Peroxisomes and peroxisomal transketolase and transaldolase enzymes are essential for xylose alcoholic fermentation by the methylotrophic thermotolerant yeast, *Ogataea (Hansenula) polymorpha*

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**Abstract**

**Background:** *Ogataea (Hansenula) polymorpha* is one of the most thermotolerant xylose-fermenting yeast species reported to date. Several metabolic engineering approaches have been successfully demonstrated to improve high-temperature alcoholic fermentation by *O. polymorpha*. Further improvement of ethanol production from xylose in *O. polymorpha* depends on the identification of bottlenecks in the xylose conversion pathway to ethanol.

**Results:** Involvement of peroxisomal enzymes in xylose metabolism has not been described to date. Here, we found that peroxisomal transketolase (known also as dihydroxyacetone synthase) and peroxisomal transaldolase (enzyme with unknown function) in the thermotolerant methylotrophic yeast, *Ogataea (Hansenula) polymorpha*, are required for xylose alcoholic fermentation, but not for growth on this pentose sugar. Mutants with knockout of *DAS1* and *TAL2* coding for peroxisomal transketolase and peroxisomal transaldolase, respectively, normally grow on xylose. However, these mutants were found to be unable to support ethanol production. The *O. polymorpha* mutant with the *TAL1* knockout (coding for cytosolic transaldolase) normally grew on glucose and did not grow on xylose; this defect was rescued by overexpression of *TAL2*. The conditional mutant, *pYNR1-TKL1*, that expresses the cytosolic transketolase gene under control of the ammonium repressible nitrate reductase promoter did not grow on xylose and grew poorly on glucose media supplemented with ammonium. Overexpression of *DAS1* only partially restored the defects displayed by the *pYNR1-TKL1* mutant. The mutants defective in peroxisome biogenesis, *pex3Δ* and *pex6Δ*, showed normal growth on xylose, but were unable to ferment this sugar. Moreover, the *pex3Δ* mutant of the non-methylotrophic yeast, *Scheffersomyces (Pichia) stipitis*, normally grows on and ferments xylose. Separate overexpression or co-overexpression of *DAS1* and *TAL2* in the wild-type strain increased ethanol synthesis from xylose 2 to 4 times with no effect on the alcoholic fermentation of glucose. Overexpression of *TKL1* and *TAL1* also elevated ethanol production from xylose. Finally, co-overexpression of *DAS1* and *TAL2* in the best previously isolated *O. polymorpha* xylose to ethanol producer led to increase in ethanol accumulation up to 16.5 g/L at 45 °C; or 30–40 times more ethanol than is produced by the wild-type strain.
Background

Peroxisomes are defined as organelles that typically contain the H₂O₂-generating enzymes, oxidase, and catalase. Most peroxisomes from yeasts to humans contain enzymes conducting fatty acid β-oxidation. Peroxisomes in fungi and plants also harbor some enzymes from the glyoxylate acid cycle [1–3]. However, the peroxisome is a metabolically versatile organelle containing enzymes catalyzing numerous catabolic and some biosynthetic reactions. In filamentous fungi, peroxisomes contain some enzymes conferring biotin and penicillin biosynthesis [4, 5]. In yeasts, peroxisomes are involved in the catabolism of many carbon and nitrogen sources, such as methanol, n-alkanes, purines, D-amino acids, methyamine, ethylamine, piperolic acid, sarcosine, glycolate and spermidine [3, 6]. Involvement of peroxisomes in sugar catabolism has been described for some parasitic Kinetoplastida protists, where these organelles are also known as glycosomes, contain enzymes catalyzing key glycolytic reactions [7]. Regarding yeast and filamentous fungi, until recently it has been accepted that the enzymes of glucose catabolism (i.e., glycolysis and pentose phosphate pathway) are located in the cytosol [8]. However, recent studies on Cryptococcus neoformans and Ustilago maydis showed that some of the glycolytic enzymes exhibit dual localization in both cytosol and peroxisomes [9, 10]. Importantly, defects in peroxisomal localization of glycolytic proteins or deficient peroxisome biogenesis impaired growth of these organisms on glucose. Candida albicans and related species showed cytosolic and peroxisomal localization of enzymes from the oxidative branch of the pentose phosphate pathway, namely glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [11]. Both cytosolic and peroxisomal isoforms of these proteins are encoded by different paralogs [12]. It is also known that peroxisomal enzymes are involved in the so-called xylulose monophosphate pathway for formaldehyde assimilation during growth on methanol. It has been suggested that this pathway for formaldehyde assimilation relies solely on the peroxisomal (and not cytosolic) enzymes of the non-oxidative branch of the pentose phosphate pathway (e.g., transketolase, transaldolase, ribulose phosphate epimerase, and ribose phosphate isomerase). Putative functions of these peroxisomal isoforms in the xylulose metabolism of K. phaffii were not studied.

Here, we addressed the metabolism and ethanol fermentation of the second most abundant sugar in nature, xylose. Xylose is the major component of the hemicellulose found in grasses and hard wood trees. For this study, we used the methylotrophic yeast, Ogataea (Hansenula) polymorpha, which is capable of fermenting glucose, cellobiose, and xylose [13]; as well as converting glycerol to ethanol [14]. Moreover, this organism is the most thermotolerant yeast known to date with a maximal growth and fermenting temperature of 50 °C, making it suitable for the process of simultaneous saccharification and fermentation [15]. Recombinant O. polymorpha strains expressing amyloid- and xylanolytic enzymes grow on starch and xylan, respectively. They also ferment these polymers to ethanol [16]. Unfortunately, the efficiency of xylose alcoholic fermentation by wild-type O. polymorpha strains is very low [17]. However, the efficiency of xylose to ethanol fermentation was significantly improved by a combination of classical selection and novel approaches in metabolic engineering. Interestingly, although it is possible to efficiently express bacterial xylose isomerase in O. polymorpha and the recombinant enzyme shows high specific activity, the ethanol yield remained low [17, 18]. Instead, an increase in ethanol production from xylose was achieved upon overexpression of the engineered xylose reductase [19] or pyruvate decarboxylase [20]. In addition, O. polymorpha strains with substantially improved ethanol production from xylose were obtained by overexpression of the modified XYL1 gene and native XYL2 and XYL3 genes coding for enzymes used by the initial reactions of xylose metabolism [21]. Further increase in ethanol production from xylose was achieved by deletion of the CAT8 gene coding for a putative transcription factor [22]. The best available strains accumulated nearly 12.5 g ethanol/L in xylose medium at 45 °C, which is 20 times greater than the wild-type strain, and yet inferior to recombinant strains of Saccharomyces cerevisiae and Scheffersomyces (Pichia) stipitis (which are mesophilic

**Conclusions:** Our results indicate the importance of the peroxisomal enzymes, transketolase (dihydroxyacetone synthase, Das1), and transaldolase (Tal2), in the xylose alcoholic fermentation of O. polymorpha.

**Keywords:** Xylose, Ogataea (Hansenula) polymorpha, Peroxisomes, Ethanol, High-temperature alcoholic fermentation
Further improvement of the high-temperature ethanol fermentation could be achieved by identification of the bottlenecks likely to exist in the xylose conversion to ethanol in the thermotolerant yeast, *O. polymorpha*.

Xylose metabolism in yeast consists of three parts: conversion to xylulose-5-phosphate, reactions of pentose phosphate pathway, and the glycolysis reactions (Fig. 1) [23]. In this case, the presence of all three parts is strongly confirmed by both biochemical and genetic evidence. It is also assumed that all corresponding enzymes are in the cytosol. However, it has been known for many years that methylotrophic yeast contains peroxisomal transketolase, known also as dihydroxyacetone synthase [24]. This enzyme is involved in the assimilation of formaldehyde during methylotrophic growth and also catalyzes the classical transketolase reaction (Fig. 1) [25]. Most of the peroxisomal proteins are located in peroxisomes due to the Peroxisome Targeting Signal I (PTS1), which represents the evolutionarily conserved amino acid sequence S–K–L [3, 26]. Due to the PTS1 sequence, dihydroxyacetone synthase from *O. polymorpha* is also located in the peroxisomes of the heterologous system for the non-methylotrophic yeast, *Saccharomyces cerevisiae* [27].

The newly available complete sequence of *O. polymorpha* genome (http://genom e.jgipsf.org/Hanpo2/Hanpo 2.home.html; [28]) has allowed identification of genes presumably encoding peroxisomal transaldolase [29]; as well as ribulose phosphate epimerase (K. Dmytruk, A. Sibirny, unpublished). Peroxisomal transaldolase, designated here as Tal2, contains a classical PTS1 signal S–K–L. Peroxisomal localization of *O. polymorpha* Tal2 was not directly determined before; however, its ortholog (Tal1-2) was found to be localized in peroxisomes in a closely related methylotrophic species, *K. phaffii* [12]. Homology in protein sequences between Tal2 *O. polymorpha* and Tal1-2 *K. phaffii* is rather high (63% identities, 79% positives) and the Tal2 protein of *O. polymorpha* also contains PTS1 signal. These data suggest that Tal2 of *O. polymorpha* is most likely to be a peroxisomal protein. However, direct evidence for peroxisomal localization of Tal2 was absent before this work.

Methylotrophic yeasts possess well-developed peroxisomes which could occupy up to 80% of cellular volume during growth on methanol [30]. We hypothesized that peroxisomal enzymes of the pentose phosphate pathway

![Fig. 1](attachment:image.png)
could be important not only for methanol metabolism [12], but also for xylose to ethanol fermentation (Fig. 1). We also decided to test the effect of pex mutations, which block peroxisome biogenesis on xylose growth and ethanol fermentation. We found that the corresponding knockouts in das1, tal2, pex3, or pex6, severely damaged xylose ethanol fermentation but did not affect growth on this sugar. Moreover, defects in expression of TKL1 (cytosolic transketolase) suppressed growth on both xylose and glucose, whereas knockout of TAL1 (cytosolic transaldolase) impaired only xylose growth. However, xylose fermentation was only partially suppressed in conditional TKL1 (pYNRI-TKL1) and knockout TAL1 (tal1Δ) mutants. In line with these observations, overexpression of the genes coding for cytosolic and peroxisomal transketolase and transaldolase (TKL1, TAL1, DAS1, TAL2) had an opposite effect and activated xylose alcoholic fermentation. Finally, simultaneous overexpression of DAS1 and TAL2 in the background of the recently isolated “best ethanol producer from xylose” [22] activated xylose alcoholic fermentation and enabled the construction of further improved ethanol producers from xylose at 45 °C.

Methods

Strains, media, and culture conditions

Ogataea polymorpha cells were grown on YPD (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) or mineral medium (6.7 g/L YNB without amino acids, 20 g/L of glucose or xylose, or 10 g/L of methanol) at 37 °C. For theNCYC495 leu1-1 strain, leucine (40 mg/L) was added to the medium. For selection of yeast transformants on YPD 0.1 g/L of nourseothricin, 0.3-0.5 g/L of geneticin, 0.075-0.1 g/L of zeocin, or 0.35 g/L of hygromycin were added. Ethanol fermentation of O. polymorpha strains was tested as described previously [22]. Fermentation experiments were performed in at least triplicate to ensure the results were reproducible. The bars in the figures indicate the ranges of the standard deviation.

Strains pex3Δ and pex6Δ O. polymorpha were kindly provided by Prof. Ida van der Klei, Groningen University, the Netherlands. S. stipitis strains—parental ku80 [31] and pex3Δ mutants were grown on YNB medium supplemented with 40 mg/mL of histidine and 2 g/L of glucose or 1 g/L of oleate at 30 °C. Ethanol fermentation of S. stipitis strains was tested (as mentioned above) for O. polymorpha strains under oxygen-limited conditions (100 rpm) at 30 °C.

The E. coli DH5α strain (p80lacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(rK−, mK+), supE44, relA1, deoR, Δ(lacZYA-argF)U169) was used as a host for plasmid propagation. Strain DH5α was grown at 37 °C in LB medium as described previously [32]. Transformed E. coli cells were maintained on a medium containing 100 mg/L of ampicillin.

Molecular biology techniques

Standard cloning techniques were carried out as described [32]. Genomic DNA of O. 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). DNA fragments were separated on a 0.8% agarose (Fisher Scientific, Fair Lawn, NJ, USA) gel. Isolation of fragments from the gel was carried out with a DNA Gel Extraction Kit (Millipore, Bedford, MA, USA). PCR amplification of the fragments of interest was done with Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, USA) according to the manufacturer’s specifications. PCRs were performed in GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Transformation of the yeasts O. polymorpha and S. stipitis was carried out as described previously [33].

Construction and analysis of das1Δ, tal2Δ, tal1Δ,
pYNRI-TKL1 O. polymorpha mutants

Genomic DNA of O. polymorpha NCYC495 leu1-1 strain was used as a template for PCR amplification of 5′ and 3′ non-coding regions of DAS1 using primers OL207/OL208 and OL209/OL210 (Additional file 1). The resulting 5′ DAS1 (926 bp) and 3′ DAS1 (1257 bp) fragments were HindIII/EcoRI or EcoRI/XbaI digested and cloned into HindIII/XbaI-linearized vector pBluescript II KS(−). The resulting recombinant construct was named pLRDAS. Gene zeoR (1132 bp) conferring resistance to zeocin was amplified using vector pPICZB (Thermo Fisher Scientific) as a template and primers Ko64/Ko65. The obtained fragment was EcoRI digested and cloned into EcoRI-linearized plasmid pLRDAS. The constructed plasmid was designated as pΔDAS-Zeo.

The same approach was used for construction of the TAL2 deletion cassette. The 5′ (779 bp) and 3′ (1440 bp) non-coding regions of TAL2 were PCR amplified with primers OL211/OL212 and OL213/OL214. Amplified 5′ and 3′ non-coding regions were double digested with HindIII/EcoRI and EcoRI/BamHI and cloned into pBluescript II KS(−) digested with HindIII/BamHI. Selective marker gene conferring resistance to zeocin was amplified from pPICZB with Ko64/Ko65 and cloned between 5′ and 3′ non-coding regions of TAL2 as EcoRI digested fragment. The resulting plasmid was named pΔTAL-Zeo.
The constructed plasmids were transformed into *O. polymorpha* NCYC495 leu1-1 recipient strain using an standard electroporation method [33]. Transformants were selected on the solid YPD medium supplemented with 75 mg/L of zeocin after 4 days of incubation. The obtained transformants were examined by PCR using genomic DNA of recombinant strains as a template. Transformants with confirmed deletion of **DAS1** and **TAL2** were stabilized by altering cultivation in non-selective and selective media and once again examined by PCR. Fragments with predicted size were amplified using pairs of primers homologous to the sequence of selective marker and regions outside from the fragments used for recombination (Ko533/Ko534, Ko557/Ko559 for **DAS1** and Ko535/Ko534, Ko557/Ko558 for **TAL2**) (Additional file 2).

The deletion cassette for isolation of **tal1Δ** mutant on the background of the wild-type strain was constructed as follows. Genomic DNA of *O. polymorpha* NCYC495 leu1-1 strain was used as template for isolation of 5′ and 3′ non-coding regions of **TAL1** gene by PCR amplifications using primers OK74/OK75 and OK76/OK77. The obtained DNA fragments were fused by overlap PCR using primers OK74/OK75 and OK76/OK77. The resulting fragment (1.8 kb) was **BamHI**/**KpnI** digested and cloned into the appropriate sites of pUC57 vector. The **hphNT1** gene (1777 bp), conferring resistance to hygromycin, was amplified from plasmid pRS42H [34] as a template and primers Ko446 and Ko450. The resulting plasmid was designated as pΔ**TAL1−Hygr**.

The constructed plasmid was transformed into *O. polymorpha* NCYC495 recipient strain using the electroporation method given above [33]. Transformants were selected on solid YPD medium supplemented with 0.35 g/L of hygromycin after 4 days of incubation.

The correct replacement of **TKL1** gene promoter was confirmed by PCR in obtained transformants using primers OK176/OK123 and OK172/OK102 (Additional file 4).

**Construction and analysis of *O. polymorpha* strains with overexpression of **DAS1**, **TAL2**, **TAL1**, and **TKL1** genes**

The recombinant plasmids, pGLG61/**DAS1** and pGLG61/**TAL2**, bearing the *O. polymorpha* genes **DAS1** and **TAL2**, respectively, were constructed on the basis of the plasmid, pGLG61 [35].

Genomic DNA from *O. polymorpha* was used as a template for PCR amplifications of the **DAS1** and **TAL2** genes. Native promoters from these genes were substituted with a strong constitutive promoter, **GAPI**, from glyceraldehyde-3-phosphate dehydrogenase (GAP). First, the **GAPI** promoter and ORF of the **DAS1** gene with terminator sequence were amplified from *O. polymorpha* genomic DNA using the primers, Ko277/Ko278 and Ko279/Ko280. Then two fragments were fused by overlap PCR using primers, Ko277 and Ko280. The resulting fragment (2.9 kb) was digested with **BamHI** endonuclease and ligated with **BamHI**-linearized plasmid, pGLG61. The resulting recombinant construct was named pGLG61/**DAS1** (Additional file 5A). At the next step, the **GAPI** promoter and ORF of the **TAL2** gene with terminator sequence were amplified from *O. polymorpha* genomic DNA using the primers, Ko405/Ko411 and Ko412/Ko414, respectively. Then two fragments were fused by overlap PCR using the primers, Ko405 and Ko414. The resulting fragment (1.8 kb) was digested with **BamHI** and **BglII** endonuclease and ligated with the **BamHI**/**BglII**-linearized plasmid, pGLG61. The resulting recombinant plasmid was named pGLG61/**TAL2** (Additional file 5A). These plasmids were introduced into the genome of the *O. polymorpha* NCYC495 leu1-1 strain.
Transformants were selected on solid YPD medium supplemented with 0.3–0.5 g/L of genetin after 5 days of incubation. Selected transformants were stabilized by alternating cultivation in non-selective and selective media and examined by diagnostic PCR using the primers, Ko277/Ko278 or Ko279/Ko280, respectively.

For overexpression of the cytosolic transketolase and transaldolase recombinant plasmids, pTkZr and pTaZr, bearing the *O. polymorpha* genes (TKL1 and TAL1), respectively, were constructed. The strong constitutive GAP promoter and ORF of the TKL1 with terminator sequence were amplified by PCR from *O. polymorpha* chromosomal DNA using two pairs of primers, A58/A35 and Ko52/Ko53, respectively. The obtained PCR products were digested with *Sac*I and then ligated with *Sac*I-linearized vector pUC57, carrying the zeocin resistance gene as a selective marker (pUC57-Zr). The resulting plasmid was designated as pTkZr. For overexpression of the TAL1 gene, the DNA fragment bearing the GAP1 promoter was fused with the ORF of the TAL1 gene. This was accomplished by overlap PCR using two pairs of primers, K43/Ko77 and Ko76/Ko84. The PCR product was digested with *Bam*HI and then ligated with *Bam*HI-linearized vector, pUC57-Zr, resulting in the recombinant construct, pTaZr (Additional file 5B). The resulting plasmids were used for transformation of *O. polymorpha* NCYC495 leu1-1 strain. The transformants were selected on solid YPD medium supplemented with 0.1 g/L of zeocin after 3 days of incubation.

For simultaneous overexpression of the DAS1 and TAL2 genes, the plasmid, pUC57/DAS1/TAL2, was constructed. *Bam*HI/PstI-restriction fragment containing GAPpr-TAL2 was isolated from the plasmid, pGLG61/TAL2, and cloned into the *Bam*HI/PstI-linearized vector pUC57. The resulting recombinant construct was named pUC57/TAL2. The *Bam*HI-restriction fragment containing GAPpr-DAS1 was isolated from the plasmid, pGLG61/DAS1, and cloned into the *Bam*HI-linearized and dephosphorylated vector, pUC57/TAL2. The resulting recombinant construct was named pUC57/DAS1/TAL2. Gene natNT2, conferring resistance to nourseothricin, was amplified from the plasmid, pRS41N [33] by PCR using primers, OK42/OK43. The fragment obtained (1.3 kb) was *Nde*I-linearized and cloned into the appropriate site of pUC57/DAS1/TAL2. The resulting plasmid was named pUC57/DAS1/TAL2/NTC (Additional file 5C). Transformants were selected on solid YPD medium supplemented with 0.1 g/L of nourseothricin after 3 days of incubation. Selected transformants were stabilized by alternating cultivation in non-selective and selective media and examined by diagnostic PCR using the primers, Ko277/Ko278 and Ko279/Ko280, respectively.

**Construction and analysis of pexΔ* S. stipitis* mutants**

Genomic DNA of *S. stipitis* ku80 strain was used as template PCR amplification of 5′ and 3′ non-coding regions of *PEX3* gene using the primers, Ko770/Ko771 and Ko772/Ko773. The resulting 5′PEX3 (1682 bp) fragment was *Sac*I/*Xba*I-digested and cloned into *Sac*I/*Xba*I-linearized vector pUC57. The resulting plasmid was named pUC57-5′PEX3. As the next step, 3′PEX3 (1499 bp) fragments were *Xba*I/*Bam*HI-digested and cloned into *Xba*I/*Bam*HI-linearized vector pUC57-5′PEX3. The resulting plasmid was named pUC57-5′-3′PEX3. *HIS3* gene (1399 bp) involved in histidine biosynthesis was amplified using vector pΔatg13Sc [36] as a template and primers KB5 and KB6. The obtained fragment was *Xba*I-digested and subcloned into *Xba*I-linearized plasmid pUC57-5′-3′PEX3. As a result, the recombinant plasmid pUC57-5′-3′PEX3-HIS3 was constructed. This plasmid was *Pst*I-linearized and transformed into *S. stipitis* ku80 his3-1 recipient strain using electroporation method [33]. Transformants were selected on solid YNB medium supplemented with 40 mg/L of histidine after 5 days of incubation at 30 °C. The obtained transformants were examined by PCR using genomic DNA of recombinant strains as a template. Transformants with confirmed deletion of *PEX3* were stabilized by altering cultivation in non-selective and selective media and once again examined by PCR. Fragments with predicted size were amplified using pairs of primers homologous to the sequence of selective marker and regions outside from the fragments used for recombination (Ko774/KB9 and KB10/Ko775) (Additional file 6).

**Fluorescence microscopy visualization of peroxisomal localization of Tal2 in *O. polymorpha***

For constitutive expression of peroxisomal SKL-tagged green fluorescent protein (GFP-SKL) in *O. polymorpha*, the DNA fragments harboring the GFP-SKL gene and the promoter *ScTEF1* were PCR amplified with the primers, K0799/K0800, from the plasmid, p416TEF-GFP [37]. The terminator, *ScCYC1*, was amplified with the primers, K0801/K0802, from the genomic DNA of *S. cerevisiae* BY4742. The backbone plasmid containing *KamMX4* selective marker was amplified with primers, K0803/K0804, from the plasmid, pCFB2055 [38]. Three PCR fragments were then Gibson assembled to generate the plasmid, pGFP-SLK. The obtained plasmid was digested with *Not*I and integrated into genome of *O. polymorpha* NCYC495 leu1-1 strain. Transformants were selected on the solid YPD medium supplemented with 0.3 g/L of genetin after 4 days of incubation. One of the transformants was used as a recipient strain for localization studies of Tal2 protein. The DNA fragment harboring the gene coding for the red fluorescent protein (RFP)
was amplified with the primers, Ko888/Ko889, from the plasmid, pDsRed2 (Clontech). The backbone plasmid containing TAL2 was amplified with the primers, Ko890/Ko891, from the plasmid, pGLG61/TAL2. Two PCR fragments were then Gibson assembled to generate the plasmid pGLG61/TAL2_RFP. This plasmid was introduced into genome of \textit{O. polymorpha} wild-type strain carrying GFP-SKL. Transformants were selected on solid YNB medium after 5 days of incubation. Selected transformants were stabilized by alternating cultivation in non-selective and selective media and examined by diagnostic PCR using the primers, Ko892/Ko414. The resulting strains were grown at 37 °C in YNB medium with glucose, xylose, or methanol during 24 h; followed by microscopy analysis. Images were captured on a fluorescence microscope (Axio Imager A1; Carl Zeiss MicroImaging, Jena, Germany) coupled to a monochrome digital camera (Axio Cam MRm; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop CC software (Adobe Systems, Mountain View, CA).

\textbf{Biochemical methods}

The enzyme activity was measured directly after the preparation of cell-free extracts. Protein concentration was determined with Folin reagent [39]. The specific activities of total transketolase and transaldolase in cell extracts were determined spectrophotometrically as described before [25, 40].

All assay experiments were repeated at least twice.

\textbf{Quantitative real-time PCR (qRT-PCR)}

Expression of the \textit{XYL1}, \textit{XYL2}, \textit{XYL3}, \textit{RPE1}, \textit{TKL1}, \textit{PDC1}, \textit{ADH1}, and \textit{CYC1} genes was analyzed by real-time PCR. The qRT-PCR was performed by 7500 Fast Real-Time PCR System (The Applied Biosystems, USA) with SG OneStep qRT-PCR kit (EURx Ltd., Gdansk, Poland) using gene-specific pairs of primers, RNA as a template, and ROX reference passive dye according to the manufacturer’s instructions as described previously [22]. The primers pairs used for qRT-PCR are listed in Additional file 1. Sequences of the tested genes were taken from \textit{O. polymorpha} genome database (\textit{Ogataea polymorpha} NCYC 495 leu1.1 v2.0-JGI Genome Portal. \url{http://genom e.jgipsf.org/Hanpo2/Hanpo2.home.html}).

\textbf{Analyses}

The biomass was determined turbidimetrically with a Helios Gamma spectrophotometer (OD, 590 nm; cuvette, 10 mm) with gravimetric calibration. Concentrations of xylose and ethanol from fermentation in medium broth were analyzed by HPLC (PerkinElmer, Series 2000, USA) with an Aminex HPX-87H ion-exchange column (Bio-Rad, Hercules, USA). A mobile phase of 4 mM H\textsubscript{2}SO\textsubscript{4} was used at a flow rate 0.6 mL/min and the column temperature was 30 °C. Alternatively, concentrations of ethanol in the medium were determined using alcohol oxidase/peroxidase-based enzymatic kit “Alcotest” [41]. Experiments were performed at least twice.

\textbf{Results}

\textit{Ogataea polymorpha} is a methylotrophic yeast and thus it contains a xylulose monophosphate pathway for formaldehyde assimilation, i.e., its conversion to three carbon compound dihydroxyacetone [42]. Work in \textit{K. phaffii} showed that the key (if not all) enzymes of this pathway are located in peroxisomes [12, 29]. However, the functions of peroxisomal transketolase (dihydroxyacetone synthase), the key enzyme in the xylulose monophosphate pathway for xylose growth and fermentation have not been studied. As was pointed above, genome of \textit{O. polymorpha} also contains the transaldolase paralog gene (designated here as TAL2), which contains codons for peroxisome targeting signal PTS1 (amino acids SKL). However, localization of this protein was not determined earlier. To study localization of the Tal2 protein, the TAL2 gene was fused with the gene coding for RFP. The vector containing the TAL2-RFP fusion was introduced into the \textit{O. polymorpha} wild-type strain, which expresses GFP containing the PTS1 signal. The GFP recipient strain we constructed possesses green fluorescent peroxisomes due to the targeting of GFP to this organelle, exactly as was described before [43] (not shown). Transformants of the GFP-expressed strain, which contained the TAL2-RFP fused gene, have been cultivated in the media with one of three carbon sources: glucose, xylose, and methanol. Cell fluorescence was also studied. Cells grown on each carbon source displayed both green and red fluorescence. Zones of green fluorescence corresponding to peroxisomes were the smallest in glucose-grown cells and the largest in methanol-grown cells, as expected. Most importantly, the red fluorescence of transaldolase Tal2 coincided perfectly with the green fluorescence of peroxisomes (Fig. 2). These data show that indeed transaldolase Tal2 containing PTS1 targeting signal is located only in peroxisomes, as expected.

The role of this peroxisomal transaldolase, Tal2; as well as the peroxisomal transketolase, Das1, in xylose metabolism and fermentation (and in this case of Tal2, also in methanol metabolism) was not studied. Furthermore, the roles of the cytosolic transketolase and transaldolase in xylose metabolism and fermentation in \textit{O. polymorpha} have not been investigated as well. In this study, we have engineered knockout and overexpression strains for each corresponding gene (\textit{DAS1}, \textit{TAL2}, \textit{TKL1}, \textit{TAL1}).
and studied their growth and fermentation on xylose and glucose. Mutants with knockout of genes coding for the peroxisomal transketolase, \(DAS1\), and presumably the peroxisomal transaldolase, \(TAL2\), did not differ from the wild-type strain regarding growth on glucose and xylose. Of note, mutant \(das1\Delta\) failed to grow on methanol, whereas growth of \(tal2\Delta\) mutant on methanol was normal (Fig. 3). However, the ability to ferment xylose was severely damaged in \(tal2\Delta\) and \(das1\Delta\) mutants (Fig. 4a, b). They also normally fermented glucose (Fig. 4c). The role of peroxisomal transketolase in methanol utilization has been known for many years [30]. Importantly, using \(O. polymorpha\) \(tal2\Delta\) mutant, we did not observe the involvement of peroxisomal transaldolase in methanol metabolism (Fig. 3), unlike what was recently hypothesized for \(K. pastoris\) [12].

Subsequently, we decided to create deletion mutants in the genes coding the cytosolic transketolase and transaldolase enzymes. We found that the isolated \(tal1\Delta\) mutant, in contrast to \(tal2\Delta\) mutant, failed to grow on xylose, whereas growth on glucose was normal and growth on methanol was delayed (Fig. 3). Interestingly, despite complete inability to grow on xylose, \(tal1\Delta\) mutant was still able to produce ethanol from this pentose (Fig. 4a). Next, we decided to test whether or not overexpression of the \(TAL2\) gene coding for peroxisomal transaldolase (under control of strong constitutive promoter, \(GAP1\), for the gene coding for glyceraldehyde-3-phosphate dehydrogenase) could rescue \(tal1\Delta\) growth on xylose. Indeed, transformants showed a restored ability to grow on xylose (Fig. 3). Moreover, xylose fermentation of the tested transformant was the same as in the wild-type strain (Fig. 4a).

Our multiple attempts to obtain mutants with knockout of the \(TKL1\) gene, encoding cytosolic transketolase were unsuccessful; even upon selection in the presence of aromatic and branched chain amino acids [44]. We speculated that \(tkl1\Delta\) mutants of \(O. polymorpha\) could be lethal. Therefore, we decided to generate a conditional mutant expressing the \(TKL1\) gene under control of strongly inducible promoter and substituted the native

| Strain             | Glucose\(^a\) | Xylose\(^b\) | Methanol\(^b\) |
|--------------------|---------------|--------------|----------------|
|                   | 1             | 10\(^{-1}\)  | 10\(^{-2}\)    |
|                   | 10\(^{-3}\)  | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
|                   |               | 10\(^{-2}\)  | 10\(^{-3}\)    |
|                   |               | 1            | 10\(^{-1}\)    |
TKL1 promoter with that of O. polymorpha YNR1 promoter of nitrate reductase gene, known to be repressed by ammonium and strongly induced by nitrate [45]. The obtained transformants grew (although poorly) on the mineral medium (with ammonium sulfate) and glucose as carbon source. The addition of aromatic and branched amino acids did not affect the growth of isolated transformants in the medium with glucose and ammonium sulfate (not shown). We then confirmed the inducible TKL1 gene regulation under control of YNR1 promoter by qRT-PCR. As expected, it was induced by nitrate and repressed by ammonium ions (Fig. 5).

However, in the medium with ammonium sulfate and xylose, transformants failed to grow at all (Fig. 3). Growth of the transformants on xylose and glucose media with nitrate as nitrogen source was normal (not shown). We then confirmed the inducible TKL1 gene regulation under control of YNR1 promoter by qRT-PCR. As expected, it was induced by nitrate and repressed by ammonium ions (Fig. 5).

However, suppression was not complete as growth started after long lag-phase and was delayed (Fig. 3). We also constructed the shortened version of DAS1 gene lacking C-terminal PTS1 motif. Overexpression of this presumably cytosolic Das1 protein more efficiently restored growth of pYNR1-TKL1 mutant in the medium with xylose and ammonium sulfate (Fig. 3). Xylose fermentation of rescue strains pYNR1-TKL1/DAS1 and pYNR1-TKL1/DAS1(-PTS1) is shown in Fig. 4b. Like growth,
ethanol production was also better rescued by the peroxisomal Das1 than its cytosolic counterpart. Still, ethanol production by both rescued transformants was approximately twofold lower on the second day of fermentation as compared to that of the wild-type strain (Fig. 4).

Altogether, we conclude that peroxisomal transketolase and peroxisomal transaldolase are compulsory for xylose alcoholic fermentation, but not for growth on this pentose, whereas their cytosolic counterparts are required for xylose utilization.

Next, we studied xylose growth and fermentation of the mutants defective in peroxisome biogenesis. We used mutants defective in the core membrane peroxisomal protein, Pex3, as well as the AAA-family ATPase, Pex6. The cells of pex3Δ and pex6Δ mutants O. polymorpha do not contain peroxisomes at all or contain only their remnants [3]. We found that growth of pex3Δ and pex6Δ mutants on xylose was normal, whereas xylose fermentation was totally abolished. However, ethanol production from glucose in pex3Δ mutants was close to that in the wild-type strain (Fig. 6). Thus, characteristics of xylose growth and fermentation of O. polymorpha pex3Δ and pex6Δ mutants were like those of das1Δ and tal2Δ mutants.

Inability of the mutants defective in peroxisome biogenesis to ferment xylose could be a consequence of mis-localization of peroxisomal transketolase and transaldolase into the cytosol. Alternatively, there could be an additional intrinsic requirement of peroxisome for xylose fermentation. To evaluate these possibilities, we decided to isolate peroxisome-deficient mutants in non-methylotrophic xylose-fermenting yeast, S. stipitis, whose genome does not contain genes of presumably peroxisomal transketolase and transaldolase. We created S. stipitis mutants with the PEX3 knockout and they were unable to grow on oleic acid as a sole carbon source (Fig. 7).
Crucially, we found that unlike the _O. polymorpha_ pex3Δ, the mutant of _S. stipitis_ did not differ from the wild-type strain in terms of growth and fermentation of glucose and xylose (Fig. 8). Thus, in the non-methylo-trophic yeast, _S. stipitis_, peroxisomes are not required for xylose fermentation, in contrast to the methylotrophic yeast, _O. polymorpha_. Possibly, this difference stems from the involvement of peroxisomal transketolase and transaldolase in regulation of xylose fermentation in _O. polymorpha_. Indeed, in _O. polymorpha_ pex3Δ or pex6Δ mutants, these enzymes are mis-localized to the cytosol, where they presumably cannot fulfil their functions. On the other hand, in _S. stipitis_, an organism lacking peroxisomal transketolase and transaldolase, xylose fermentation does not require any peroxisomal enzymes.

Based on the data for the requirements of the peroxisomal transketolase (Das1) and the peroxisomal transaldolase (Tal2), we hypothesized that overexpression of _DAS1_ and/or _TAL2_ would have beneficial effects on ethanol production from xylose. To test this, we overexpressed each of the mentioned genes separately or together under control of the strong constitutive promoter, GAP. Overexpression of these genes led to an increase in the total activities of the corresponding enzymes, cytosolic and peroxisomal transketolase and transaldolase (Table 1).

Transformants with overexpression of _TAL2_ doubled their ethanol production from xylose, whereas overexpression of _DAS1_ led to tripled ethanol production. Moreover, co-overexpression of _DAS1_ and _TAL2_ led to fourfold increase in ethanol production from xylose relative to that of the wild-type strain (Fig. 9a).

For comparison, we also overexpressed the genes, _TKL1_ and _TAL1_, coding for cytosolic transketolase and transaldolase, respectively. We found that overexpression of these genes also stimulated ethanol production from xylose during 3 days of fermentation (Fig. 9b).

One may assume that Das1 and Tal2 proteins, in addition to their catalytic reactions, also affect the expression of other genes. We tested expression of several genes involved with xylose metabolism and ethanol fermentation (_XYL1, XYL2, XYL3, TKL1, TAL1, RPE1, CYC1, FBP1, PCK1, PDC1, ADH1_) in the strains with knockout and overexpression conferred by the _DAS1_ and _TAL2_ genes. Interestingly, this comparison showed that deletion of _DAS1_ and _TAL2_ genes lowered the expression of the _XYL1_ gene coding for xylose reductase, whereas their overexpression, inversely, activated _XYL1_ expression (Table 2). This result suggests possible involvement of peroxisomal transketolase and transaldolase in the transcriptional regulation of the xylose reductase (i.e., the enzyme of the initial step of xylose metabolism).

We hypothesized that overexpression of _DAS1_ and _TAL2_ could be a useful approach for construction of advanced ethanol producers from xylose under elevated temperatures. To test this, we co-overexpressed the _DAS1_ and _TAL2_ genes in the background of the best previously constructed ethanol producer, BEP cat8Δ of _O. polymorpha_ [22]. The resulting strain BEP cat8Δ/_DAS1/_ _TAL2_ accumulated 29% more ethanol relative to the

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**Table 1** Specific activities of Tkl (transketolase) and Tal (transaldolase) in the cells of _O. polymorpha_ strains with overexpressed _DAS1_ and _TAL2_ genes during cultivation at 37°C

| Activity       | Tkl (mU) | Tal (mU) |
|---------------|----------|----------|
| WT            | 10 ± 1.1 | 183 ± 2.33 |
| WT/DAS1       | 21 ± 1.5 | –        |
| WT/TAL2       | –        | 292 ± 3.45 |
| WT/DAS1/TAL2  | 19 ± 0.5 | 288 ± 3.11 |

−, not determined
parental strain BEP cat8Δ during xylose fermentation producing 16.1 g/L of ethanol versus 12.5 g/L for BEP cat8Δ [22]. The BEP cat8Δ/DAS1/TAL2 strain accumulated only trace amounts of xylitol (up to 0.5 g/L) during xylose fermentation.

These transformants did not demonstrate a change in ethanol production in glucose medium (not shown). Transformants with co-overexpression of DAS1 and TAL2 were characterized by increased ethanol production from xylose; however, xylitol consumption from the medium was not activated relative to the parental strain (Fig. 10). The resulting strain exhibited a one-third increase in ethanol yield in xylose medium (Table 3) relative to the parental strain.

Discussion

The role of peroxisomes in yeast and fungal glucose metabolism was recently established for relatively limited number of species [8–11]. However, nothing was known about the role of peroxisomes in the metabolism of the major pentose sugar, xylose. In this work, we have shown the importance of peroxisomal enzymes of PPP in xylose alcoholic fermentation in the native xylose-fermenting yeast, O. polymorpha. It was known for many years that this organism contains peroxisomal transketolase (dihydroxyacetone synthase, Das1) involved in methanol metabolism at the stage of formaldehyde assimilation [24]. Here, we found in the O. polymorpha genome database a gene designated (by us), as TAL2, which contains the PTS1 signal sequence and encodes the peroxisomal transaldolase (Tal2). This protein is located in peroxisomes under cultivation on glucose, xylose, or methanol as carbon sources (Fig. 2).

It is interesting that both peroxisomal transketolase and transaldolase are not necessary for growth on xylose as a sole carbon and energy source, in contrast to their cytosolic counterparts, Tk11 and Tal1, but are required for xylose to ethanol fermentation. Inversely, the knockout of genes coding for cytosolic transketolase, TKL1, and transaldolase, TAL1, totally abolished growth on xylose, while only partially suppressing the fermentation of this sugar. It should be stated that peroxisomal transaldolase is not involved in methanol metabolism in O. polymorpha (Fig. 3). Earlier, the role of peroxisomal transaldolase in methanol metabolism was suggested for K. phaffii [12]; however, such role was not shown directly.

We have also found that overexpression of Das1 and/or Tal2 enhanced ethanol production from xylose (like the effects of Tk11 and Tal1 overexpression). Importantly, these manipulations specifically affected xylose fermentation, but not that of glucose. A crucial difference between growth and fermentation is that the latter demands limited oxygen supply. It could be that Das1 and Tal2 might be involved in responding to limited oxygen. Reactions catalyzed by transketolase and transaldolase do not involve any oxidoreductive changes; however, it is still possible that further peroxisomal metabolism decreases cell reductive state, which is favorable for xylose (but not glucose) alcoholic fermentation. Alternatively, this result could be explained by the potential involvement of the DAS1 and TAL2 genes in transcriptional regulation of XYL1, coding for the enzyme of the initial step of xylose metabolism and fermentation (Fig. 1; Table 2). Roles of enzymes in the regulation of gene expression in yeasts have been thoroughly demonstrated [46]. The specific mechanisms of Das1 and Tal2 involvements in regulation of XYL1 remain to be elucidated, however. It cannot be excluded that peroxisomes are required for xylose to ethanol fermentations due to the location of the Das1 and Tal2 enzymes in this organelle. Indeed, in S. stipitis,
| Strains         | Genes | XYL1  | XYL2  | XYL3  | PDC1  | RPE1  | TKL1  | ADH1  | CYC1  | FBP1  | PCK1  |
|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| das1∆/WT      |       | 0.295±0.072 | 1.330±0.044 | 1.491±0.111 | 1.253±0.110 | 1.261±0.267 | 1.384±0.169 | 0.861±0.032 | 1.397±0.065 | 1.738±0.056 | 1.462±0.019 |
| DAS1*/WT      |       | 2.155±0.232 | 1.044±0.134 | 0.801±0.015 | 0.713±0.006 | 0.877±0.056 | 1.337±0.041 | 0.571±0.125 | 1.099±0.088 | 0.309±0.099 | 0.447±0.098 |
| tal2∆/WT      |       | 0.124±0.062 | 1.538±0.016 | 0.909±0.181 | 0.717±0.104 | 0.830±0.072 | 0.867±0.111 | 0.609±0.018 | 1.576±0.205 | 1.901±0.049 | 1.44±0.051  |
| TAL2*/WT      |       | 1.452±0.234 | 1.301±0.246 | 1.158±0.092 | 0.580±0.101 | 0.575±0.161 | 0.667±0.122 | 1.246±0.081 | 1.689±0.133 | 1.428±0.085 | 0.837±0.114 |

The mRNA quantification was normalized to ACT1 mRNA.
which does not contain peroxisomal transketolase and transaldolase enzymes, peroxisomes are not required for xylose fermentation.

The ability to increase ethanol production in *O. polymorpha* from xylose after overexpression of peroxisomal transketolase and presumably peroxisomal transaldolase was shown both for the wild-type strain and for earlier constructed advanced ethanol producer from xylose [22]. Co-overexpression of Das1 and Tal2 in the advanced ethanol producer led to the accumulation of 16.0–16.5 g ethanol/L at 45 °C in xylose medium. This titer of ethanol is 30–40 × higher relative to that accumulated by the wild-type strain and one-third higher than in previous best *O. polymorpha* strain [22]. It is interesting to compare our best strain isolated herein with xylose-fermenting strain isolated in another species of thermotolerant yeasts, *Kluyveromyces marxianus* [47]. Ethanol yield and the productivity of our best *O. polymorpha* strain with overexpressed DAS1 and TAL2 at 45 °C are like that described for *K. marxianus* at 42 °C; however, at 45 °C, *K. marxianus* showed a drop in the mentioned parameters. Moreover, our best strain of *O. polymorpha* did not accumulate xylitol during xylose fermentation, whereas *K. marxianus* accumulated large amounts of this by-product [47]. However, the main drawback of constructed *O. polymorpha* strain is incomplete xylose consumption under fermentation condition. To address this, we plan to activate xylose uptake and manipulate the genes of glycolysis and of oxidative and non-oxidative branches of pentose phosphate pathway. Indeed, we have found recently that simultaneous overexpression of pyruvate decarboxylase and alcohol dehydrogenase in the wild-type strain of *O. polymorpha* increased ethanol accumulation from glucose, xylose, and glycerol [14]. Besides, we can expect an increase in ethanol accumulation from xylose after additional overexpression of the genes, *TKL1* and *TAL1*, coding for cytosolic transketolase and transaldolase, respectively.

**Conclusions**

Xylose to ethanol fermentation in the methylotrophic thermotolerant yeast, *Ogataea polymorpha*, depends on functional peroxisomal transketolase (Das1) and transaldolase (Tal2), whereas their cytosolic counterparts (Tk1 and Tal1) are indispensable for growth on this pentose. Defects in peroxisome biogenesis in *O. polymorpha* (but not in the non-methylotrophic yeast *S. stipitis*) strongly compromise xylose fermentation. Glucose fermentation does not depend on peroxisomes or peroxisomal and cytosolic transketolases and transaldolases.

**Table 3** Main parameters of xylose fermentation at 45 °C by the tested *O. polymorpha* strains

| Strain               | Ethanol (g/L) | Ethanol yield (g/g consumed xylose) | Rate of ethanol synthesis (g/g biomass/h) | Productivity of ethanol synthesis (g/L/h) |
|----------------------|---------------|------------------------------------|------------------------------------------|------------------------------------------|
| BEP cat8Δ            | 12.51 ± 0.134 | 0.340 ± 0.015                      | 0.091 ± 0.003                            | 0.205 ± 0.009                            |
| BEP cat8Δ/DAS1/TAL2  | 16.13 ± 0.141 | 0.393 ± 0.017                      | 0.092 ± 0.004                            | 0.210 ± 0.005                            |

**Fig. 10** Ethanol production, biomass accumulation, and xylose consumption during xyloferment fermentation at 45 °C by *O. polymorpha* strains a BEP cat8Δ and b BEP cat8Δ/DAS1/TAL2. The data represent values of typical single cultivation.
Co-overexpression of peroxisomal transketolase and transaldolase in a previously isolated, advanced ethanol from xylene producer further increased ethanol accumulation up to 16.1 g/L at 45 °C.

Additional files

**Additional file 1.** Primers used in this study.

**Additional file 2.** Scheme of DAS1 and TAL2 deletion cassettes (ZeRO – gene conferring resistance to zeocin) and PCR verification of the correct cassette integration into genome of the wild-type strain (del1Δ and tal2Δ – constructed deletion strains; WT – recipient strain NCYC495 leu1-1).

**Additional file 3.** Scheme of TAL1 deletion cassette (hphNT1 – gene conferring resistance to hygromycin) and PCR verification of the correct cassette integration into genome of the wild-type strain (pYNR1-TKL1 – constructed strains with substituted gene promoter; WT – recipient strain NCYC495 leu1-1).

**Additional file 4.** Scheme of TAL1 promoter replacement by YNR1 gene promoter (hphNT1 – gene conferring resistance to hygromycin) and PCR verification of the correct cassette integration into the genome of the wild-type strain (pYNR1-TKL1) – constructed strains with substituted gene promoter; WT – recipient strain NCYC495 leu1-1).

**Additional file 5.** Schemes of plasmids used in this study: pGLG61/DAS1, pGLG61/TAL2, pTKrZ, pTAzz. (a) Expression cassettes HpGAPpr-DAS1 and HpGAPpr-TAL2 are shown as gray and white boxes, respectively. The gene-tic resistance gene (APH), linked to the impaired constitutive gene promoter, encoding glyceraldehyde-3-phosphate dehydrogenase (HpGAPpr) and O. polymorpha LEU2 gene are shown as black and light-gray boxes, respectively. The telomeric region (TEL188) is an autonomously replicating sequence is designated with the hatched lines. Origin of replication (ori) and ampicillin resistance gene (bla) are shown as arrows. (b) Expression cassettes HpGAPpr-TAL1 and HpGAPpr-TAL2 are shown as white and gray boxes, respectively. Zeocin resistance gene (ZeoR), is shown as a light-gray box. (c) Expression cassettes HpGAPpr-TAL2 and HpGAPpr-DAS1 are shown as gray and white boxes, respectively. Nourseothricin resistance gene (nmt1NT2), is shown as a black box. Restriction sites: Rl, EcoRl; Xb, Xbol; Pi, PstI; Bl, BamHl; Kl, KpnI; BlI, BgII; Sml, Smol; Sc, Sacl; Ss, Sall; Nd, Ndel.

**Additional file 6.** Scheme of PEX3 deletion cassette (His3, gene involved in histidine biosynthesis, was used as selective marker) and PCR verification of the correct cassette integration into the genome of the wild-type strain (pex3Δ – constructed deletion strain; WT – recipient strain NCYC495 leu1-1).

Authors’ contributions

O.O., V.K., and J.R. carried out strains construction, evaluation of enzymes activity, qRT-PCR and fermentation experiments, and co-drafted the manuscript. R.V.V. participated in strain construction and fermentation experiments. O.V. commented and approved the manuscript. O.A.S. performed and approved the manuscript. O.A.S. provided guidance and suggestions for experimental design, and wrote and edited the manuscript. All authors participated in finalizing the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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References

1. van Roermund CW, Waterham HR, Jist L, Wanders RJ. Fatty acid metabolism in Saccharomyces cerevisiae. Cell Mol Life Sci. 2003;60:1838–51.
2. Michels PA, Meyeroos J, Krazy H, Galland N, Herman M, Hannavaa E. Peroxisomes, glyoxysomes and glycosomes (review). Mol Membri Biol. 2005;22:133–45.
3. Sibirny AA. Yeast peroxisomes: structure, functions and biotechnological opportunities. FEMS Yeast Res. 2016;16:fow038.
4. Magliano P, Filippi M, Sanglard D, Poirier Y. Characterization of the Aspergillus nidulans biotin biosynthetic gene cluster and use of the bioDA gene as a new transformation marker. Fungal Genet Biol. 2011;48:208–15.
5. Bartoszewska M, Opaliński Ł, Veenhuis M, van der Klei I. The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. Biotechnol Lett. 2011;33:1921–31.
6. Aksam EB, de Vries B, van der Klei IJ, Kiel JA. Preserving organelle vitality: peroxisomal quality control mechanisms in yeast. FEMS Yeast Res. 2009;9:808–20.
7. Meyeroos J, Cheo J, Fan E, Hol WG, Michels PA. Biogenesis of peroxiso- somes and glycosomes: trypanosomatid glycosome assembly is a promising new drug target. FEMS Microbiol Rev. 2004;28:603–43.
8. Stehlik T, Sandrock B, Ast J, Freitag J. Fungal peroxisomes as biosynthetic organelles. Curr Opin Microbiol. 2014;22:8–14.
9. Idrurm A, Giles SS, Perfect JR, Hetman J. Peroxisome function regulates growth on glucose in the basidiomycete fungus Cryptococcus neoformans. Eukaryot Cell. 2007;6:60–72.
10. Freitag J, Ast J, Bo M. Cryptic peroxisomal targeting via alternative splicing and stop codon read-through in fungi. Nature. 2012;485:522–5.
11. Stripski B, Visser WF, Distel B. Alternative splicing directs dual localization of Candida albicans 6-phosphogluconate dehydrogenase to cytosol and peroxisomes. FEMS Yeat Res. 2012;12:61–8.
12. Rußmayer H, Buchetics M, Gruber C, Valli M, Grillitsch K, Modarres G, et al. Systems-level organization of yeast methylotrophic lifestyle. BMC Biol. 2015;13:80.
13. Ryabova OB, Chmil OM, Sibirny AA. Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast Hansenula polymorpha. FEMS Yeast Res. 2003;4:157–64.
14. Kata I, Semkiv MV, Ruchala J, Dmytruk KV, Sibirny AA. Overexpression of the genes PDC1 and ADH1 activates glycerol conversion to ethanol in the thermotolerant yeast Ogataea (Hansenula) polymorpha. Yeast. 2016;33:471–8.
15. Ishchuk OP, Voronovsky AY, Abbas CA, Sibirny AA. Construction of Hansenula polymorpha strains with improved thermotolerance. Biotechnol Bioeng. 2009;104:911–9.

16. Voronovsky AY, Rahulya OV, Abbas CA, Sibirny AA. Development of strains of the thermotolerant yeast Hansenula polymorpha capable of alcoholic fermentation of starch and xylan. Metab Eng. 2009;11:234–42.

17. Voronovsky AY, Ryabova OB, Verba OV, Ishchuk OP, Dmytruk KV, Sibirny AA. Expression of xyA genes encoding xylose isomerases from Escherichia coli and Streptomyces coelicolor in the methylotrophic yeast Hansenula polymorpha. FEMS Microbiol Lett. 2005;5:1055–62.

18. Dmytruk OV, Voronovsky AY, Abbas CA, Dmytruk KV, Ishchuk OP, Sibirny AA. Overexpression of bacterial xylose isomerase and yeast host xylulokinase improves xylose alcoholic fermentation in the thermotolerant yeast Hansenula polymorpha. FEMS Yeast Res. 2008;8:165–73.

19. Dmytruk OV, Dmytruk KV, Abbas CA, Voronovsky AY, Sibirny AA. Engineering of xylose reductase and overexpression of xylitol dehydrogenase and xylulokinase improves xylose alcoholic fermentation in the thermotolerant yeast Hansenula polymorpha. Microb Cell Fact. 2008;7:21.

20. Ishchuk OP, Voronovsky AY, Stasyk OV, Gayda GZ, Gonchar MV, Abbas CA, Sibirny AA. Overexpression of pyruvate decarboxylase in the yeast Hansenula polymorpha results in increased ethanol yield in high-temperature fermentation of xylose. FEMS Yeast Res. 2008;8:1164–74.

21. Kurylenko OO, Ruchala J, Hryniv OB, Abbas CA, Dmytruk KV, Sibirny AA. Metabolic engineering and classical selection of the methylotrophic thermotolerant yeast Hansenula polymorpha for improvement of high-temperature xylose alcoholic fermentation. Microb Cell Fact. 2014;13:122.

22. Ruchala J, Kurylenko OO, Soontorngun N, Dmytruk KV, Sibirny AA. Transcriptional activator Cat8 is involved in regulation of xylose alcoholic fermentation in the thermotolerant yeast Ogataea (Hansenula) polymorpha. Microb Cell Fact. 2017;16:36.

23. Jeffers T, Jin Y. Metabolic engineering for improved fermentation of pentoses by yeasts. Appl Microbiol Biotechnol. 2004;63:495–509.

24. Hansen H, Didion T, Thiemann A, Veenhuis M, Roggenkamp R. Targeting sequences of the two major peroxisomal proteins in the methylotrophic yeast Hansenula polymorpha. Mol Gen Genet. 1992;235:269–78.

25. Wates M, Quayle J. The interrelation between transketolase and dihydroxyacetone synthase activities in the methylotrophic yeast Candida boidinii. J Gen Microbiol. 1981;124:309–16.

26. Gould SJ, Keller GA, Hosken N, Wilkinson J, Subramani S. A conserved tripeptide sorts proteins to peroxisomes. J Cell Biol. 1989;108:1657–64.

27. Gödecke A, Veenhuis M, Roggenkamp R, Janowicz ZA, Hollenberg CP. Biosynthesis of the peroxisomal dihydroxyacetone synthase from Hansenula polymorpha in Saccharomyces cerevisiae induces growth but not proliferation of peroxisomes. Curr Genet. 1989;16:13–20.

28. Riley R, Haridas S, Wolfe KH, Lopes MR, Hittinger CT, Goker M, et al. Comparative genomics of biotechnologically important yeasts. Proc Natl Acad Sci USA. 2016;113(35):9982–7.

29. Kurylenko O, Ruchala J, Dmytruk K, Sibirny A. New targets for improvement of xylose alcoholic fermentation in the methylotrophic yeast Hansenula polymorpha. In: Sibirny A, Fedorovych D, Gonchar M, Grabek-Lejko D, editors. Living organisms and bioanalytical approaches for detoxification and monitoring of toxic compounds. Rzeszow: University of Rzeszow; 2015. p. 247–57.

30. van der Klei UJ, Urimoto H, Sakai Y, Veenhuis M. The significance of peroxisomes in methanol metabolism in methylotrophic yeast. 2006;1763:1453–62.

31. Maassen N, Freese S, Schuff B, Passoth V, Kliner U. Nonhomologous end joining and homologous recombination DNA repair pathways in integration mutagenesis in the xylose-fermenting yeast Pichia stipitis. FEMS Yeast Res. 2008;8:735–43.

32. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 3rd ed. New York: Cold Spring Harbor Lab; 2001.

33. Faber KN, Haima P, Harder W, Veenhuis M, Ab G. Highly-efficient electrotoration of the yeast Hansenula polymorpha. Curr Genet. 1994;16:305–10.

34. Taxis C, Knop M. System of centromeric, episomal, and integrative vectors based on drug resistance markers for Saccharomyces cerevisiae. Biotechniques. 2006;40:73–8.

35. Sohn J, Choi E, Kang H, Rhee J, Agapanov M. A dominant selection system designed for copy-number-controlled gene integration in Hansenula polymorpha DL1. Appl Environ Microbiol. 1999;51:800–7.

36. Dmytruk K, Ruchala J, Grabek-Lejko D, Puchalski C, Sibirny A. Autophagy-related gene ATG13 is involved in control of xylose alcoholic fermentation in the thermotolerant methylotrophic yeast Ogataea polymorpha. FEMS Yeast Res. 2018;18(2):010.

37. Jensen ED, Ferreira R, Jakobunas T, Arsoviska D, Zhang J, Ding L, et al. Transcriptional reprogramming in yeast using dCas9 and combinatorial gRNA strategies. Microb Cell Fact. 2017;16(1):46.

38. Ferreira R, Teixeira PG, Mossing M, David F, Sievers V, Nielsen J. Metabolic engineering of Saccharomyces cerevisiae for overproduction of triacylglycerols. Metab Eng Commun. 2018;6:22–7.

39. Lowry O, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–75.

40. Bergmeyer HU, Gawehn K. Methods of enzymatic analysis, vol. 2. London: Academic Press; 1974.

41. Gonchar M, Maidan M, Pavlishko H, Sibirny A. A new oxidase-peroxidase kit for ethanol assays in alcoholic beverages. Food Technol Biotechnol. 2001;39:37–42.

42. Veenhuis M, Van Dijken JP, Harder W. The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. In: Advances in microbial physiology. Cambridge: Academic Press; 1983.

43. Monosov EZ, Wenzel TJ, Luers GH, Heyman JA, Subramani S. Labeling of peroxisomes with green fluorescent protein in living P-pastoris cells. J Histochem Cytochem. 1996;44:851–9.

44. Sundström M, Lindqvist Y, Schneidert G, Hellman U, Ronne H. Yeast kit for ethanol assays in alcoholic beverages. Food Technol Biotechnol. 1994;16:305–10.

45. Avila J, Prez MD, Brito N, Gonz C, Siverio JM. Cloning and disruption of the gene encoding xylose isomerase of the xylose-fermenting yeast Ogataea polymorpha. FEMS Yeast Res. 2008;8:199–206.

46. Santangelo GM. Glucose signaling in Saccharomyces cerevisiae. J Biol Chem. 1993;268:24346–52.

47. Zhang J, Zhang B, Wang D, Gao X, Sun L. Rapid ethanol production at high temperatures by yeasts. Biotechnol Biofuels 2018;11:197.