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Enhancing erythritol productivity in *Yarrowia lipolytica* using metabolic engineering

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ABSTRACT

Erythritol (1,2,3,4-butanetetrol) is a four-carbon sugar alcohol with sweetening properties that is used by the agrofood industry as a food additive. In this study, we demonstrated that metabolic engineering can be used to improve the production of erythritol from glycerol in the yeast *Yarrowia lipolytica*. The best results were obtained using a mutant that overexpressed *GUT1* and *TKL1*, which encode a glycerol kinase and a transketolase, respectively, and in which *EYK1*, which encodes erythrulose kinase, was disrupted; the latter enzyme is involved in an early step of erythritol catabolism. In this strain, erythritol productivity was 75% higher than in the wild type; furthermore, the culturing time needed to achieve maximum concentration was reduced by 40%. An additional advantage is that the strain was unable to consume the erythritol it had created, further increasing the process's efficiency. The erythritol productivity values we obtained here are among the highest reported thus far.

1. Introduction

Erythritol (1,2,3,4-butanetetrol) is a four-carbon sugar alcohol with sweetening properties that is used by the agrofood industry as a food additive (E968). Erythritol is noncariogenic and has been determined to be safe for human consumption even when high doses are consumed daily (Bernt et al., 1996; Kawanabe et al., 1992). It has extremely low digestibility and does not modify blood insulin levels (Munro et al., 1998). Erythritol is widespread in nature and has been found in seaweed, fungi, fruit, and fermented food, although always at low levels (Moon et al., 2010). It is also produced by osmophilic microorganisms in response to osmotic stress (Hallsworth and Magan, 1997).

Although erythritol can be produced chemically from dialdehyde starch, this process has never been industrialized due to its low efficiency. Instead, erythritol is most commonly generated from glucose via fermentation processes using osmophilic yeasts (Moon et al., 2010) namely *Aurobasidium* sp. (Ishizuka et al., 1989), *Trigonopsis variabilis* (Kim et al., 1997), *Torula* sp. (Lee et al., 2000), *Candida magnoliae* (Ryu et al., 2000), *Pseudozyma tsubakaensis* (Jeya et al., 2009), and *Moniliela* sp. (Lin et al., 2010). Despite the some of these processes have been developed at industrial scale, they suffer from the high cost of the fermentation media and the production of unwanted byproducts such as mannitol, and organic acids which renders the downstream processing more challenging. Recently, *Yarrowia lipolytica* has also been found to be an efficient erythritol producer (Rymowicz et al., 2008; Yang et al., 2014; Park et al., 2016; Mirończuk et al., 2016).

Y. lipolytica is a non-conventional model yeast species that is well-known for its unusual metabolic properties (Fickers et al., 2005; Nicaud, 2012). Because it can secrete large amounts of proteins and metabolites of biotechnological interest, *Y. lipolytica* has several industrial applications, including heterologous protein synthesis and citric acid production, and has been accorded GRAS status (Fickers et al., 2005; Zinjarde, 2014). *Y. lipolytica* is highly proficient at producing erythritol and can use raw glycerol instead of glucose as its main carbon source (Rymowicz et al., 2008; Almeida et al., 2012). Raw glycerol is a byproduct of the biodiesel production or fat industries (i.e. fat saponification, stearin synthesis) and is thus available in large quantities at lower price than glucose. Moreover, glycerol allows higher production yields as compared to glucose, making the erythritol production process more profitable. (Rymowicz et al., 2008; Tomaszewska et al., 2012; Rywińska et al., 2013). The synthesis of erythritol from glycerol is not a redox-balanced reaction, as it requires a net amount of oxidized cofactors. However it is more advantageous than using glucose since synthesis of erythritol from the latter consumes

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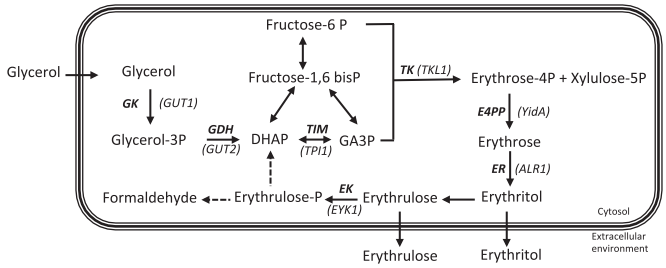

Fig. 1. Pathway used by Y. lipolytica to synthesize erythritol from glycerol. DHAP: dihydroxyacetone phosphate; GA3P: glyceraldehyde-3-phosphate; GK: glycerol kinase; GDH: glycerol-3P dehydrogenase; TIM: triosephosphate isomerase; TK: transketolase; E4PP: erythrose-4P phosphatase; ER: erythrose reductase; and EK: erythrulose kinase. Dotted arrows represent the hypothetic catabolism pathway of erythrulose-P according to Paradowska and Nitka (2009).

reduced cofactors that must be replenished through glucose oxidation.

When erythritol is synthesized from glycerol, the latter is first phosphorylated by a glycerol kinase (GK) before subsequently being dehydrogenated by a glycerol-3P-dehydrogenase (GDH), giving rise to dihydroxyacetone phosphate (DHAP) (Fig. 1). DHAP is then converted by a triosephosphate isomerase (TIM) into glyceraldehyde-3-phosphate, which enters into the pentose phosphate pathway, where a transketolase (TK) converts it into erythrose-4-phosphate. The latter is dephosphorylated by an erythrose-4P phosphatase (E4P**P**) and reduced by an erythrose reductase (ER) to become erythritol. Depending on experimental conditions, *Y. lipolytica* can also use erythritol as its main carbon source. We have recently highlighted that this catabolic pathway in *Y. lipolytica* is similar to those present in other yeasts, including *Lipomyces starkeyi* (Carly et al., submitted for publication) (Fig. 1). Furthermore, we identified the gene *EYK1* (YALI0F01606g), which encodes an erythrulose kinase (EK). In *Y. lipolytica*, disruption of *EYK1* impairs growth on erythritol.

Although most of the genes involved in erythritol synthesis have been described, little is known about their regulation at finer scales or about the pathway's limiting steps. To date, most studies seeking to improve erythritol production have used wild type strains or randomly generated mutants and have focused on optimizing the culture medium or culturing conditions (Rymowicz et al., 2008; Yang et al., 2014; Mirończuk et al., 2015; Rakicka et al., 2016). However, some recent research using *Y. lipolytica* has underscored the potential utility of genetic engineering approaches. Mirończuk et al. (2016) found that the constitutive expression of genes encoding GK and GDH (i.e., *GUT1* and *GUT2*, respectively) in the *Y. lipolytica* A101 strain led to a significant increase in glycerol uptake capacity. Overexpression of these genes resulted in significant increases in erythritol productivity (23% for *GUT1* only and 35% for both) in the mutants as compared to the wild type strain; in contrast, overexpression of *GUT2* alone led to a 28% decrease in erythritol productivity. Other enzymes merit some attention as well. Transketolase (TK) has been described as a key enzyme in erythritol synthesis in *Trichosporonoides megachiliensis* (Sawada et al., 2009) similarly to erythrose reductase (ER) in *Candida magnolia* (Ghezelbash et al., 2014). Furthermore, using proteomics, Yang et al. (2015) highlighted the importance of triose-phosphate isomerase (TIM) in erythritol synthesis in *Y. lipolytica*.

In this study, we sought to identify additional limits acting on erythritol production and thus further enhance this process. To this end, we constructed a set of strains that overexpressed genes encoding for key enzymes in the erythritol synthesis pathway. We then studied the effects of different gene combinations on erythritol synthesis. In the most productive mutants, *EYK1* was also disrupted to further increase erythritol productivity.

2. Materials and methods

2.1. Strains, media, and culture conditions

The *Escherichia coli* and *Y. lipolytica* strains used in this study are listed in Supplementary table 1. The *E. coli* strains were grown at 37 °C in Luria-Bertani medium supplemented with kanamycin sulfate (50 mg/L; Sigma-Aldrich). The *Y. lipolytica* strains were grown at 28 °C in YNB medium supplemented to meet the requirements of auxothrophs (Barth and Gaillardin, 1996); EG medium (glycerol 50 g/L, yeast extract 5 g/L and peptone 5 g/L); EPF medium (glycerol 100 g/L, yeast extract 1 g/L, NH_4Cl 4.5 g/L, $CuSO_4$ 0.7 mg/L, $MnSO_4{\cdot}H_2O$ 32 mg/L, and 0.72 M phosphate buffer pH 4.3) or EPB medium (glycerol 150 g/L, NH_4Cl 2 g/L, KH_2PO_4 0.2 g/L, $MgSO_4{\cdot}7H_2O$ 1 g/L, NaCl 25 g/L and yeast extract 1 g/L). For solid media, agar (15 g/L) was added. Shake-flask cultures were performed in triplicate for eight days in a rotary shaker at 28 °C and 190 RPM. After 72 h of preculturing in 35 mL of EG medium, cells were transferred to a 250 mL-flask containing 35 mL of EPF medium; initial optical density at 600 nm (OD_{600}) was 0.4. Bioreactor cultures were performed in duplicate in a 2-L Biostat B-Twin fermentor (Sartorius) containing 1 L of EPB medium and kept at 28 °C. Stirrer speed was set to 800 RPM, and the aeration rate was 1 L/min. The pH was automatically maintained at 3.0 by the addition of 20% (w/v) NaOH or 40% (w/v) H_3PO_4.

2.2. Quantifying biomass, glycerol concentration, and erythritol concentration

Biomass was determined gravimetrically after the cells had been washed and dried at 105 °C for 24 h. Glycerol and erythritol concentrations were determined using an HPLC (Agilent 1200 series; Agilent Technologies) equipped with a refractive index detector and an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad). Elution was performed using 15 mM trifluoroacetic acid as the mobile phase at a flow rate of 0.5 mL/min and a temperature of 65 °C.

2.3. General molecular biology techniques

Standard media and techniques were used for *E. coli* (Sambrook et al., 1989), and the media and techniques used for *Y. lipolytica* have been described elsewhere (Barth and Gaillardin, 1996). The restriction enzymes, DNA polymerases, and ligase were supplied by Thermo Fisher Scientific. Genomic DNA from *Y. lipolytica* was prepared in accordance with Querol et al. (1992). PCR was performed using the primers listed in Supplementary table 2. DreamTaq DNA polymerase (Thermo Scientific) was used for cloning, and ExTaq DNA polymerase (Takara) was used to verify genomic structure. The PCR fragments were purified from the agarose gels using a GeneJet Gel Extraction Kit (Thermo Scientific). DNA sequencing was performed by GATC Biotech (https://www.gatc-biotech.com), and the primers were synthetized by Eurogentec (https://secure.eurogentec.com/).

2.4. Strain construction

Details on the genes that were overexpressed in *Y. lipolytica* are provided in Table 1. The *Y. lipolytica* genes (namely, *GUT1*, *GUT2*, *TPI1*, *TKL1*) were amplified from the genomic DNA of JMY2900. Gene *YidA* was amplified from *E. coli* strain BL21, and gene *ALR* was amplified from *C. magnolia* strain CBS2800. A synthetic, codon-optimized version of *ALR (ALR_CO)* specific to *Y. lipolytica* was obtained from GeneArt (https://www.thermofisher.com/geneart). Codon content of gene *ALR* was compared to *Y. lipolytica* CLIB122 codon usage table using Graphical Codon Usage Analyzer CGUA (http://gcua.schoedl.de/) and rare codons (below 20% in frequency in *Y. lipolytica*) were replaced with the most common codons coding for the same amino acid (Supplementary Fig. 1). All the gene-amplification primers were

Table 1
Genes overexpressed in Y. lipolytica.

Reference	Name	Enzyme encoded	Origin	Modification
YALI0F00484g	*GUT1*	Glycerol kinase	*Y. lipolytica*	*BamHI* site removed
YALI0B13970g	*GUT2*	Glycerol-3P dehydrogenase	*Y. lipolytica*	*BglII* site removed
YALI0F05214g	*TPI1*	Triose-P isomerase	*Y. lipolytica*	
YALI0E06479g	*TKL1*	Transketolase	*Y. lipolytica*	Intron removed, *ClaI* site removed
EG11195	*YidA*	Erythrose-4P phosphatase	*E. coli*	
FJ550210	*ALR*	Erythrose reductase	*C. magnoliae*	

designed to introduce an *Avr*II site at the 3′ end and either a *Bam*HI or a *Bgl*II restriction site at the 5′ end (Supplementary table 2). Introns and undesirable restriction sites were removed by overlap extension PCR and site-directed mutagenesis (Higuchi et al., 1988; see Table 1 for details). Amplicons were purified from the agarose gel and then digested using *BamH*I/*Avr*II or *Bgl*II/*Avr*II restriction enzymes. The corresponding fragments were subsequently cloned into *BamH*I/*Avr*II digested JMP1047 or JMP2563 vectors to obtain *URA3* or *LEU2*-selectable plasmids, respectively (Supplementary table 1). The correctness of the resulting constructs was verified by DNA sequencing.

Strain RIY203 was constructed by disrupting the *EYK1* gene in the Po1d strain as described elsewhere (Vandermies et al., 2017, in press). *EYK1* P and T fragments were amplified from Po1d genomic DNA using primer pairs EYK1-PF/EYK1-PR and EYK1-TF/EYK1-TR, respectively. The *URA3* marker was amplified from the JMP113 plasmid using the primer pair LPR-F/LPR-R. Amplicons were digested with *Sfi*I before being purified and ligated, using T4 DNA ligase. The ligation products were amplified via PCR using the primer pair EYK1-PF/EYK1-TR. They were then purified and used to transform the Po1d strain. The result was strain RIY147, from which the *URA3* marker was popped out using the *Cre*-lox recombination system and the replicative vector pRIP132 (Vandermies et al., 2017, in press); this process yielded strain RIY203.

Expression cassettes for the *GUT1*, *GUT2*, *TPI1*, *TKLI*, *YidA*, *ALR*, and *ALR_CO* genes were rescued from corresponding vectors by *Not*I digestion. They were then purified from the agarose gel and used to transform *Y. lipolytica* strains Po1d or RIY203 (Supplementary Fig. 2). Transformants were selected using YNB medium supplemented with uracil or leucine, depending on the nature of their auxotrophy. The correctness of the strain construction was verified by performing analytical PCR on genomic DNA; depending on the marker, primer pair URA3F/61stop or LEU2F/61stop was employed. Prototrophic stains were obtained by transforming the mutants with either the JMP1047 or JMP2563 plasmid, depending on the nature of the strain's auxotrophy.

2.5. RNA isolation and transcript quantification

Shake-flask cultures were grown in EPF medium for 24 h. Cells were then collected at an OD_{600} of 0.5 and stored at −80 °C. RNA extraction and cDNA synthesis were performed as previously described (Sassi et al., 2016). Primers for RT-qPCR are listed in Supplementary table 2. Gene expression levels were standardized using the expression level of the actin gene as the reference (ΔCT method). For genes *GUT1*, *GUT2*, *TPI1*, and *TKL1*, the fold difference in gene expression between the mutants and the JMY2900 strain were calculated as $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). Samples were analyzed in duplicate.

2.6. Statistical analysis

Statistical signification of results was assessed by analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Alpha value was set at 0.05. All statistical tests were performed using GraphPad Prism 6.0.2 software.

Table 2
Dynamics of erythritol production and glycerol consumption in different strains grown in EPF medium in shake flasks for eight days.

Strain	Overexpressed genes	Biomass (g_{DCW}/L)	q_{ERY} (g/g_{DCW}.h)	q_{GLY} (g/g_{DCW}.h)	$Y_{P/S}$ (g/g)
JMY2900 (WT)	–	5.30	0.035	0.076	0.46
FCY205	*GUT1*	4.83	0.051*	0.091*	0.56*
FCY206	*GUT2*	4.83	0.038	0.068	0.56
FCY207	*TPI1*	5.02	0.039	0.071	0.54
FCY208	*TKL1*	5.36	0.040	0.068	0.59*
FCY209	*YidA*	4.96	0.031	0.066	0.44
FCY210	*ALR*	6.24*	0.028	0.049*	0.57*
FCY213	*GUT1-GUT2*	3.62*	0.056*	0.102*	0.54
FCY214	*GUT1-TKL1*	4.81	0.058*	0.095*	0.61*
FCY215	*GUT1-ALR*	4.48	0.058*	0.094*	0.61*
FCY216	*GUT1-TPI1*	4.65	0.048*	0.085	0.56*

The values provided are the means of three independent replicates; the standard deviations represented less than 10% of the means. The abbreviations are as follows: q_{ERY}: specific erythritol production rate; q_{GLY}: specific glycerol consumption rate; and $Y_{P/S}$: erythritol/glycerol conversion yield.
* Significantly different from JMY2900.

3. Results and discussion

3.1. Overexpression of glycerol kinase increases glycerol consumption rate and erythritol productivity

With the goal of enhancing erythritol productivity, we first attempted to improve glycerol consumption by overexpressing the genes *GUT1* (which codes for GK; YALI0F00484g) and *GUT2* (which codes for GDH; YALI0B13970g) either separately or simultaneously in the *Y. lipolytica* strain Po1d (Fig. 1 and Supplementary table 1). For strain FCY205 (pTEF-*GUT1*), the specific glycerol consumption rate (q_{GLY}) was 20% higher than that of the wild type strain JMY2900 (0.091 and 0.076 g/g_{DCW}.h respectively; Table 2). This increase is similar to that obtained for the *Y. lipolytica* A101 mutant that overexpressed *GUT1* (Mirończuk et al., 2016). In contrast, strain FCY206 (pTEF-*GUT2*) showed a slightly lower glycerol consumption rate as compared to the wild type strain (Fig. 1) even though *GUT2* expression was six times greater in FCY206 than in the wild type (Supplementary Fig. 3). Furthermore, strain FCY213, which overexpressed both *GUT1* and *GUT2*, demonstrated just a slight increase in specific glycerol consumption rate compared to strain FCY205 (pTEF-*GUT1*) (0.102 and 0.091 g/g_{DCW}.h, respectively). In *Y. lipolytica* strain A101, an 11% increase in glycerol consumption was observed when both *GUT1* and *GUT2* were coexpressed as compared to when just *GUT1* was overexpressed (Mirończuk et al., 2016). For strains FCY205 (pTEF-*GUT1*) and FCY213 (pTEF-*GUT1-GUT2*), a similar increase of q_{GLY} was observed (12%; Table 2). Therefore, the simultaneous overexpression of *GUT1* and *GUT2* seems to have a combined effect on glycerol consumption under our experimental conditions. However, strain FCY213 (pTEF-*GUT1-GUT2*) had a 30% lower maximum biomass as compared to the wild type (3.62 and 5.3 g_{DCW}/L, respectively; Table 2).

In FCY205 (pTEF-*GUT1*), the specific erythritol production rate (q_{ERY}) was 45% greater than in the wild type (0.051 and 0.035 g/g_{DCW}.h, respectively), while the glycerol/erythritol conversion yield ($Y_{P/S}$) was 21% higher (0.56 and 0.46 g/g, respectively). Surprisingly, and in contrast to the findings of Mirończuk et al. (2016), the overexpression of *GUT2* (strain FCY206) had no effect on specific erythritol production rate (0.038 g/g_{DCW}.h). In strain FCY213 (pTEF-*GUT1-GUT2*), there was only a slight increase (9%) in the specific erythritol production rate as compared to that in strain FCY205 (*p*TEF-*GUT1*) (0.056 and 0.051 g/g_{DCW}.h, respectively). Since the overexpres-

sion of *GUT1* led to an increase in both the specific glycerol consumption rate and the specific erythritol production rate, an additional strain, FCY212, was constructed that contained two copies of the pTEF-*GUT1* expression cassette. However, it did not display further improvement in erythritol productivity (data not shown).

3.2. Overexpression of triose isomerase and transketolase leads to an increase in erythritol productivity

Genes such as *TPI1*, *TKL1*, *E4PP*, and *ER* encode key enzymes involved in the pathway by which erythritol is synthesized from DHAP, the end product of glycerol catabolism (Fig. 1). They were overexpressed separately in the Po1d strain to assess their effects on erythritol productivity. Genes encoding TIM (*TPI1*; YALI0F05214g) and TK (*TKL1*; YALI0E06479g) were identified in the *Y. lipolytica* genome and used to construct strains FCY207 and FCY208, respectively. Unfortunately, erythrose-4P-phosphatase (E4P**P**) has yet to be characterized in yeast. However, Kuznetsova et al. (2006) reported that, in *E. coli*, a member of the haloacid dehalogenase-like hydrolase superfamily—HAD13—showed a high degree of phosphatase activity directed toward erythrose-4-phosphate (K_{cat}/K_m value of 10^6). Therefore, the corresponding *YidA* gene (EG11195) was cloned, assessed for codon compatibility in *Y. lipolytica*, and used to construct strain FCY209. We were also interested in ER. Past research has suggested that gene JX885666 in *Y. lipolytica* strain DSMZ70562 encodes an ER (Ghezelbash et al., 2014). However, a BlastN search using the gene sequence as a query did not find a corresponding gene in the genome of strain Po1d. Moreover, our attempts to amplify that particular gene using the primers designed by Ghezelbash and colleagues (ER1 and ER2; Supplementary table 2) were unsuccessful for both Po1d and DSMZ70562 (data not shown). In contrast, we did find a corresponding gene (FJ550210) in *C. magnolia* JH110 (Lee et al., 2010) using a BlastN search. Consequently, it was amplified from the genomic DNA of *C. magnolia* CBS2800 and used to construct strain FCY210.

These engineered strains were grown in EPF medium for eight days (i.e., until glycerol near-exhaustion). Strains FCY207 (pTEF-*TPI1*) and FCY208 (pTEF-*TKL1*) showed increased erythritol production compared to the wild type (5% and 16%, respectively; Fig. 2 and Supplementary Fig. 3). Strain FCY208 (pTEF-*TKL1*) had a higher conversion yield than did strain FCY205 (pTEF-*GUT1*) (0.59 and 0.56 g/g, respectively; Table 2). However, glycerol consumption was somewhat lower in the former (0.068 g/g_{DCW}.h) than in the wild type strain (0.076 g/g_{DCW}.h). Strain FCY210, which overexpressed the *ALR* gene taken from *C. magnolia*, did not have increased erythritol production (Fig. 2). To circumvent any potential problems that may have occurred in the translation of *ALR* mRNA in strain FCY210, a codon-optimized version of *ALR* (*ALR*_CO) was designed and used to construct strain FCY211. The latter did not show significantly improved erythritol productivity relative to strain FCY210 (data not shown). Strain FCY209, which over expressed E4PP (*YidA*), had a significantly lower level of erythritol production than the wild type (30 and 35 g/L, respectively) and a slower specific erythritol production rate (0.031 and 0.035 g/g_{DCW}.h, respectively). This negative effect of *YidA* overexpression could be linked to the ability of the *YidA* gene product to hydrolyze DHAP, as suggested by Kuznetsova et al. (2006).

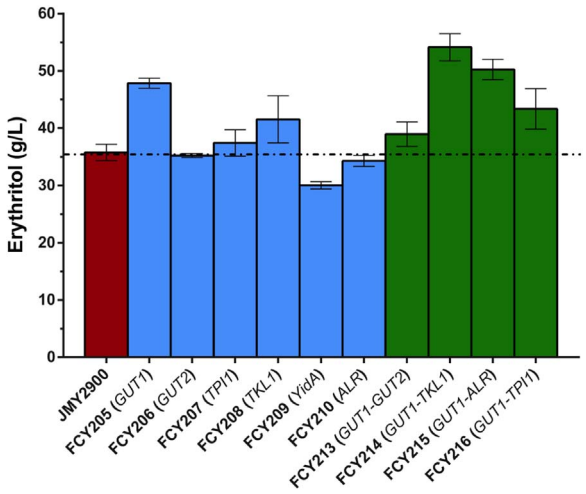

Fig. 2. Erythritol production of the study strains grown in EPF medium in shake flasks for eight days.

3.3. The pull and push strategy to enhance erythritol production

As mentioned above, strain FCY205 (pTEF-*GUT1*) showed a significant increase in glycerol uptake, while strain FCY208 (pTEF-*TKL1*) displayed the highest conversion yield. Hence, to further increase erythritol productivity, *GUT1* and *TKL1* were coexpressed in strain FCY214 (pTEF-*GUT1-TKL1*; Supplementary Fig. 3). In shake-flask cultures, this strain performed significantly better than the wild type, displaying a 65% increase in erythritol productivity. It combined the higher glycerol uptake capacity of strains FCY205 (pTEF-*GUT1*, 0.095 and 0.091 g/g_{DCW}.h, respectively) and the higher conversion yields of FCY208 (pTEF-*TKL1*, 0.61 and 0.59 g/g, respectively). To further expand this push and pull strategy, strains FCY215 and FCY216 were constructed; they coexpressed *GUT1-ALR* and *GUT1-TPI1*, respectively. Although these two strains performed better than wild type, their erythritol production was not significantly greater than that of strain FCY214 (pTEF-*GUT1-TKL1*) (Fig. 2, Table 2). This suggests that glycerol assimilation and the redirection of glyceraldehyde-3-phosphate towards the non-oxidative part of pentose phosphate pathway are the main limiting steps for the synthesis of erythritol. Therefore, increasing the rate of these reactions could contribute to increasing the erythritol productivity and yield.

Next, we investigated the behavior of strain FCY214 (pTEF-*GUT1-TKL1*) and the wild type under bioreactor conditions. The strains were cultured for 96 h in EPD medium, and cell growth, glycerol consumption, and erythritol production were monitored (Fig. 3). For strain FCY214 (pTEF-*GUT1-TKL1*), the final erythritol concentration in the culture supernatant was 79.4 g/L (Table 3). This value was 42% greater than that obtained for the wild type (55.8 g/L; Table 3). Under bioreactor conditions, erythritol was produced at a high, constant rate (0.84 g/L.h) between 24 h and the end of culture (Table 3 and Fig. 3). This result contrasts with those obtained for *Y. lipolytica* strain AJD pADUTGut1/2 (constitutive *GUT1/GUT2* coexpression), whose erythritol production differed from that of the wild type (strain A101) only during the four last hours of culture (Mirończuk et al., 2016). Here, erythritol specific production rate was similar for the bioreactor and shake-flask cultures of strain FCY214 (0.057 and 0.058 g/g_{DCW}.h, respectively), while glycerol uptake was slightly higher in the bior-

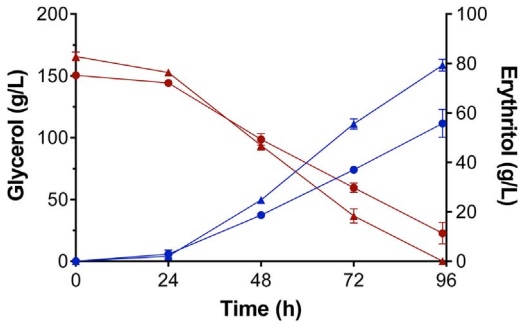

Fig. 3. Glycerol uptake (red line) and erythritol production (blue line) for FCY214 (pTEF-GUT1-TKL1 triangles) and JMY2900 (WT; circles) grown in EPB medium in a bioreactor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 3
Dynamics of erythritol production and glycerol consumption for bioreactor cultures of JMY2900 (WT), FCY214, and FCY218. The standard deviations represented less than 10% of the means. The abbreviations are the same as in Table 2.

	JMY2900	FCY214	FCY218
Erythritol production (g/L)	55.8	79.4	80.6
Erythritol productivity (g/L.h)	0.59	0.84	1.03
q_{ERY} (g/g_{DCW}.h)	0.046	0.057	0.073
q_{GLY} (g/g_{DCW}.h)	0.105	0.119	0.138
$Y_{P/S}$ (g/g)	0.44	0.48	0.53
Final biomass (g_{DCW}/L)	12.8	14.7	14.6

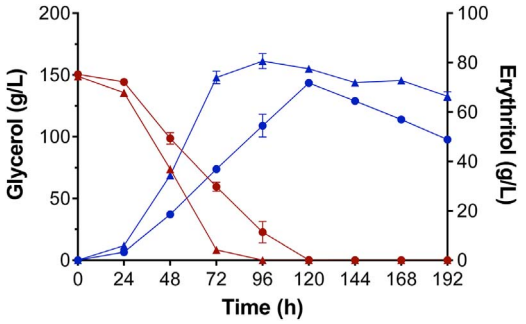

Fig. 4. Glycerol uptake (red line) and erythritol production (blue line) for FCY218 (pTEF-GUT1-TKL1-Δeyk1; triangles) and JMY2900 (WT; circles) grown in EPB medium in a bioreactor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

eactor (0.119 vs. 0.095 g/g_{DCW}.h).

3.4. Disruption of EYK1 in strain FCY214 further increases erythritol productivity

The ability of *Y. lipolytica* to catabolize erythritol alongside glycerol negatively affects erythritol productivity and conversion yield. Recently, we discovered that the gene *EYK1* (YALI0F01606g) is involved in erythritol catabolism (Carly et al., submitted for publication). Therefore, this gene was disrupted in a strain overexpressing *GUT1* and *TKL1* (Supplementary Fig. 2). As expected, the resulting strain, FCY218, was unable to consume erythritol, especially after the glycerol source had been exhausted in the culture medium (Fig. 4). In those conditions, erythrulose, the byproduct of erythritol oxidation, started to accumulate in the culture medium after glycerol depletion, however in small amount (less than 3% of the produced erythritol was converted after 24 h of glycerol depletion). Consequently, strain FCY218 performed better than strain FCY214 (glycerol specific consumption rate: 0.138 vs. 0.119 g/g_{DCW}.h; erythritol specific production rate: 1.03 vs. 0.84 g/L.h; and conversion yield: 0.53 vs. 0.48 g/g; Table 3). Moreover, the maximum concentration of erythritol was obtained in 40% less time than in the wild type strain, which further emphasizes the potential benefits of this system.

4. Conclusion

In the past, erythritol productivity in *Y. lipolytica* has largely been improved by classical approaches that consisted of optimizing either the culture medium or culturing conditions (Rymowicz et al., 2008, Mirończuk et al., 2014, Yang et al., 2014). Recently, however, Mirończuk et al. (2016) reported that metabolic engineering could increase the rate of glycerol catabolism, resulting in a 35% increase in erythritol productivity. In this study, we attempted to make further progress by increasing the flow of carbon through the erythritol synthesis pathway, notably in the transitions from erythrose phosphate to erythritol. Our approach resulted in a 65% increase in erythritol specific production rate relative to the wild type. A major challenge in the development of an efficient process for producing erythritol is the ability of *Y. lipolytica* to catabolize erythritol alongside glycerol. Here, we also constructed a strain whose erythritol catabolism was impaired. In this mutant, erythritol productivity was increased 78% relative to the wild type and maximum concentrations were obtained in 40% less time. Moreover, we achieved these values using an inexpensive medium and without having optimized culturing conditions. The next step is to develop a glycerol fed-batch fermentation method that can further increase the specific erythritol production rate as well as yield.

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Conflict of interest

The authors declare that they have no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2017.05.002.

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