particularly to fermentation of xylose containing sources, more particularly to recombinant *H. polymorpha* strains useful for the production of ethanol by fermentation of xylose, and still more particularly to strains of *H. polymorpha* that overexpress a mutant *H. polymorpha* xylose reductase having altered affinity for NADPH together with either endogenous or recombinant xylose dehydrogenase and xylulokinase, that achieve enhanced ethanol production by fermentation on xylose containing media.

Background

- [0003] Ethanol produced from lignocellulosics is an environmentally friendly alternative to fossil fuels. As a substantial fraction of lignocellulose material consists of xylose, it is necessary to ferment efficiently xylose to ethanol to make the process cost-effective [1].
- [0004] Some yeasts, filamentous fungi and bacteria are able to convert xylose to ethanol. Yeasts and most of other fungi first reduce xylose to xylitol using xylose reductase, which strongly prefers NADPH as coenzyme, EC 1.1.1.21 (XR). Then they oxidize xylitol to xylulose with strictly NAD-dependent xylitol dehydrogenase, EC 1.1.1.9 (XDH) [2]. The difference in cofactor specificity results in redox imbalance that leads to decreasing ethanol production and accumulation of xylitol [3, 4, 5, 6, 7, 8]. The xylitol production has been reduced by metabolic engineering directed to optimize the expression levels of XR and XDH [9, 10, 11, 12], change the cofactor specificity of XR from NADPH to NADH [13, 13a], or modify the redox metabolism of the host cell [14, 15, 16]. The other used approach to bypass redox imbalance during xylose fermentation was based on expression of fungal or bacterial xylose isomerase, EC 5.3.1.5 (XI) which converts xylose directly to xylulose and does not require redox cofactors [17, 18]

- [0005] The additional overexpression of xylulokinase, EC 2.7.1.17 (XK) (the third enzyme in the xylose metabolism) that converts xylulose to xylulose-5-phosphate, which enters the pentose phosphate pathway and then into the central metabolism, has been shown to enhance both aerobic and anaerobic xylose utilization in XR-XDH- as well as XI carrying strains [12, 19]. Overexpression of XK is necessary to overcome the naturally low expression level of this enzyme [3, 5]. The overexpression resulted in more efficient conversion of xylose to ethanol [5, 20].
- [0006] The thermotolerant methylotrophic yeast *Hansenula polymorpha* is capable of alcoholic fermentation of xylose at elevated temperatures (45 - 48 °C) [21, 22, 23]. This property of *H. polymorpha* makes it a good candidate for use in an efficient process of simultaneous saccharification and fermentation (SSF). SSF combines enzymatic hydrolysis of lignocellulosic material with subsequent fermentation of released sugars in the same vessel. Major advantages of exploring the utility of *H. polymorpha* for ethanol production from cellulosic material using are this yeast has (i) well developed methods of molecular genetics and (ii) the availability of a whole genome sequence for a model strain CBS4732 [24; 25].

Summary

- [0007] The present disclosure describes the construction of recombinant *H. polymorpha* strains that overexpress a modified XR (K341R N343D) together with native XDH and XK on the $\Delta xy11$ background. Xylose consumption, ethanol and xylitol production of the strain in comparison with those of strains overexpressing the native XR, XDH and XK are presented for comparison to demonstrate that overexpression of the altered XR enzyme enhances ethanol yield when the *H. polymorpha* strain is grown on media containing xylose. Related recombinant nucleic acids, the mutant enzyme, and methods of using the same for ethanol fermentation on xylose are also disclosed herein.

Description of the Drawings

- [0008] **Figure 1.** Linear schemes of the plasmids used in this disclosure: pX1M-Z, pX1N-Z, pX1M-Z-X2, pX1N-Z-X2 and pGLG61/HpXYL3. Expression cassettes prGAP-XYL1-trAOX, prGAP-XYL2-trXYL2 and prGAP-XYL3-trAOX are shown as white, gray and dotted boxes, respectively. The modified version of XYL1 ORF is shown as black box. The zeocin resistance gene (*Zeo'*) and geneticin resistance gene (*APH*), linked to an impaired constitutive gene promoter, encoding glyceraldehydephosphate dehydrogenase

- (*GAP*) are designated with the hatched lines. *H. polymorpha* *LEU2* gene and the telomeric region (TEL188) [29] as an autonomously replicating sequence are shown as cross-hatched lines. Origin of replication ORI and ampicillin resistance gene (*bla*) - arrows. Restriction sites: P, *Pst*I; H, *Hind*III; S, *Sac*I; Sl, *Sal*I; B, *Bam*HI; ScII, *Sac*II; Xb, *Xba*I; RI, *Eco*RI; NdI, *Nde*I; N, *Not*I.
- [0009] **Figure 2.** Alignment of XRs cofactor binding site sequences from several xylose-utilizing yeasts against the *H. polymorpha* *XYL1* sequence. Conserved sequences are in bold. Underlined amino acids were changed (K→R and N→D).
- [0010] **Figure 3.** Comparison of xylose fermentations at 48 °C by CBS4732 (A), XRn/XDH/XK (B) and XRm/XDH/XK (C). Symbols: ethanol (■), xylitol (●), biomass (▲) and xylose (◆).
- [0011] **Figure 4.** Table 1 showing strains and plasmids used in the present disclosure.
- [0012] **Figure 5.** Table 2 showing XR, XDH, XK activities, as well as the ethanol and xylitol productivity of *H. polymorpha* transformants described herein in comparison to a control strain.
- [0013] **Figure 6.** Table 3 showing a comparison of xylose fermentations at 48 °C by 2Et-/2xPDC1 and 2Et-/2xPDC1/XRm/XDH.
- [0014] **Figure 7.** Figure 7A shows the DNA sequence for the cloned *H. polymorpha* *xyll* gene encoding xylose reductase with the start and stop codons highlighted. Figure 7B shows the protein sequence for the cloned *H. polymorpha* xylose reductase protein with the NADPH binding site prior to mutagenesis being highlighted.

Detailed Description of Methods, Strains, and Results

Strains and media

- [0015] Yeast strains *H. polymorpha* CBS4732s (*leu2-2*) [26], *Δxyll* [22] and transformants (Table 1) were grown on YPD (0.5% yeast extract, 1% peptone, 2% glucose) or minimal medium (0.67% YNB without amino acids, 4% xylose or 2% glucose) at 37 °C. For the CBS4732s strain leucine (40 mg L⁻¹) was supplemented into the medium. For selection of yeast transformants on YPD, 130 – 150 mg L⁻¹ of zeocin or 0.5 – 0.6 mg L⁻¹ of G418 were added.
- [0016] The *E. coli* DH5α strain (Φ80d*lacZ*Δ*M15*, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_K⁻, m_K⁺), *supE44*, *relA1*, *deoR*, Δ(*lacZYA-argF*)U169) was used as a host for propagation of plasmids. The strain DH5α was grown at 37 °C in LB medium as described previously [27]. Transformed *E. coli* cells were maintained on a medium containing 100 mg L⁻¹ of ampicillin.

Molecular-biology techniques

- [0017] Standard cloning techniques were applied [27]. Genomic DNA of *H. polymorpha* was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Restriction endonucleases and DNA ligase (Fermentas, Vilnius, Lithuania) were used according to the manufacturer specifications. Plasmid isolation from *E. coli* was performed with the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). PCR-amplification of the fragments of interest was done with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) according to the manufacturer specification. PCRs were performed in GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Transformation of the yeast *H. polymorpha* by electroporation was carried out as described previously [28].

Plasmid construction

- [0018] Recombinant plasmids pX1N-Z and pX1M-Z bearing native and modified version of XR, respectively, were constructed on the basis of the plasmid pUC57 (Fermentas, Vilnius, Lithuania). *Bam*HI/*Sac*I fragment with the *HpGAP* promoter and *HpAOX* terminator from the plasmid pKO8-GAPpr [22] was cloned into the *Bam*HI-*Sac*I digested plasmid pUC57 with preliminary eliminated restriction sites *Nde*I and *Hind*III. In the resulting plasmid restriction sites *Nde*I and *Not*I located between the *HpGAP* promoter and *HpAOX* terminator were removed and the unique *Hind*III site arose. The ORF of *XYL1* was PCR-amplified from genomic DNA of CBS4732 using pair of primers HpX1for (CCC AAG CTT ATG CAC ACG CAG ATT AGC AAA AAT CTT G) and HpX1rev (CGC AAG CTT TTA GAT AAA GGT TGG AAT TTC GTT CCA GGT CC) and cloned into the *Hind*III site to create expression cassette pr*GAP-XYL1-trAOX* (restriction sites are underlined in all primers). Modification of XR gene was performed via the overlap PCR. The pairs of primers HpX1Mfor (CAT CTT GGT CAT TCC AAG GTC CGA CCA AAA GGA GAG ACT G) and HpX1Mrev (CAG TCT CTC CTT TTG GTC GGA CCT TGG AAT GAC CAA GAT G) were used to produce K341→R and N343→D substitutions in resulting modified XR (mismatched bases for the mutation are shown in bold). Primers HpX1for and HpX1rev were used for cloning of modified version of XR gene as described above for the native gene. The yeast selective marker conferring resistance to zeocin was PCR-amplified from the plasmid pPICZB (Invitrogen) using pair of primers Ko58 (CGG GGT ACC TG CAG ATA ACT TCG

- TAT AGC ATA C) and Ko59 (CGG GGT ACC TG CAG TAA TTC GCT TCG GAT AAC) and cloned into the *Pst*I linearized vectors creating pX1N-Z or pX1M-Z (Fig. 1).
- [0019] The *H. polymorpha* *XYL2* gene with own terminator and the *HpGAP* promoter were amplified from the genomic DNA of CBS4732 using the corresponding pairs of primers L1 (CTC GGA TCC CAA TTA TCA TTA ATA ATC) / Ko135 (CAG CAG AAG GAT TGT TCA TTT TGT TTC TAT ATT ATC) and Ko134 (GAT AAT ATA GAA ACA AAA TGA ACA ATC CTT CTG CTG) / Ko133 (ACA GGA TCC ATC CAT GAG AAA CG). Primers L1 and Ko133 were used for obtaining the fragment containing the *XYL2* gene with own terminator driven with the *HpGAP* promoter by the overlap PCR. This fragment was cloned into the *Bam*HI linearized plasmids pX1N-Z i pX1M-Z, resulting in the recombinant constructs pX1N-Z-X2 and pX1M-Z-X2, respectively (Fig. 1).
- [0020] The expression cassette containing *prGAP-XYL3-trAOX* was obtained as *Sac*II restriction fragment from the plasmid pKO8/GAP/HpXYL3 [23] and cloned into the *Sac*II linearized plasmids pGLG61 [29]. The resulting plasmid was designated pGLG61/HpXYL3 (Fig. 1). The accuracy of constructed plasmids was verified by sequencing. Constructed plasmids are presented in Table 1.

Biochemical methods

- [0021] The XR activity in cell extracts was determined spectrophotometrically at 37 °C. The XR assay mixture contained: Tris-HCl buffer (pH 7.0) 100 mM, NADPH 0.15 mM and xylose 350 mM. The reaction was started with cell extract addition [3]. To evaluate K_M towards NADPH or NADH the XR activities were measured with four different concentrations of cofactors 20, 50, 100 and 150 μ M (each in triplicate).
- [0022] The XDH activity in cell extracts was determined spectrophotometrically at 37 °C. The XDH assay mixture contained: Tris-HCl buffer (pH 8.8) 100 mM, $MgCl_2$ 10 mM, NAD 3 mM and xylitol 300mM. The reaction was started with cell extract addition [3].
- [0023] The XK activity in cell extracts was determined spectrophotometrically at 37 °C as was described before [30], with some modifications. The XK assay mixture contained: Tris-HCl buffer (pH 7.8) 50 mM, $MgCl_2$ 5 mM, NADH 0.2 mM, phosphoenolpyruvate 1 mM, D-xylulose 8.5 mM, lactate dehydrogenase (EC 1.1.1.27) (Fluka, St. Louis, MO, USA) 10 U, pyruvate kinase (EC 2.7.1.40) (Fluka, St. Louis, MO, USA) 0.05 U, and ATP 2 mM. The reaction was started with addition of cell extract. For the XK assay, another blank without pyruvate kinase and lactate dehydrogenase was used to minimize the interference of XDH activity in *H. polymorpha*.

[0024] All assay experiments were repeated at least twice.

Analyses

[0025] Cells of transformants were grown in the rich YPX medium (1% yeast extract, 2% peptone, 4% xylose) during 2 days and inoculated into the YNB medium with 12% xylose. Fermentation was carried out at the temperature of 48 °C with limited aeration (140 revolutions $\times$ min⁻¹). Concentrations of ethanol in medium were determined using alcohol oxidase/peroxidase-based enzymatic kit “Alcotest” [31]. Concentrations of xylitol in medium were determined enzymatically as described earlier (Enzymatic determination of D-sorbitol and xylitol, R-Biopharm GmbH, Darmstadt, Germany) with slight modifications. Nitrotriazolium Blue (NTB) 12 mM and phenazine methosulfate 15 mM were used instead of iodinitrotriazolium chloride and diaphorase, respectively. The absorbance of the reduced NTB was measured at 570 nm. Concentrations of xylose from fermentation in mineral medium were analyzed by chemical method as was described before [32].

[0026] Experiments were performed at least twice.

Results

Engineering of XR

[0027] To improve alcoholic fermentation of xylose and decrease xylitol formation, XR of *H. polymorpha* has been subjected to site-specific mutagenesis to reduce its affinity for NADPH. The amino acid sequence of the cofactor binding site of *H. polymorpha* XR (SEQ ID NO: 1) shows strict homology to the corresponding site of other xylose-utilizing yeasts (Fig. 2). In the present work the inventors substitute lysine and asparagine for arginine and aspartic acid at amino-acid positions 341 and 343, respectively to obtain a mutated *H. polymorpha* XR protein having the cofactor binding site of SEQ. ID NO: 2. These substitutions resemble those developed for successful modification of XR cofactor specificity in *Candida tenuis* [33].

Strain construction

[0028] To generate strains with overexpression of native or modified versions of XR or strains with simultaneous overexpression of native or modified XR together with XDH, the *H. polymorpha* $\Delta xyl1$ [22] strain was transformed with *SacI* linearized plasmids pX1N-Z and pX1M-Z or pX1N-Z-X2 and pX1M-Z-X2, respectively. The transformants were grown on YPD medium supplemented with zeocin. The presence of expression cassettes

in the transformants was examined by PCR using corresponding primers. To express the XK, the recombinant plasmid pGLG61/HpXYL3 was transformed into the recipient strain *H. polymorpha* overexpressing native or modified versions of XR and XDH. The transformants were grown on YPD medium in the presence of increasing concentrations of G418. The highest concentration of G418, which allows the transformants to grow, was $0.4 \text{ mg} \times \text{mL}^{-1}$. Colonies able to grow on the selective medium appeared after 3 days of incubation with frequency of up to 20 transformants $\times \text{mg}^{-1}$ DNA. The transformants were stabilized by cultivation in non-selective media for 10–12 generations with further shifting to the selective media with G418. The presence of recombinant *XYL3* gene driven by the *HpGAP* promoter in genomic DNA of stable transformants was proven by PCR. As pGLG61-based plasmids promote multiple integration into the genome of recipient strains [29], constructed strains were examined by Southern hybridisation to select recombinant strains with equal amount of XK expression cassette. The strains bearing 3 copies of XK expression cassette were selected (data not shown). Constructed yeast strains are represented in Table 1.

Biochemical analysis of constructed strains

[0029] Biochemical properties of XR in one of constructed recombinant strains (designated XRm) were studied. Specific activities of XR (with both cofactors NADPH and NADH), XDH and XK as well as affinities of native XR (XRn) and engineered XRm were measured (Table 2). XRm was characterized by K_M of $152 \text{ } \mu\text{M}$ for NADPH using xylose as a substrate, which is 17 times higher than the K_M for NADPH of the native XR ($9 \text{ } \mu\text{M}$). The K_M of engineered XRm for NADH remained nearly unchanged ($112 \text{ } \mu\text{M}$). Specific activity of XR with NADPH in the XRm strain decreased 4.8 times compared with the strain overexpressing native XR. The specific activity of XR with NADH in both strains remained unchanged. Strains XRn/XDH and XRm/XDH with additional overexpressing XDH possessed two-fold increase in the specific activity of XDH compared with the wild type strain. Overexpression of XK in strains XRn/XDH/XK and XRm/XDH/XK resulted in up to 2.4-fold increase in specific activity of XK as compared to CBS4732 (Table 2).

Xylose fermentation

[0030] Xylose fermentation by the constructed strains was compared in batch cultures with limited aeration. A mineral medium containing xylose (12%) and initial biomass concentration $2 \text{ g (dry weight)} \times \text{L}^{-1}$ were used. Results of ethanol and xylitol production

by the constructed strains are shown in Table 2. Ethanol productivity of the XRm strain was $9.8 \text{ mg} \times (\text{L} \times \text{h})^{-1}$, which is 1.5- and 1.3-fold higher than the productivity of the XRn and the wild-type strain CBS4732, respectively. Xylitol production of these strains varied insignificantly. Ethanol productivity of the strain XRm/XDH ($18.4 \text{ mg} \times (\text{L} \times \text{h})^{-1}$) was increased 1.5 and 2.4 times as compared to XRn/XDH and CBS4732, respectively. Strain XRm/XDH possessed 1.3- and 2.6-fold reduction in xylitol production compared with XRn/XDH and CBS4732 strains. Ethanol productivity of the strain XRm/XDH/XK ($54.7 \text{ mg} \times (\text{L} \times \text{h})^{-1}$) was 4- and 7.4-fold higher compared to those of the strain XRn/XDH/XK ($13.8 \text{ mg} \times (\text{L} \times \text{h})^{-1}$) and CBS4732 ($7.5 \text{ mg} \times (\text{L} \times \text{h})^{-1}$). The xylitol production of the strain XRm/XDH/XK was significantly reduced to $0.9 \text{ mg} \times (\text{L} \times \text{h})^{-1}$, which is 4.7- and 3-fold lower than those of the XRn/XDH/XK and control strain, respectively. Representative fermentation profiles for the strains XRn/XDH/XK, XRm/XDH/XK and CBS4732 are shown in Figure 3. It has to be mentioned, that the consumption of xylose by *H. polymorpha* strains during the fermentation is low. Ethanol produced in the initial stage of xylose fermentation is reutilized after 1-2 days of the fermentation.

Expression of the XR mutant, XD, and XK genes in a *H. polymorpha* strain that over expresses pyruvate decarboxylase for improved alcoholic fermentation of xylose at elevated temperature (48° C).

- [0031] To improve alcoholic fermentation of xylose the engineered version of xylose reductase (XR) together with native xylitol dehydrogenase (XDH) were overexpressed in a recombinant *H. polymorpha* strain with an elevated level of pyruvate decarboxylase activity. Construction and biochemical characteristics of the initial strain 2Et⁻/2xPDC1 have been described earlier [35, 36].
- [0032] To generate strains with simultaneous overexpression of modified versions of XR together with XDH, the *H. polymorpha* 2Et⁻/2xPDC1 strain was transformed with *SacI* linearized plasmid pX1M-Z-X2 (Fig. 1) [37]. Transformants were grown on YPD medium supplemented with zeocin ($150 \text{ mg} \times \text{L}^{-1}$). Colonies able to grow on the selective medium appeared after 3 days of incubation with frequency of up to 40 transformants $\times \text{mg}^{-1}$ DNA. The transformants were stabilized by cultivation in non-selective medium for 10–12 generations with further shifting to the selective medium with zeocin. The presence of expression cassettes in the transformants was examined by PCR using primers L1/HpX1rev for the expression cassette bearing modified *XYL1* gene under the control of strong constitutive promoter *HpGAP* and primers L1/Ko133 for the

- expression cassette bearing *XYL2* gene under the same promoter. The sequences of used primers as well as modification of *XYL1* gene are described in the article Dmytruk O. et al. [37].
- [0033] Xylose fermentation by the constructed strain was examined in batch cultures with limited aeration at 48°C. A mineral medium containing xylose (12%) and initial biomass concentration of 2 g (dry weight)×L⁻¹ were used. Results of ethanol production by the constructed strains are shown in Table 3. Ethanol productivity of the 2Et⁻/2xPDC1/XRm/XDH strain was 0.11 g×(L×h)⁻¹, which is 2.7-fold higher than the productivity of the initial strain 2Et⁻/2xPDC. Representative fermentation profiles for the strains 2Et⁻/2xPDC1 and 2Et⁻/2xPDC1/XRm/XDH are shown in Figure 5.
- [0034] Overexpression of engineered XR together with native XDH obviously neutralize redox imbalance in constructed recombinant strain of *H. polymorpha*, leading to the significant improvement of ethanol productivity during xylose fermentation.

Discussion

- [0035] As was described earlier [6], natural xylose-utilizing yeasts display alcoholic fermentation only when their XR possessed NADH-linked activity. The XR of *H. polymorpha* belongs to enzymes with dual cofactor specificity, however NADPH is strongly preferred (>10-fold). In the present case, we focused our efforts on engineering the XR with increased K_M for NADPH. Lysine and asparagine residues were substituted for arginine and aspartic acid, respectively, at the positions 341 and 343 in the frame of cofactor binding site using the site-specific mutagenesis, according to data for the XR gene of *C. tenuis* [33]. The modified version of XR gene under control of the strong constitutive *HpGAP* promoter was overexpressed on the $\Delta xyl1$ background. It resulted in significant increase of K_M for NADPH, while K_M for NADH remained nearly unchanged. Obtained results are in good agreement with reported features of modified XR from *C. tenuis* [33]. The constructed XRm strain showed a slight increase in ethanol productivity as compared to the wild type strain, while the overexpression of native XR had no positive effect. Xylitol production of these strains varied insignificantly. It has to be emphasized that mutated XR reveals significantly lower specific activity with NADPH which resulted in increase of ethanol productivity of the XRm. For further improvement of ethanol production, XDH was expressed together with the modified XR. Overexpression of enzymes for initial two stages of xylose utilization pathway resulted in the 2.4-fold improvement of ethanol productivity accompanied by the 2.6-fold decrease of xylitol production.

[0036] In our previous work we developed *H. polymorpha* strains co-overexpressing *E. coli* XI and own XK. The strains were characterized with significant improvement of ethanol production during xylose fermentation [23]. In the present study, the constructed strain XRm/XDH/XK overexpressing the modified XR together with XDH and XK is characterized with significant increase in ethanol productivity (up to 7.4 times) as compared to the wild type strain. Importantly, xylitol production by this strain is reduced considerably: $0.9 \text{ mg} \times (\text{L} \times \text{h})^{-1}$ versus $4.2 \text{ mg} \times (\text{L} \times \text{h})^{-1}$ by the wild type strain. Additional overexpression of XDH and XDH together with XK led to a gradual increase in ethanol productivity and simultaneously a decrease in xylitol production. It may be assumed that the initial stages of xylose utilization are limiting in alcoholic fermentation of xylose in *H. polymorpha*. In Figure 3, the fermentation profiles of XRm/XDH/XK and XRn/XDH/XK are represented. The consumption of xylose by both constructed *H. polymorpha* strains and the wild type strain during the fermentation was rather low (Fig. 3). This may suggest that xylose uptake in *H. polymorpha* is quite inefficient and corresponding genes coding putative xylose transporters should be cloned and overexpressed. In addition, bottlenecks downstream of XR cannot be excluded and arrant further investigation. The fermentation profile revealed reutilization of synthesized ethanol. The reason of this phenomenon is not understood. In another study the present inventors have isolated a *H. polymorpha* 2EtOH- mutant which is characterized by significant decrease in synthesized ethanol consumption (Ishchuk et al., unpublished). The molecular nature of the corresponding mutation, however, remains unknown.

[0037] Recombinant strains of *H. polymorpha* constructed in this study showed significant increase in ethanol productivity during high-temperature xylose fermentation. On the other hand, ethanol production from xylose is still very low as compared to the best current xylose fermenting strains [13a, 17, 34]. Therefore, further efforts have to be applied to improve the xylose alcoholic fermentation in the thermotolerant yeast *H. polymorpha*.

Conclusion

[0038] In the present work, co-overexpression of mutated form of XR (K341→R N343→D) together with native XDH and XR in a strain carrying the Δxyl deletion resulted in significant increase (7.4 fold) in ethanol productivity with simultaneous reduction of xylitol production during high-temperature xylose fermentation. The same construction integrated into the chromosome of a *H. polymorpha* strain that a over expresses the *H.*

polymorpha pyruvate decarboxylase gene *PDC1*, but lacking the $\Delta xy1$ deletion also increases ethanol fermentation from xylose by nearly a factor of three. It is therefore expected that the combination of overexpression of the *PDC1* gene and the mutated XR gene in a *H. polymorpha* strain also carrying the $\Delta xy1$ deletion will enhance ethanol production from xylose even further.

Incorporation by Reference

[0039] Each of the following references are cited herein to provide a better understanding of the inventions disclosed herein and to provide descriptions of techniques, sources and materials that will further enable one of ordinary skill in the art to make and use the materials and processes described herein. Accordingly, each of the following references are incorporated herein by reference in their entirety, unless any disclosure provided herein conflicts with the incorporated reference, in which case the conflicting subject matter disclosed herein controls over the cited reference.

Terms in the Claims

[0040] In the claims that follow the Reference section hereafter, the term “gene” is a shorthand expression that means any polynucleotide encoding the enzyme identified by the gene named. “Unless expressly stated in the context of the claims,” “gene” may, but does not necessarily include, non-coding sequences. Unless otherwise stated, the polynucleotide may have the same primary structure as the named gene that is endogenous in the genome of an organism, or be a recombinant form of the named gene linked to other polynucleotide elements, or be a synthetic form of the named gene, or be a mutated form where various elements in the named gene have been changed but the gene still encodes an operable form of the identified enzyme. The term “mutated” means any change in the named gene that makes it different from the endogenous form of the gene. “Native” means the endogenous structure of the gene as it exists in the genome of the organism.

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We claim:

1. A recombinant *H. polymorpha* strain comprising at least one recombinant nucleic acid that singularly or in combination, includes at least one gene encoding a xylitol dehydrogenase enzyme, at least one gene encoding a xylose reductase enzyme having a higher binding affinity for NAD(H) than for NADP(H), and at least one gene encoding a xylulokinase enzyme, each of said genes being operably linked to at least one promoter that overexpresses said genes in said *H. polymorpha* strain and where said recombinant *H. polymorpha* strain produces a greater amount of ethanol when fermented in a media containing xylose than a parent *H. polymorpha* strain not containing said genes.
2. The recombinant *H. polymorpha* strain of claim 1 wherein each of said genes of the recombinant nucleic acid is integrated into the *H. polymorpha* chromosome.
3. The recombinant *H. polymorpha* strain of claim 1 wherein the *H. polymorpha* strain has a mutation rendering at least one native gene encoding a xylose reductase inoperable.
4. The recombinant *H. polymorpha* strain of claim 1 wherein each of said genes is over expressed in the *H. polymorpha* strain relative to the parent *H. polymorpha* strain.
5. The recombinant *H. polymorpha* strain of claim 1 further comprising at least one sequence encoding a pyruvate decarboxylase enzyme operably linked to a promoter that overexpresses said pyruvate decarboxylase enzyme in the recombinant *H. polymorpha* strain.
6. The recombinant *H. polymorpha* strain of claim 1 wherein at least one promoter comprises a HpGAP promoter obtained from *H. polymorpha*.
7. The recombinant *H. polymorpha* strain of claim 1 wherein the recombinant xylose reductase gene is a mutant of a native *H. polymorpha* xylose reductase gene.
8. The recombinant *H. polymorpha* strain of claim 7 wherein the xylose reductase gene has a mutation in the native *H. polymorpha* xylose reductase gene that changes a first codon encoding lysine at position 341 to a codon that encodes arginine and a mutation that changes a second codon expressing asparagine at position 343 to a codon the encodes aspartic acid.
9. A process for making ethanol comprising growing the recombinant *H. polymorpha* strain of any one of claims 1-8 in a media comprising xylose under conditions that cause the recombinant *H. polymorpha* to make ethanol.
10. A recombinant nucleic acid comprising a sequence encoding a xylose reductase gene from *H. polymorpha*.

11. The recombinant nucleic acid of claim 10 wherein the sequence encoding the xylose reductase encodes an NADPH/NADH binding domain according to SEQ. ID NO: 1.
12. The recombinant nucleic acid of claim 10 wherein the sequence encoding the xylose reductase encodes an NADPH/NADH binding domain according to SEQ. ID NO: 2.
13. The recombinant nucleic acid of claim 10 according to SEQ. ID NO: 3.
14. The recombinant nucleic acid of claim 10 that encodes a protein according to SEQ. ID NO: 4.
15. The recombinant nucleic acid of claim 10 that encodes a protein according to SEQ. ID NO: 4 except having a mutation in the NADPH/NADH binding domain according to SEQ. ID NO: 2.

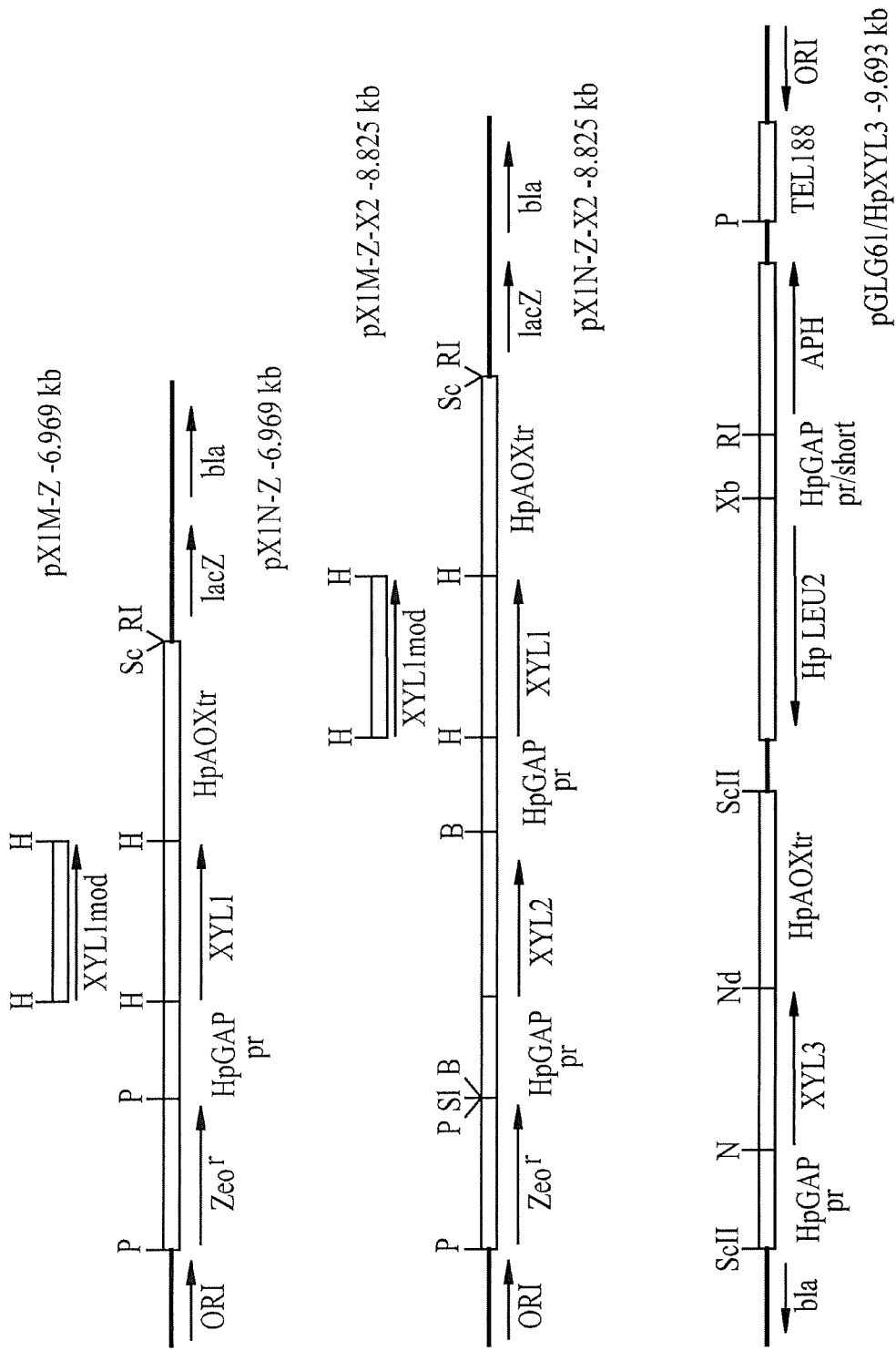


Fig. 1

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Hansenula polymorpha-- 332-TQRDILV**I**PKSNQKERLV (SEQ.ID NO: 1)
Candida shehatae----- 265-AQ**R**GIAV**I**PKSNLPERLV
Pichia guilliermondii-- 256-TQ**R**GLAV**I**PKSNNPDRL
Candida parapsilosis-- 259-TQ**R**GIAV**I**PKSNNPDRLA
Pichia stipitis----- 260-SQ**R**GIA**I**PKSNTVPRL
Candida tenuis----- 264-AQ**R**GIAV**I**PKSNLPERLV

H.p mutant XR----- 332-TQRDILV**I**PRSDQKERLV (SEQ.ID NO: 2)

Fig. 2

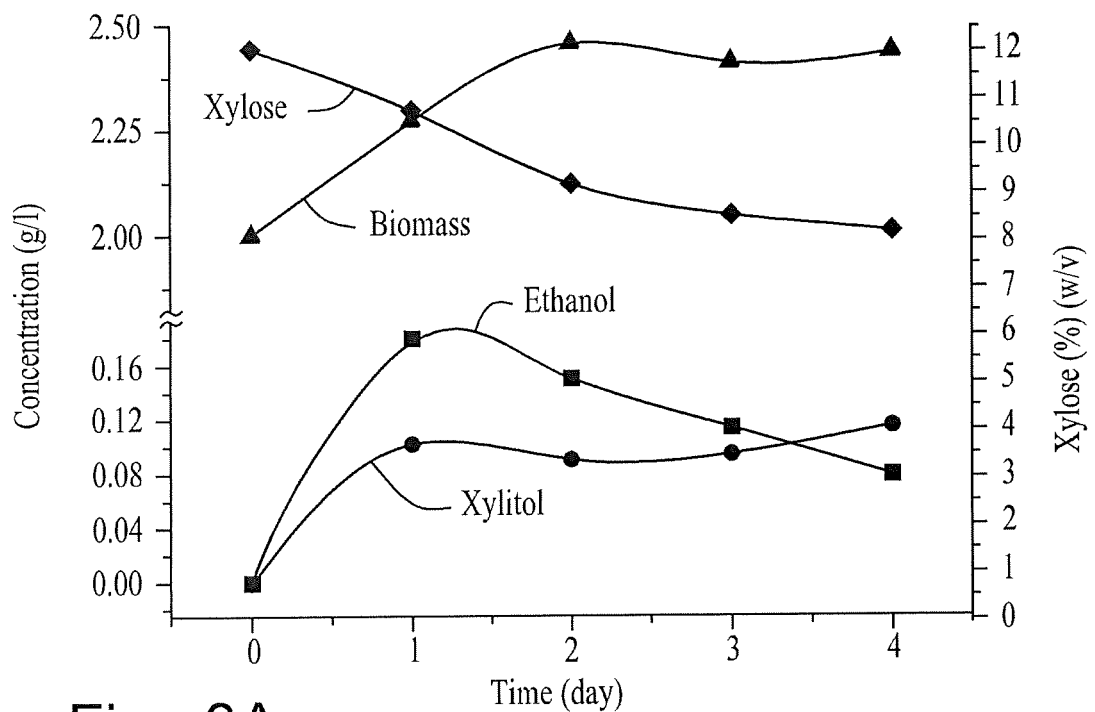


Fig. 3A

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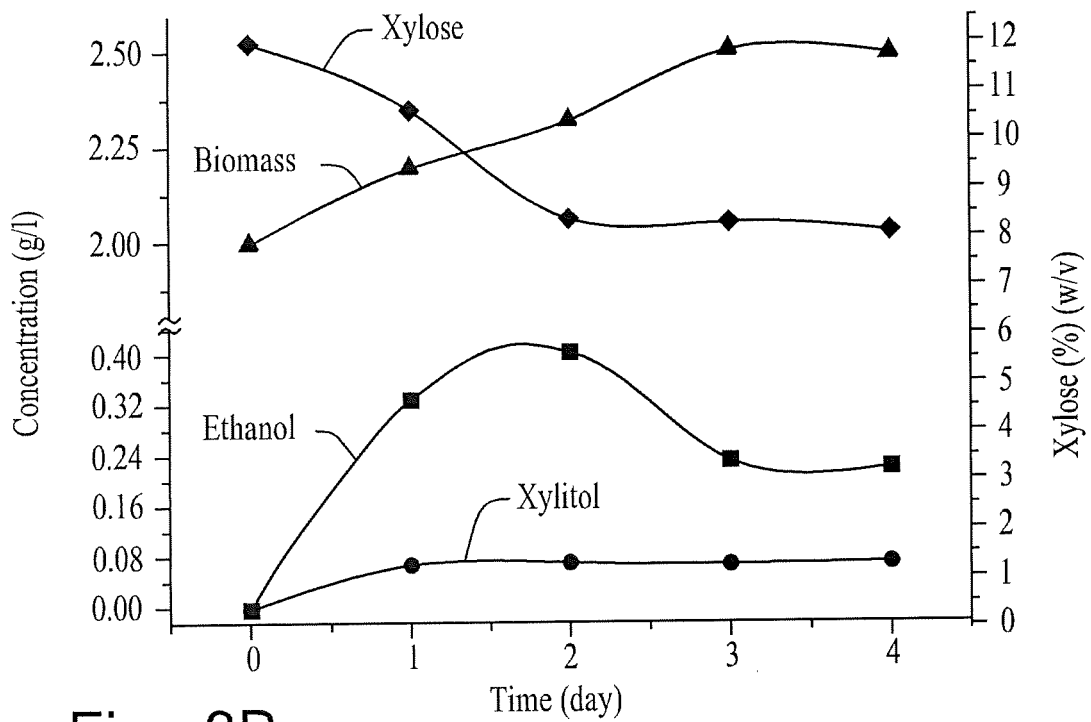


Fig. 3B

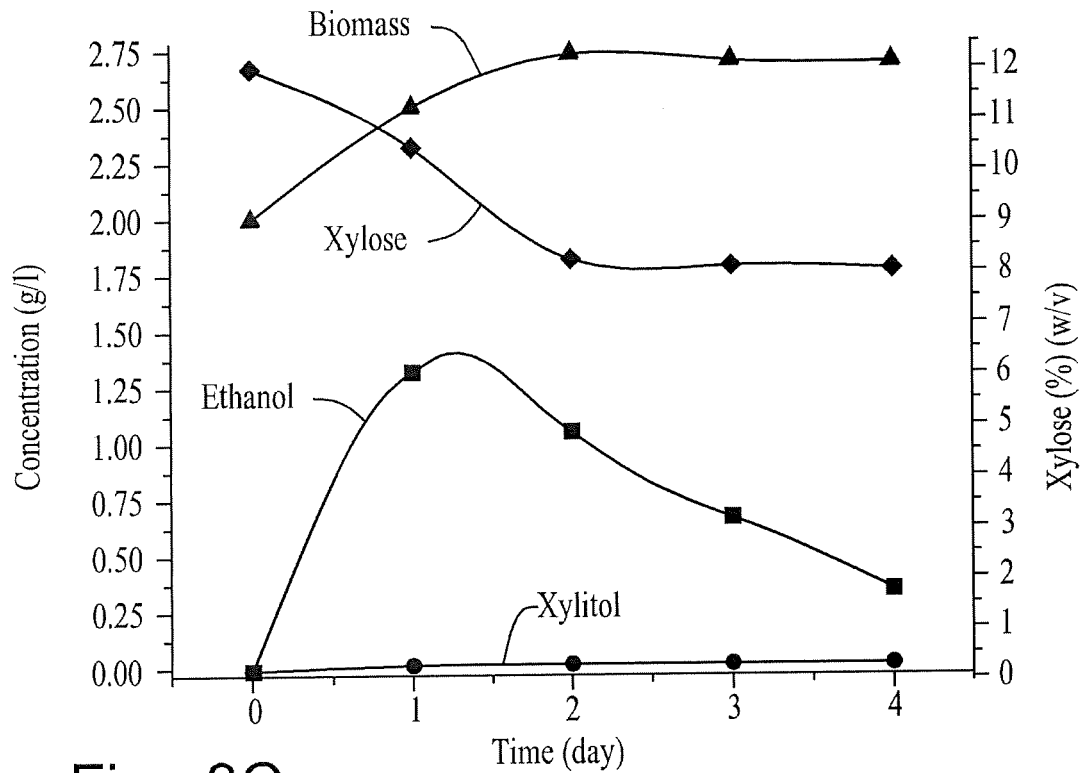


Fig. 3C

Table 1. Strains and plasmids used in the present investigation.

Strains	Genotype	Reference
CBS4732	<i>leu 2-2</i>	[26]
$\Delta xyl1$	$\Delta xyl1::LEU2Sc$	[22]
XRn	$\Delta xyl1$, pX1N-Z (<i>GAPp-XYL1-AOXt</i>)	This study
XRm	$\Delta xyl1$, pX1M-Z (<i>GAPp-XYL1mod-AOXt</i>)	This study
XRn/XDH	$\Delta xyl1$, pX1N-Z-X2 (<i>GAPp-XYL1-AOXt</i> , <i>GAPp-XYL2-XYL2t</i>)	This study
XRm/XDH	$\Delta xyl1$, pX1M-Z-X2 (<i>GAPp-XYL1mod-AOXt</i> , <i>GAPp-XYL2-XYL2t</i>)	This study
XRn/XDH/XK	$\Delta xyl1$, pX1N-Z-X2 (<i>GAPp-XYL1-AOXt</i> , <i>GAPp-XYL2-XYL2t</i>), pGLG61/HpXYL3 (<i>GAPp-XYL3-AOXt</i>)	This study
XRm/XDH/XK	$\Delta xyl1$, pX1M-Z-X2 (<i>GAPp-XYL1mod-AOXt</i> , <i>GAPp-XYL2-XYL2t</i>), pGLG61/HpXYL3 (<i>GAPp-XYL3-AOXt</i>)	This study

Fig. 4

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Table 2. XR, XDH, XK activities, ethanol and xylitol productivity of *H. polymorpha* transformants and control strain

Strain	Activity [U (mg protein ⁻¹)]					Productivity [mg (L h) ⁻¹]	
	XR			XDH	XK	Ethanol	Xylitol
	NADPH / K _M (μM)	NADH / K _M (μM)					
XRn	0.091±0.005 /9±0.5	0.016±0.001 /100±5.5	0.551±0.035	-	6.3	3.6	
XRm	0.01±0.001 /152±8.4	0.014±0.001 /112±6.5	0.504±0.028	-	9.8	3.2	
XRn/XDH	-	-	1.3±0.074	-	12	3.1	
XRm/XDH	-	-	1.485±0.087	-	18.4	1.6	
XRn/XDH/XK	-	-	-	0.375±0.021	13.8	2.8	
XRm/XDH/XK	-	-	-	0.366±0.019	54.7	0.9	
CBS4732	0.034±0.025 /7±0.4	0.012±0.001 /85±4.7	0.697±0.049	0.156±0.009	7.5	4.2	

XRn – transformant bearing normal *XYL1* gene; XRm – transformant bearing modified *XYL1* gene (*XYL1m*).

- not determined

Fig. 5

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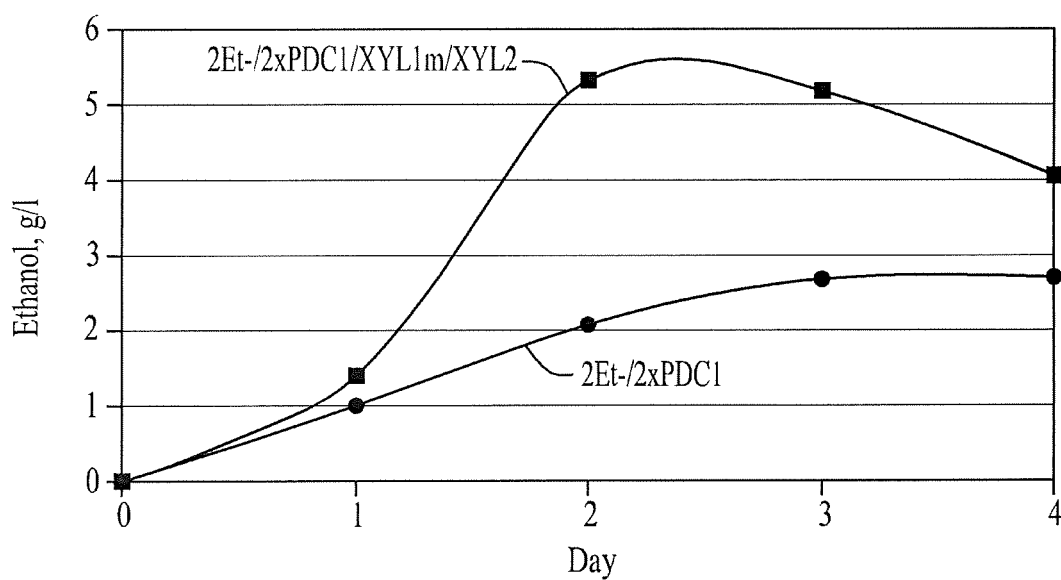


Fig. 6A

Table 3. Ethanol productivity of *H. polymorpha* constructed and initial strain

Strain	Ethanol productivity [g (L h) ⁻¹]
2Et-/2xPDC1	0.04
2Et-/2xPDC1/XRm/XDH	0.11

Fig. 6B

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H. polymorpha Xyl1 - DNA sequence for XYLOSE REDUCTASE

SEQ. ID NO: 3

1934 bp

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1 TTTTTTGTGT ATCTGTTCTGA TTTTCTTCTT GAGTTGTAGG CGTTCTTTAG TCTTCATTTT
61 TACTCGAGGC TTCTGCTCTG CTTTGCTATT GAACTCAGCT TTTGAAACCC GAATGGGTAG
121 TTCATCTGCA GGTCTGAAGTT TTGTTTCATC CAATAGCTCA ATTGCGAGGT CACAGCTCTC
181 CTCTTTTAGA TATTCTATTA GACAATCACC TTTGAAGTGG CCTCCGTCGT CTTTATACAT
241 TTTGATTTTG GAGGACCCAG TGCSCAAATC CTGTGCAAGA ACTCCATAGT TACCGAAAAC
301 TGCCTCCAGT TCCTCGTAGG TGATATCTTG GGGGATCTGA CTTACATAAA TGCAAGTATT
361 TCTGTTCCTC CGAAACTGCT TCAATTCCTC TTTCTCTTGC TTCAATTTCAT CGAGCCGCTG
421 ACGCTTCTCG CGTTTAAGAT ATTCAGAAGT GGTACCTCA TCATGAGGCC TCTTCTCGGG
481 CAGGTAATCC TGTATAACTG GTACCCAACG CTCTAGAATT GGATTGAACT CAAAGCACTC
541 CTTTTCCTCG GGATTTTCAA TCACCCATCG AGCACGTACT TTATCAAAGA ATAAGTATGT
601 TGCCAGATTC TGTGGCAGAT CCTCCGGAAG AGTGTTCTGT ATCTCATCGA TCAAGGGCAT
661 GAGAAATATGC ACACGCAGAT TAGCAAAAAT CTGTATTAT TTTTAGTGG CTGCACACCT
721 CACACCGATT TCCCCGAAAT CGCTGCGAAT GAAGGCAGTG TGGGGGTACG TAATGTTGAG
781 CTGAAACAGC AAACCTATAA ATTTGTGGGG GAATTTCTTA GAAAAAAGCA CCCTTGCTTA
841 AAATTAGTTT TTAACCTTTT TCACTTTTTA GGAGAGATGG CTTCCAAGAC AATTAAGTTG
901 AATAATGGCA TTGAAATCCC ACTAGTTGGG TTCGGCTGCT GGAAAGTCGA AAAGTCAATC
961 TGTGCTGAGC AGATTTACGA GGCAATTAAA GTTGGTTACC GTCTGTTTGA TGGCGCAATG
1021 GACTATGGTA ATGAAAAGCA AATTGGCCAG GGAATTGCCA AGGCCATTGA AGATGGTTTG
1081 GTCAAGAGAG AGGAGCTGGT TATTGTTTCC AAGCTATGGA ACAATTATCA CAAGCCGGAG
1141 AATGTGAAGA AGGCAATTAG AAGAGTTCTT GACGACCTAA AATTGGATTA TTTGGATATT
1201 TACTACATCC ACTTCCCAAT TGCTCAAAAA TTTGTCCCAT TCGAGGAGAA ATACCCACCA
1261 GGGTTTTACT GTGGCCCTAA TGGTTGGGAG TTCGAGGACG TTCCGCTTGC CGTGACTTGG
1321 AGGGCAATGG AAGAACTTGT TGAGGAGGGG CTTGTCAAGT CAATTGGTAT CTCCAACCTC
1381 TCAGGTGCTT TGATCCAGGA CCTGCTGAGA GGTTCGAAGA TTAAGCCTCA ATTGCTTCAG
1441 ATTGAGCACC ATCCTTACCT CACTCAAGAG AAATTGATCA AGTACGTTCA GGCTCAGGAC
1501 ATTGCTGTTG TTGCCTACTC TTCATTTGGA CCACAGTCCT TTGTGGAGCT AGACCATGCT
1561 AAGGCCAAGA ACACGTGTTT TTTGCTCAAG CACGATCTGA TCAACAGCAT TGCTTCTGCT
1621 CACAAGGTCA CCCCTGCTCA GGTCTCTCTG AGATGGGCTA CCCAAAGAGA CATCTTGGTC
1681 ATTCCAAAGT CCAACCAAAA GGAGAGACTG GTCCAAAACC TGAAGGTCAA TGACTTCAAT
1741 CTGTCTGAGG AAGAGATCAA GCAAATCAGC TCTTTGAACC AGGATCTGAG ATTCAATGAT
1801 CCTTGGACCT GGAACGAAAT TCCAACCTTT ATCTAAACCT TTGAAGGAAT AGAGTGGTTT
1861 ACCCACAAAC TATCATTGTA GGATGGTGCG TAAATTTCCT TCAGTATGGC GAACGCAAGG
1921 TCTGTCCGCG AAAA

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Fig. 7A

Figure *H. polymorpha* Xyl1 - protein sequence for XYLOSE REDUCTASE

SEQ. ID NO: 4

389 aa

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.....1 MHTQISKNLV LFFSGCTPHT DFPEIAANEG SGGVRNVELK QQTYKFVGEF PRKKHPCLKL
61 VFNFFHFLGE MASKTIKLN GIEIPLVGFG CWKVEKSICA EQIYEAIKVG YRLFDGAMDY
121 GNEKQIGQGI AKAIEDGLVK REELVIVSKL WNNYHKPEN V KKAIRRVLDD LKLDYLDIYY
181 IHFPQAQKQV PFEEKYPPGF YCGPNWGEFE DVPLAVTWRA MEELVEEGLV KSIGISNFSG
241 ALIQDLLRGC KIKPQLLQIE HHPYLTQEKI IKYVQAQDIA VVAYSSFGPQ SFVELDHAKA
301 KNTVSLKHD LINSIASAHK VTPAQVLLRW ATQRDILVIP KSNQKERLVQ NLKVNDFNLS
361 EEEIKQISSL NQDLRFNDPW TWNEIPTFI

```

Fig. 7B