Functional overexpression of genes involved in erythritol synthesis in the yeast *Yarrowia lipolytica*

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**Abstract**

**Background:** Erythritol, a four-carbon polyol synthesized by microorganisms as an osmoprotectant, is a natural sweetener produced on an industrial scale for decades. Despite the fact that the yeast *Yarrowia lipolytica* has been reported since the 1970s as an erythritol producer, the metabolic pathway of this polyol has never been characterized. It was shown that erythritol synthesis in yeast occurs via the pentose phosphate pathway (PPP). The oleaginous yeast *Y. lipolytica* is a good host for converting inexpensive glycerol into a value-added product such as erythritol. Glycerol is a renewable feedstock which is produced on a large scale as a waste product by many branches of industry.

**Results:** In this study, we functionally overexpressed four genes involved in the pentose phosphate pathway (PPP): gene YALI0E06479g encoding transketolase (*TKL1*), gene YALI0F15587g encoding transaldolase (*TAL1*), gene YALI0E22649g encoding glucose-6-phosphate dehydrogenase (*ZWF1*), and gene YALI0B15598g encoding 6-phosphogluconate dehydrogenase (*GND1*). Here, we show that the crucial gene for erythritol synthesis in *Y. lipolytica* is transketolase. Overexpression of this gene results in a twofold improvement in erythritol synthesis during a shake-flask experiment (58 g/L). Moreover, overexpression of *TKL1* allows for efficient production of erythritol independently from the supplied dissolved oxygen. Fermentation conducted in a 5-L bioreactor at low agitation results in almost 70% higher titer of erythritol over the control strain.

**Conclusion:** This work presents the importance of the PPP in erythritol synthesis and the feasibility for economic production of erythritol from glycerol by the yeast *Y. lipolytica*.

**Keywords:** *Yarrowia lipolytica*, Erythritol, Glycerol, Transketolase, Pentose phosphate pathway
ribitol, mannitol, and organic acids. For economic reasons, an alternative, low-value carbon source for erythritol biosynthesis is sought. Good substitutes of glucose for industrial applications are agricultural wastes, biodiesel byproducts, or other renewable feedstocks such as glycerol. Every year, crude glycerol is produced in huge quantities by many industrial branches, e.g., in biodiesel production, fat saponification, stearin production, and alcoholic beverage production units [10]. Despite the fact that crude glycerol is contaminated by many undesired compounds such as methanol, salts, and matter organic non-glycerol (MONG), some microorganisms are capable of metabolizing it; one of them is *Y. lipolytica* [11].

The unconventional yeast *Y. lipolytica* is a well-studied oleaginous model organism for investigating lipid production and accumulation in eukaryotic cells. However, it also possesses capability for production of polyols and organic acid [12–18]. Despite many studies on erythritol synthesis using *Y. lipolytica*, the metabolic pathway of this phenomenon has never been described. It was shown that in fungus and yeast, erythritol synthesis occurs via the pentose phosphate pathway (PPP) [19, 20] and in the final step, erythrose reductase (ER) reduces erythrose to erythritol with concomitant NAD(P)H oxidation [1].

The aim of this study was to identify the key genes involved in erythritol synthesis. In this study, we overexpressed genes involved in PPP, transketolase (*TKL1*, *YALI0E06479g*), transaldolase (*TAL1*, *YALI0F15587g*), and two dehydrogenases: *ZWF1* (*YALI0E22649g*) and *GND1* (*YALI0B15598g*), responsible for production of reducing agents in the cell, to verify their roles in erythritol biosynthesis. Moreover, we found that the engineered strains are able to produce erythritol at low aeration, this being beneficial for downstream processing. The gene which has the highest impact on erythritol synthesis is transketolase.

**Methods**

**Microorganisms, media, and culture conditions**

The *Y. lipolytica* strains used in this study were derived from the strain *Y. lipolytica* MK1 [21]. All the strains used in this study are listed in Table 1. In Table 2, the gene name, gene symbol, and abbreviation are summarized.

*Escherichia coli* strains were cultivated in LB medium according to standard protocols. Rich yeast extract peptone glucose (YPD) medium was used for the yeast inoculum preparation and contained 1% (w/v) yeast extract, 1% (w/v) peptone, and 2% (w/v) glucose.

### Table 1 Strains and plasmids used in this study

| Strain      | Genotype or plasmid                                      | Source               |
|-------------|---------------------------------------------------------|----------------------|
| *E. coli*   |                                                         |                      |
| DH5α        | F− endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR rupG     | [33]                 |
|             | @80lacZ ΔΔM15 Δ(lacZYA-argF)J169, hsdR17(k-mK+)λ–       |                      |
| DH5α        | pAD, UAS1SG1TEF promoter                                | This study           |
| DH5α        | pAD-TKL1, *YALI0E06479g*                               | This study           |
| DH5α        | pAD-TAL1, *YALI0F15587g*                               | This study           |
| DH5α        | pAD-GDN1, *YALI0B15598g*                               | This study           |
| DH5α        | pAD-ZWF1, *YALI0E22649g*                               | This study           |
| Dh5α        | phrGFP II-C                                            | Agilent              |
| Dh5α        | pAD-hrGFP                                              | This study           |
| Dh5α        | P*TKL1*-gfp                                            | This study           |
| Dh5α        | P*TAL1*-gfp                                            | This study           |
| Dh5α        | P*GDN1*-gfp                                            | This study           |
| Dh5α        | P*ZWF1*-gfp                                            | This study           |
| *Y. lipolytica* |                                             |                      |
| AMM         | MATA, MK1: ura3-302                                     | [28]                 |
| MK1         | MATA, UV-mutant                                         | [21]                 |
| AMM pAD-TKL1| MATA, MK1: ura3-302, overexpression *YALI0E06479g*     | This study           |
| AMM pAD-TAL1| MATA, MK1: ura3-302, overexpression *YALI0F15587g*     | This study           |
| AMM pAD-GDN1| MATA, MK1: ura3-302, overexpression *YALI0B15598g*     | This study           |
| AMM pAD-ZWF1| MATA, MK1: ura3-302, overexpression *YALI0E22649g*     | This study           |
| AMM P*TKL1*-gfp | MATA, MK1: ura3-302, promoter TKL1 fused to hrGfp     | This study           |
| AMM P*TAL1*-gfp | MATA, MK1: ura3-302, promoter TAL1 fused to hrGfp    | This study           |
| AMM P*GDN1*-gfp | MATA, MK1: ura3-302, promoter GDN1 fused to hrGfp    | This study           |
| AMM P*ZWF1*-gfp | MATA, MK1: ura3-302, promoter ZWF1 fused to hrGfp    | This study           |
Table 2 Description of genes overexpressed in Y. lipolytica

| Gene | Number      | Function                      |
|------|-------------|-------------------------------|
| TAL1 | YALI0F15587g| Transaldolase                 |
| ZWF1 | YALI0E22649g| Glucose-6-phosphate dehydrogenase |
| GND1 | YALI0B15598g| 6-Phosphogluconate dehydrogenase |

During shake-flask experiments, the cultures were grown in 0.3-L Erlenmeyer flasks or 0.25-L baffled flasks containing 0.03 L medium on a rotary shaker (CERTOMAT IS, Sartorius Stedim Biotech) at 28 °C at 280 rpm. Erythritol production was conducted in Erythritol Synthesis Medium (g/L): 100 glycerol (Chempur, Poland), 2.3 (NH₄)₂SO₄ (Chempur), 1 MgSO₄ × 7H₂O (Chempur), 0.23 KH₂PO₄ (Chempur), NaCl 26.4 (Chempur), 1 yeast extract (Merck, Germany), and 3 CaCO₃, pH 3.0.

The Control Medium was as follows: (g/L): 100 glucose (Merck, Germany), 2.3 (NH₄)₂SO₄ (Chempur), 1 MgSO₄ × 7H₂O (Chempur), 0.23 KH₂PO₄ (Chempur), NaCl 26.4 (Chempur), 1 yeast extract (Merck, Germany), and 3 CaCO₃, pH 3.0.

Bioscreen C

The yeast strain was grown in 100-well plates in 200 µL of YNB supplemented with glycerol 2% (w/v) or glucose 2% (w/v). First, the strains were grown for 24 h in YPD medium containing 1% (w/v) yeast extract, 1% (w/v) peptone, and 2% (w/v) glucose. The cells were washed and inoculated to an OD₆₀₀ of 0.15 in each well. Quintuple experiments were performed at 28 °C under constant agitation with a Bioscreen C (Oy Growth Curves Ab Ltd., Finland). Growth was monitored by measuring the optical density at 420–560 nm every 30 min for 72 h.

Bioreactor studies

To prepare an inoculation culture for fermentation in a bioreactor, the cultures were grown in 0.3-L Erlenmeyer flasks (containing 0.1 L of YPD medium) on a shaker at 28 °C for 72 h at 140 rpm. Erythritol production was conducted in a medium consisting of (g/L): 150 glycerol, 2.3 (NH₄)₂SO₄, 1 MgSO₄ × 7H₂O, 0.23 KH₂PO₄, NaCl 26.4, and 1 yeast extract, pH 3.0. An inoculum of 0.2 L was introduced into the bioreactor containing the production medium. The cultivations were performed in a 5-L jar bioreactor (Biostat B Plus, Sartorius, Germany) with a working volume of 2 L at 28 °C. The aeration was fixed at 0.8 L/min. The stirrer speed was adjusted to 500 rpm. The pH was maintained automatically at 3.0 via the addition of NaOH (40% w/v). The amount of the supplied NaOH has been taken into account during calculations of the metabolite concentrations. In order to limit evaporation during the batch cultures, the exhaust gases were passed into the exhaust condenser in which the moisture was removed and returned to the vessel. The cultures were performed in three replicates.

Cloning and transformation protocols

All restriction enzymes were purchased from FastDigest Thermo Scientific (USA), and all of the digestions were performed according to standard protocols. The PCR reactions were set up using recommended conditions and Phusion high-fidelity DNA polymerase (Thermo Scientific). The ligation reactions were performed for 10 min at room temperature using T4 DNA Ligase (Thermo Scientific). The gel extractions were performed using the Gel Out gel extraction kit purchased from A&A Biotechnology (Poland). The E. coli minipreps were performed using the Plasmid Mini Kit (A&A Biotechnology). Transformation of E. coli strains was performed using standard chemical protocols [22]. Genomic DNA (gDNA) was extracted from Y. lipolytica using the Genomic Mini AX Yeast Spin kit (A&A Biotechnology, Poland). The obtained plasmids were digested with MsiI to create linear expression cassettes devoid of E. coli DNA and surrounded by Y. lipolytica rDNA for targeted integrations. The transformants were plated out on selective media [23] and were confirmed via gDNA extraction and three distinct PCR confirmations.

Construction of overexpression plasmids

First, plasmid pAD carrying UAS1B 16-TEF promoter was formed. Primers pAD-NotI-F and pAD-NotI-R amplified linear plasmid pAD-UTGut1 [23] but devoid of the rDNA for targeted integrations. The transformants were performed according to standard protocols. The PCR reactions were set up using recommended conditions and Phusion high-fidelity DNA polymerase (Thermo Scientific). The ligation reactions were performed for 10 min at room temperature using T4 DNA Ligase (Thermo Scientific). The gel extractions were performed using the Gel Out gel extraction kit purchased from A&A Biotechnology (Poland). The E. coli minipreps were performed using the Plasmid Mini Kit (A&A Biotechnology). Transformation of E. coli strains was performed using standard chemical protocols [22]. Genomic DNA (gDNA) was extracted from Y. lipolytica using the Genomic Mini AX Yeast Spin kit (A&A Biotechnology, Poland). The obtained plasmids were digested with MsiI to create linear expression cassettes devoid of E. coli DNA and surrounded by Y. lipolytica rDNA for targeted integrations. The transformants were plated out on selective media [23] and were confirmed via gDNA extraction and three distinct PCR confirmations.

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Construction of hrGFP plasmids
First, the gene hr-GFP from plasmid phrGFP II-C (Agilent) using primers hrGFP-AscI-F/hr-Nhel-R was amplified, digested with the enzymes, and cloned into previously digested pAD, yielding pAD-hrGFP. Construction of the promoter-gfp fusion plasmids P_{TKL1-}\text{-gfp}, P_{TAL1-}\text{-gfp}, P_{ZWF1-}\text{-gfp}, P_{GND1-}\text{-gfp} was carried out by amplifying the entire promoter region using primers Ptk-Bsp119I-F/Ptk-AscI-R, Ptal-Bsp119I-F/Ptal-AscI-R, Pzwf-Bsp119I-F/Pzwf-AscI-R, Pgd-Bsp119I-F/Pgd-AscI-R. Subsequently, promoters were cloned into gel-extracted pAD (promoterless) digested with Ascl/ Bsp119I, yielding P_{TKL1-}\text{-gfp}, P_{TAL1-}\text{-gfp}, P_{ZWF1-}\text{-gfp}, P_{GND1-}\text{-gfp}. The obtained plasmids were digested with MssI and transformed into the strain Y. lipolytica AMM.

RNA isolation and transcript quantification
The shake-flask cultures were grown for 24 h in Erythritol Synthesis Medium supplemented with glycerol (100 g/L) or in the Control Medium supplemented with glucose (100 g/L). Next, the cultures were collected and centrifuged for 5 min at 12,000g. The RNA was extracted using a Total RNA Mini Plus kit (A&A Biotechnology, Poland). Each sample was treated with DNAse I (Thermo Scientific) and the Eco Real-Time PCR System manufacturer’s instructions. We measured RNA quantities using a Biochrom WPA Biowave II spectrophotometer (Biochrom Ltd., UK) equipped with a TrayCell (Hellma Analytics, Germany), and the samples were stored in a −80 °C freezer. We conducted cDNA synthesis using Maxima First Strand cDNA. Synthesis kits for RT-qPCR (Thermo Scientific) were used according to the manufacturer’s instructions. We measured RNA quantities using a Biochrom WPA Biowave II spectrophotometer (Biochrom Ltd., UK) equipped with a TrayCell (Hellma Analytics, Germany), and the samples were stored in a −80 °C freezer. We conducted cDNA synthesis using Maxima First Strand cDNA. Synthesis kits for RT-qPCR (Thermo Scientific) were used according to the manufacturer’s instructions. We carried out qRT-PCR analyses using a DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific) and the Eco Real-Time PCR System (Illumina, USA). Primers for RT-PCR were designed as follows: gene TKL1 (YALI0E06479g) encoding the transketolase, gene TAL1 (YALI0F15987g) encoding transaldolase, gene GND1 (YALI0B15598g) encoding NADP⁺-dependent 6-phosphogluconate dehydrogenase, gene ZWF1 (YALI0E22649g) encoding NADP⁺-dependent glucose-6-phosphate dehydrogenase were used as a template. Primers qTket-F and qTket-R bind to the first exon at 25 bp and to the second exon at 478 bp of the TKL1 gene, respectively, resulting in a 111 bp qRT-PCR product. Next, primers qTald-F and qTald-R bind to the first exon at 42 bp and to the second exon at 478 bp of the TAL1 gene, respectively, resulting in a 107 bp qRT-PCR product. Gene GND1 possesses three introns; thus primer qGND1-F binds to the second exon at 257 bp, and primer qGND1-R binds to the second exon at 595 bp. The obtained PCR product in qRT-PCR is 105 bp. Primers qZWF1-F and qZWF1-R bind to the first exon at 5 bp and to the second exon at 595 bp of the ZWF1 gene, respectively, resulting in a 151 bp qRT-PCR product. The results were normalized to the actin gene ACT-F/ACT-R and analyzed using the ddCT method [24]. Samples were analyzed in triplicate.

Analytical methods
Samples (10 mL) from the cultures were centrifuged (10 min; 4 °C; 5500×g), harvested by filtration on 0.45-μm pore membranes and washed twice with distilled water. The biomass was determined gravimetrically after drying at 105 °C. The concentrations of the metabolites were determined using HPLC equipped with a HyperRez Carbohydrate H⁺ Column (Thermo Scientific, Waltham, MA) coupled to a UV (λ = 210 nm) ( Dionex, Sunnyvale, USA) and a refractive index (RI) detector (Shodex, Ogi-machi, Japan). The column was eluted with 25 mM of trifluoroacetic acid (TFA) at 65 °C and a flow rate of 0.6 mL/min.

Fluorescence microscopy
Yeast cells were visualized using an Axio Scope A1 Zeiss microscope (Zeiss, Germany). A GFP filterset (Zeiss), operated at excitation of 470/40 nm, and emission of 525/50 nm, was used to detect green fluorescence. Microscopy data were stored using ZEN lite (Zeiss).

Calculation of fermentation parameters
To consider medium dilution due to the addition of NaOH required for pH control, the amounts of erythritol and byproducts in the culture broth were used to calculate the mass yield of erythritol (Y_{ERY}), and the volumetric erythritol productivity (Q_{ERY}). The mass yield of erythritol (Y_{ERY}) was expressed in g/g from glycerol and was calculated by the equation

\[ Y_{ERY} = \frac{P}{S}. \]

The volumetric erythritol productivity (Q_{ERY}) expressed in g/L/h was calculated from

\[ Q_{ERY} = \frac{P}{V \cdot t}, \]

where P is the amount of erythritol in the culture liquid at the end of cultivation (g); S is the total amount of glycerol consumed (g); V is the initial volume of culture liquid (l); and t is the fermentation time (h).

Results and discussion
Quantification of gene expression during erythritol synthesis
Production of erythritol by yeast is a well-known phenomenon; however, the metabolic pathway of this process for Y. lipolytica has never been characterized. Our previous study showed the hypothetical pathway of
erythritol from glycerol in this oleaginous yeast [21]. In this study, we focused on four genes to investigate their role in erythritol biosynthesis. Here, we choose genes belonging to the pentose phosphate pathway (PPP), since it was shown that erythritol synthesis occurs via this pathway [25]. In this study, we selected genes encoding transketolase (TKL1, YALI0E06479g), transaldolase (TAL1, YALI0F15587g), NADP$^+$-dependent 6-phosphogluconate dehydrogenase (GND1, YALI0B15598g), and NADP$^+$-dependent glucose-6-phosphate dehydrogenase (ZWFI, YALI0E22649g). The first (oxidative) phase of PPP starts with dehydrogenation of glucose 6-phosphate, by 6-phosphogluconate dehydrogenase, resulting in NADPH and 6-phosphoglucono-δ-lactone synthesis (Fig. 1). Next, 6-phosphoglucono-δ-lactone is hydrolyzed by a specific lactonase to produce 6-phosphoglucuronate. Consequently, this sugar is oxidatively decarboxylated by 6-phosphogluconate dehydrogenase, yielding ribulose-5-phosphate along with cogeneration of NADPH. In the second, nonoxidative phase of PPP, transketolase converts ribose-phosphate and xylose-5-phosphate into glyceraldehyde 3-phosphate (GAP) and sedoheptulose 7-phosphate. Next, these compounds are catalyzed by transaldolase to form fructose 6-phosphate and erythrose 4-phosphate. Transketolase converts glyceraldehyde-3-phosphate with fructose-6-phosphate into xylulose-5-phosphate and erythrose-4-phosphate (E4P). The last step of erythritol synthesis is beyond PPP, and it is a reduction of erythrose to erythritol by erythrose reductase with codominant NADPH.

The standard carbon source for yeast Y. lipolytica is glucose, and it is commonly used in research and in industry. However, during our study, we observed that the level of erythritol synthesis by strain Y. lipolytica MK1 depends on the carbon source, and it is higher when glycerol is applied (Additional file 2). Probably it is due to higher osmotic stress caused by glycerol. Moreover, in agreement with another study, we observed that in the medium containing glucose as a carbon source, strain MK1 forms mycelium [26]. In medium with glycerol, only yeast cells were observed; this suggests that mycelia cannot synthesize erythritol efficiently. Therefore, to verify the activity of the PPP genes during erythritol synthesis, we compared the relative expressions of the mentioned

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**Fig. 1** Overview of the principal metabolic pathway for erythritol synthesis in Y. lipolytica. Function of the genes names of which are given in bold characters was verified experimentally in this or in the previous study [23].
genes under two different conditions. First, the strain MK1 was grown in the Erythritol Synthesis Medium (see “Methods”) in the baffled flasks; second, MK1 strain was grown in the Control Medium. Remarkably, immediately after 24 h, gene TKL1 exhibited a sevenfold increase in expression over the control, and we also observed enhanced expressions for ZWF1 and TAL1, by 3.0- and 1.8-fold respectively (Additional File 3: Figure S1).

Interestingly, the expressions of the GND1 gene remain at the same level under both conditions (Fig. 2a). To confirm the qRT-PCR results, we fused the promoters of the mentioned genes with hrGFP protein, to visualize their activities under both conditions. In agreement with previous experiment, we observed enhanced GFP expressions for TLK1, ZWF1, and TAL1 in Erythritol Synthesis Medium (Fig. 2b). Strain AMM P_{GND1-gfp} showed similar fluorescence under both conditions, suggesting that this dehydrogenase is active, independent of the applied carbon source. It is worth noting that we did not observe mycelium in the AMM-derived strains.

Previously, the activities of the enzymes encoded by these genes were studied for Moniliella megachiliensis (formerly Trichosporonoides megachiliensis) [20]. In that study, all enzymes belonging to PPP showed high activity during erythritol synthesis; however, transketolase was found as a crucial enzyme for the process. The results obtained in our study confirm that in Y. lipolytica, transketolase is expressed at an enhanced level in comparison with the other genes in the PPP.

**Overexpression of PPP genes in Y. lipolytica**

First, we sought to verify the influence of the PPP genes’ overexpression on Y. lipolytica growth. Thus, we constructed overexpression cassettes containing the genes TKL1, TAL1, ZWF1, and GND1 under a hybrid TEF promoter [27], and transformed the cassettes into the AMM strain, derived from MK1 [28], resulting in strains listed in Table 1.

Next, we monitored the growth of the engineered strains and the wild type in YNB media supplemented with glucose or glycerol. As seen in Fig. 3, all the engineered strains grew better on medium supplemented with glycerol than the control. In the first 24 h, the growth of the all strains was similar; however, after this period, when the maximum activity of the TEF promoter started, we noted significant enhancement in growth of the engineered strains. The control strain grew on YNB with glycerol in the similar way as in the previous study [11]. We did not observe any significant differences in growth between the engineered strains, either on YNB with glucose (data not shown) or on YNB with glycerol.

![Fig. 2](image-url)
Taking into account this result, the effects of TKL1, TAL1, ZWF1, and GND1 overexpressions on erythritol synthesis were first assessed in the shake-flask experiment (Erlenmeyer flask). The conditions of the experiment were established previously [21]. In this study, we monitored two parameters: glycerol consumption and erythritol synthesis (Fig. 4a, b). After 24 h, we did not observe any significant differences in substrate assimilation or in erythritol biosynthesis. However, after 48 h, all the engineered strains showed elevated erythritol synthesis coupled with more rapid glycerol utilization. The most conspicuous effect was observed for the strain overexpressing the TKL1 gene; this strain produced 51.09 ± 1.97 g/L of erythritol within 94 h. Erythritol titers for strains AMM pAD-TAL1, AMM pAD-ZWF1, and AMM pAD-GND1 were 46.69 ± 1.59, 42.53 ± 2.62, and 40.16 ± 2.15 g/L, respectively. The control strain

**Fig. 3** Growth curves of various Y. lipolytica strains: MK1 (red), AMM pAD-TKL1 (black), AMM pAD-TAL1 (green), AMM pAD-GND1 (purple), and AMM pAD-ZWF1 (blue). The strains were grown on a YNB/glycerol medium. Quintuple experiments were performed at 28 °C under constant agitation using Bioscreen C

**Fig. 4** Results of the shake-flask experiment in the Erlenmeyer flasks (a, b) and in the baffled flasks (c, d). Parameters of erythritol synthesis by various Y. lipolytica strains in shake-flask experiments at the end of the process; $Q_{\text{ERY}}$ erythritol productivity; $Y_{\text{ERY}}$ erythritol yield. The cultures were performed in three biological replicates. The error bars represent the standard deviation. Tables summarize the parameters of erythritol synthesis at the end of the processes
produced only 25.30 ± 1.83 g/L. Although glycerol incorporation into the cell was not modified, we observed enhancement of glycerol utilization. In the cultures containing the overexpressing TKL1, ZWF1, and GND1 strains, after 94 h, glycerol was totally depleted, whereas in the control strain, 28 g/L of glycerol was detected. This phenomenon might be a result of pulling the metabolism toward erythritol synthesis by overexpression of the genes involved in this pathway. This has an enormous impact on the erythritol productivity (QERY); the engineered strains achieved twofold higher productivity over the control strain. In addition, the yield of erythritol (YERY) was significantly improved (Fig. 4). The best impact on YERY was noted for strain AMM pAD-TKL1, where the yield achieved was 0.51 g/g, whereas for the control strain, 48 MK1 0.40 ± 0.01 0.32 ± 0.06 2.60 ± 0.13 0.55 ± 0.02
AMM pAD-ZWF1 1.33 ± 0.10 1.99 ± 0.23 8.58 ± 1.33 0.53 ± 0.08
AMM pAD-TKL1 0.87 ± 0.10 1.52 ± 0.07 6.32 ± 2.18 0.77 ± 0.19
AMM pAD-TAL1 1.04 ± 0.07 1.12 ± 0.03 5.05 ± 1.27 0.41 ± 0.04
48 MK1 0.43 ± 0.13 0.39 ± 0.09 3.05 ± 0.01 0.66 ± 0.06
AMM pAD-GND1 1.12 ± 0.01 3.00 ± 0.09 11.50 ± 0.25 0.45 ± 0.20
AMM pAD-ZWF1 1.58 ± 0.10 2.82 ± 0.15 10.33 ± 1.51 0.68 ± 0.15
AMM pAD-TKL1 1.09 ± 0.01 2.48 ± 0.02 7.64 ± 2.51 1.11 ± 0.38
AMM pAD-TAL1 1.52 ± 0.11 2.11 ± 0.45 5.85 ± 1.05 0.66 ± 0.13
54 MK1 0.64 ± 0.01 0.85 ± 0.04 4.54 ± 0.14 0.75 ± 0.05
AMM pAD-GND1 1.32 ± 0.12 5.45 ± 0.22 16.24 ± 0.71 0.64 ± 0.30
AMM pAD-ZWF1 2.01 ± 0.20 5.07 ± 0.01 13.62 ± 3.31 0.84 ± 0.32
AMM pAD-TKL1 1.35 ± 0.05 4.41 ± 0.05 10.33 ± 2.82 1.60 ± 0.61
AMM pAD-TAL1 1.70 ± 0.16 3.11 ± 0.65 7.92 ± 0.69 0.76 ± 0.01
72 MKI 0.89 ± 0.04 1.71 ± 0.16 5.98 ± 0.10 0.79 ± 0.08
AMM pAD-GND1 1.79 ± 0.12 7.63 ± 0.48 18.54 ± 1.67 0
AMM pAD-ZWF1 2.61 ± 0.16 7.02 ± 0.38 15.32 ± 4.28 0
AMM pAD-TKL1 1.53 ± 0.39 6.66 ± 0.78 12.70 ± 2.18 0.31 ± 0.10
AMM pAD-TAL1 2.30 ± 0.13 6.23 ± 0.36 11.16 ± 1.41 1.14 ± 0.52

Table 3 Side-metabolites of Y. lipolytica strains in shake-flask experiments in Erlenmeyer flasks

| Time (h) | Strain         | Arabitol (g/L) | Mannitol (g/L) | Citric acid (g/L) | α-Ketoglutaric acid (g/L) |
|---------|----------------|----------------|----------------|------------------|--------------------------|
| 24      | MK1            | 0              | 0              | 0                | 0                        |
|         | AMM pAD-GND1   | 0.20 ± 0.01    | 0.05 ± 0.01    | 1.13 ± 0.01      | 0                        |
|         | AMM pAD-ZWF1   | 0.24 ± 0.03    | 0.01 ± 0.01    | 0.78 ± 0.01      | 0                        |
|         | AMM pAD-TKL1   | 0              | 0              | 0.84 ± 0.01      | 0                        |
|         | AMM pAD-TAL1   | 0              | 1.12 ± 0.03    | 0                | 0                        |
| 48      | MK1            | 0.40 ± 0.05    | 0.32 ± 0.06    | 2.60 ± 0.13      | 0.55 ± 0.02               |
|         | AMM pAD-GND1   | 0.93 ± 0.05    | 2.15 ± 0.02    | 9.94 ± 0.11      | 0.44 ± 0.05               |
|         | AMM pAD-ZWF1   | 1.33 ± 0.10    | 1.99 ± 0.23    | 8.58 ± 1.33      | 0.53 ± 0.08               |
|         | AMM pAD-TKL1   | 0.87 ± 0.10    | 1.52 ± 0.07    | 6.32 ± 2.18      | 0.77 ± 0.19               |
|         | AMM pAD-TAL1   | 1.04 ± 0.07    | 1.12 ± 0.03    | 5.05 ± 1.27      | 0.41 ± 0.04               |
| 54      | MK1            | 0.43 ± 0.13    | 0.39 ± 0.09    | 3.05 ± 0.01      | 0.66 ± 0.06               |
|         | AMM pAD-GND1   | 1.12 ± 0.01    | 3.00 ± 0.09    | 11.50 ± 0.25     | 0.45 ± 0.20               |
|         | AMM pAD-ZWF1   | 1.58 ± 0.10    | 2.82 ± 0.15    | 10.33 ± 1.51     | 0.68 ± 0.15               |
|         | AMM pAD-TKL1   | 1.09 ± 0.01    | 2.48 ± 0.02    | 7.64 ± 2.51      | 1.11 ± 0.38               |
|         | AMM pAD-TAL1   | 1.52 ± 0.11    | 2.11 ± 0.45    | 5.85 ± 1.05      | 0.66 ± 0.13               |
| 72      | MK1            | 0.64 ± 0.01    | 0.85 ± 0.04    | 4.54 ± 0.14      | 0.75 ± 0.05               |
|         | AMM pAD-GND1   | 1.32 ± 0.12    | 5.45 ± 0.22    | 16.24 ± 0.71     | 0.64 ± 0.30               |
|         | AMM pAD-ZWF1   | 2.01 ± 0.20    | 5.07 ± 0.01    | 13.62 ± 3.31     | 0.84 ± 0.32               |
|         | AMM pAD-TKL1   | 1.35 ± 0.05    | 4.41 ± 0.05    | 10.33 ± 2.82     | 1.60 ± 0.61               |
|         | AMM pAD-TAL1   | 1.70 ± 0.16    | 3.11 ± 0.65    | 7.92 ± 0.69      | 0.76 ± 0.01               |
| 94      | MK1            | 0.89 ± 0.04    | 1.71 ± 0.16    | 5.98 ± 0.10      | 0.79 ± 0.08               |
|         | AMM pAD-GND1   | 1.79 ± 0.12    | 7.63 ± 0.48    | 18.54 ± 1.67     | 0                        |
|         | AMM pAD-ZWF1   | 2.61 ± 0.16    | 7.02 ± 0.38    | 15.32 ± 4.28     | 0                        |
|         | AMM pAD-TKL1   | 1.53 ± 0.39    | 6.66 ± 0.78    | 12.70 ± 2.18     | 0.31 ± 0.10               |
|         | AMM pAD-TAL1   | 2.30 ± 0.13    | 6.23 ± 0.36    | 11.16 ± 1.41     | 1.14 ± 0.52               |

It has been shown that oxygen concentration in the medium has a huge impact on erythritol synthesis by M. megachiliensis [20]. Therefore, to further characterize the engineered strains and explore their abilities of erythritol synthesis, the shake-baffled flask fermentation was conducted. Again, we monitored glycerol assimilation and erythritol synthesis (Fig. 4c, d). In agreement with our assumption, the process was much more efficient than when conducted in Erlenmeyer flasks. First, erythritol production started already after a few hours; therefore, at 24 h of growth, this polyol was detected for all tested strains, including the control. Total time of fermentation
was only 72 h for all the engineered strains; for the control, residual glycerol was still detected (3.34 g/L). Increased aeration has the highest influence on the MK1 strain, because the titer of erythritol at the end of the process (51.15 ± 1.16 g/L) increased twofold in comparison with the previous experiment (25.3 ± 1.83 g/L). Nevertheless, changes of the condition also improved production of the TKL1 overexpressing strain. Here, the erythritol titer was the highest among the tested strains, and it achieved 58.0 ± 0.87 g/L. This leads to enhancement of the process parameters; \( Y_{\text{ERY}} \) achieved 0.58 g/g and \( Q_{\text{ERY}} \) 0.81 g/L h. This is one of the highest amounts of erythritol titers obtained in the shake-flask experiments.

A previously conducted experiment for \( Y. \text{lipolytica} \) in a shake-flask experiment showed a lower titer levels (20.03–35.53 g/L), \( Y_{\text{ERY}} \) (0.33–0.42 g/g), and \( Q_{\text{ERY}} \) (0.08–0.15 g/L h) [29]. Also in experiments using \( Torula \) sp. supplementation of culture media resulted in enhanced amount of erythritol titer, which was 56.6 g/L, whereas \( Y_{\text{ERY}} \) achieved was only 0.28 g/g [30].

Surprisingly, overexpressions of \( ZWF1 \) and \( GND1 \) at higher aeration resulted in higher production of erythritol than at low aeration (Fig. 4); however, after glycerol depletion, we noted rapid polyol assimilation. As a result, both stains showed a slightly lower erythritol titer than at low aeration (Fig. 4); however, after glycerol depletion, we noted rapid polyol assimilation. As a result, both stains showed a slightly lower erythritol titer than the control at the end of the process. These data suggest that both dehydrogenases might play a role in erythritol assimilation as a potential carbon source. Next, strain AMM pAD-TAL1 under high aeration conditions produced less erythritol than in low aeration condition. However, we noted that carbon flux was pushed toward citric acid synthesis. This strain produced 23.37 ± 0.92 g/L of citric acid in baffled flasks, which was a twofold improvement compared with Erlenmeyer flasks, where the citric acid level achieved was 11.16 ± 1.41 g/L. Moreover, it was the highest titer of citric acid among the tested strains (Table 4). This undesired effect for the downstream process can be eliminated by optimization of the fermentation conditions. It is worth noting that the optimization of fermentation conditions is important and has the same high impact on the process as metabolic engineering. Therefore, all genetic modifications should be coupled with the optimization of the fermentation conditions. In accordance with the aim of this study, and to further characterize the AMM pAD-TKL1 transformant and explore its erythritol production characteristics, a large-scale experiment was performed using a 5-L bioreactor.

The effect of TKL1 overexpression in bioreactor fermentations

Given the results of the study, we sought to cultivate AMM pAD-TKL1 strain in a bioreactor fermentation, consistent with experiments previously conducted [23]. This strain was chosen since TKL1 was found to be a crucial gene for erythritol production, and a transformant

Table 4  Side-metabolites of \( Y. \text{lipolytica} \) strains in shake-flask experiments in baffled flasks

| Time (h) | Strain     | Arabitol (g/L) | Mannitol (g/L) | Citric acid (g/L) | \( \alpha \)-Ketoglutaric acid (g/L) |
|----------|------------|----------------|----------------|-------------------|-----------------------------------|
| 24       | MK1        | 0.24 ± 0.03    | 0              | 2.38 ± 0.03       | 0.23 ± 0.01                       |
|          | AMM pAD-GND1 | 0.15 ± 0.04    | 0.29 ± 0.01    | 8.05 ± 0.05       | 0.25 ± 0.00                       |
|          | AMM pAD-ZWF1 | 0.35 ± 0.01    | 0.37 ± 0.05    | 9.00 ± 1.30       | 0.33 ± 0.04                       |
|          | AMM pAD-TKL1 | 0.26 ± 0.01    | 0.32 ± 0.03    | 7.61 ± 0.21       | 0.47 ± 0.02                       |
|          | AMM pAD-TAL1 | 0              | 3.20 ± 0.05    | 7.10 ± 0.50       | 0                                  |
| 48       | MK1        | 0.77 ± 0.00    | 1.19 ± 0.01    | 9.53 ± 0.36       | 0.44 ± 0.01                       |
|          | AMM pAD-GND1 | 0.70 ± 0.02    | 3.14 ± 0.02    | 14.26 ± 0.08      | 1.12 ± 0.08                       |
|          | AMM pAD-ZWF1 | 1.05 ± 0.01    | 3.22 ± 0.13    | 15.37 ± 3.54      | 1.31 ± 0.24                       |
|          | AMM pAD-TKL1 | 0.52 ± 0.00    | 2.43 ± 0.07    | 13.64 ± 0.54      | 2.10 ± 0.11                       |
|          | AMM pAD-TAL1 | 0.93 ± 0.02    | 3.20 ± 0.05    | 17.03 ± 2.82      | 0.27 ± 0.19                       |
| 54       | MK1        | 0.76 ± 0.05    | 1.35 ± 0.27    | 9.99 ± 0.28       | 0.45 ± 0.01                       |
|          | AMM pAD-GND1 | 0.74 ± 0.02    | 3.70 ± 0.05    | 15.60 ± 0.24      | 1.10 ± 0.08                       |
|          | AMM pAD-ZWF1 | 1.08 ± 0.02    | 3.79 ± 0.13    | 16.64 ± 4.04      | 1.29 ± 0.24                       |
|          | AMM pAD-TKL1 | 0.54 ± 0.00    | 2.87 ± 0.05    | 15.61 ± 0.54      | 2.04 ± 0.13                       |
|          | AMM pAD-TAL1 | 1.16 ± 0.07    | 4.73 ± 0.15    | 13.67 ± 1.77      | 0.00 ± 0.00                       |
| 72       | MK1        | 0.71 ± 0.07    | 1.90 ± 0.08    | 10.69 ± 0.27      | 0.37 ± 0.02                       |
|          | AMM pAD-GND1 | 0.75 ± 0.04    | 4.26 ± 0.10    | 15.95 ± 0.34      | 0                                  |
|          | AMM pAD-ZWF1 | 1.08 ± 0.10    | 4.27 ± 0.09    | 15.97 ± 3.83      | 0                                  |
|          | AMM pAD-TKL1 | 0.55 ± 0.01    | 3.40 ± 0.06    | 17.35 ± 1.60      | 0                                  |
|          | AMM pAD-TAL1 | 0.96 ± 0.25    | 4.72 ± 0.40    | 23.37 ± 0.92      | 0                                  |
overexpressing TKL1 showed the highest capacity for erythritol synthesis. During cultivation of Y. lipolytica on an industrial scale, one of the important issues is the high cost of energy consumption for continuous and intense agitation. This yeast involves high demand for oxygen; otherwise the fermentation parameters significantly decrease. In the shake-flask experiment, as we observed that low aeration did not have a negative influence on erythritol synthesis, we sought to conduct the fermentation process at lower agitation (500 rpm) than that applied in the standard procedures (800 rpm). The aeration was fixed at 0.8 L/min, and again as a control strain, MK1 was used.

In agreement with the other studies [31], the dissolved oxygen fell to below 1% after 24 h of growth. As seen in Fig. 5a for the strain overexpressing TKL1, low oxygen was not an obstacle to growth. At the end of the process, the biomass of AMM pAD-TKL1 strain reached 20 g/L, which is a standard biomass titer in the erythritol synthesis medium [23]. In contrast, the control strain produced a maximum 14.1 g/L biomass after 96 h of growth, and it decreased to 10 g/L at the end of the process. The first detection of erythritol was after 24 h for both stains, and we did not notice any difference between the two strains. However, after the next 24 h, when the highest activity of the TEF promoter started, we observed rapid production of erythritol for the engineered strain (Fig. 5a). The engineered strain produced 62.5 g/L of erythritol within 102 h, resulting in $Q_{ERY} = 0.62 \pm 0.05$ g/L h and $Y_{ERY} = 0.42 \pm 0.05$ g/g. Interestingly, production of the side-metabolites was significantly inhibited in comparison with the shake-flask experiment, and the maximum content of each of the undesired products did not exceed 5 g/L (Table 5). This effect was caused by the established and fully controlled fermentations process in the bioreactor; pH and the oxygen supply were maintained at constant levels. A similar effect during erythritol production was observed previously [21].

The low concentration of side-metabolites is important for industrial applications, since it simplifies the purification process and consequently reduces production costs. The engineered strain assimilated glycerol within 102 h of fermentation, whereas the control strain was not able to deplete glycerol during 168 h of cultivation. Moreover, biosynthesis of erythritol was limited compare to

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### Table 5 Side-metabolites of Y. lipolytica strains in bioreactor study

| Time (h) | Strain       | Arabitol (g/L) | Mannitol (g/L) | Citric acid (g/L) | α-Ketoglutaric acid (g/L) |
|----------|--------------|----------------|----------------|------------------|--------------------------|
| 24       | MK1          | 0              | 0              | 0                | 0                        |
|          | AMM pAD-TKL1 | 0              | 0              | 0.02 ± 0.02      | 0.25 ± 0.07              |
| 48       | MK1          | 0.35 ± 0.07    | 0              | 0.15 ± 0.2       | 0.70 ± 0.14              |
|          | AMM pAD-TKL1 | 0.76 ± 0.08    | 1.00 ± 0.01    | 0.14 ± 0.10      | 1.21 ± 0.13              |
| 72       | MK1          | 0.70 ± 0.01    | 0.35 ± 0.19    | 0.40 ± 0.17      | 0.90 ± 0.14              |
|          | AMM pAD-TKL1 | 1.21 ± 0.16    | 2.72 ± 0.03    | 0.93 ± 0.52      | 2.93 ± 0.38              |
| 96       | MK1          | 0.85 ± 0.07    | 1.20 ± 0.42    | 0.70 ± 0.57      | 0.90 ± 0.42              |
|          | AMM pAD-TKL1 | 1.33 ± 0.46    | 4.01 ± 1.42    | 1.63 ± 0.67      | 4.07 ± 0.90              |
| 102      | MK1          | 0.90 ± 0.14    | 1.15 ± 0.49    | 0.80 ± 0.51      | 0.95 ± 0.49              |
|          | AMM pAD-TKL1 | 1.40 ± 0.57    | 5.00 ± 0.28    | 4.00 ± 1.97      | 3.95 ± 1.77              |
| 168      | MK1          | 0.15 ± 0.21    | 0.25 ± 0.20    | 1.20 ± 0.28      | 0                        |
|          | AMM pAD-TKL1 | –              | –              | –                | –                        |
the engineered strain, and the titer was 37.3 g/L at the end of the process, resulting in $Q_{\text{ERY}}$ 0.27 g/L h and $Y_{\text{ERY}}$ 0.25 g/g. Due to weak growth of the control strain, production of byproducts was highly limited (Table 5).

The process conducted for the control strain at low agita-
tion was inefficient and time consuming compared with
the previous studies [5, 21, 32]. These data confirm that
oxygen limitation has a huge impact on erythritol syn-
thesis by Y. lipolytica. These results showed that transket-
olase plays a crucial role in erythritol synthesis and its
overexpression allows for robust growth independent of
oxygen supply. The engineered strain AMM pAD-TKL1
synthesized erythritol efficiently in a short period of time
at a low oxygen concentration. It has a huge potential for
industrial application.

**Conclusions**

Until now, the metabolic pathway of erythritol synthesis
in Y. lipolytica has been never described. In this study, we
showed that erythritol production in Y. lipolytica occurs
via the pentose phosphate pathway (PPP) and the gene
YALI0E06479g encoding transketolase plays a crucial role
in this process. Moreover, gene YALI0E22649g encoding
glucose-6-phosphate dehydrogenase (ZWF1) provides
the reducing agent which is required in the last step of
the process. Overexpression of the genes belonging to the
PPP enhances the level of erythritol titer. An increased
level of TKL1 transcription results in significantly higher
erythritol synthesis under conditions of oxygen limita-
tion. During the shake-flask experiment, the engineered
strain increased erythritol production twofold over the
control. Over the course of the bioreactor study, strain
AMM pAD-TKL1 produced 67% more erythritol than the
test strain, and production of the by-product was signifi-
cantly inhibited. Moreover, these results were obtained
using a low-cost medium in which the single carbon
source was glycerol and at low agitation; this situation is
beneficial for downstream processing.

**Abbreviations**

TKL1: gene YALI0E06479g encoding transketolase; TAL1: gene YALI0F15587g
encoding transaldolase; ZWF1: gene YALI0E22649g encoding glucose-6-phos-
phate dehydrogenase; GND1: gene YALI0B15598g encoding 6-phosphogluco-
ate dehydrogenase.

**Authors’ contributions**

AMM designed the study, constructed the plasmids and the strains, per-
formed qRT-PCR study, took microscopy pictures, analyzed the data, and
wrote the manuscript. AB participated in the fermentation experiments. AD
participated in the design of the study, helped to analyze the data, and revised
the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

The authors thank Magdalena Rakicka for HPLC analysis.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of supporting data**

The authors promise the availability of supporting data.

**Consent for publication**

The authors have consented for publication.

**Funding**

This work was financed by the Polish National Centre for Research and Devel-
opment under project LIDER/010/207/L-5/13/NCBR/2014.

Received: 19 January 2017 Accepted: 22 March 2017
Published online: 24 March 2017

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