Overexpression of YALI0B07117g Encoding Erythrose Reductase Homolog Results in Enhanced Erythritol Synthesis From Glycerol by the Yeast Yarrowia Lipolytica.

Mateusz Szczepańczyk
Wroclaw University of Environmental and Life Sciences: Uniwersytet Przyrodniczy we Wroclawiu

Dorota A. Rzechonek
Wrocław University of Environmental and Life Sciences Faculty of Biology and Animal Science:
Uniwersytet Przyrodniczy we Wroclawiu Wydzialu Biologii i Hodowli Zwierzat

Adam Dobrowolski
Wrocław University of Environmental and Life Sciences Faculty of Biology and Animal Science:
Uniwersytet Przyrodniczy we Wroclawiu Wydzialu Biologii i Hodowli Zwierzat

Aleksandra Maria Mirończuk (✉ aleksandra.mironczuk@upwr.edu.pl)
Uniwersytet Przyrodniczy we Wroclawiu  https://orcid.org/0000-0003-1604-1635

Research

Keywords: Yarrowia lipolytica, erythritol, glycerol, erythrose reductase, sweetener

DOI: https://doi.org/10.21203/rs.3.rs-706459/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background

Polyols are a group of sweet alcohols, frequently used as food additives. The constantly rising demand for polyols requires the application of new strategies to increase the production. Erythritol is synthesized by the yeast *Yarrowia lipolytica* under high osmotic pressure as an osmoprotectant. The metabolic pathway resulting in erythritol production remains partially unknown. However, the last reaction resulting in erythritol synthesis is conducted by an erythrose reductase (ER).

Results

The *Y. lipolytica* strain was genetically modified to increase the erythritol yield and productivity, using glycerol as a sole carbon source. The modification focused on the ER homologue *YALI0B07117g* after the *in silico* analysis of the protein sequences of all reported ER homologues. Initial results in shake-flask experiments proved the influence of the gene *YALI0B07117g* in erythritol synthesis. Deletion of the gene resulted in 3-fold and 2-fold increased production of mannitol and arabitol, respectively. Overexpression of the native ER homologue gene showed a positive influence on erythritol production. Bath cultures were conducted and the obtained strain reached the yield of 0.4 g/g. The specific consumption rate (qs) increased from 5.83 g/g/L for the WT strain to 8.49 g/g/L for the engineered strain, while the productivity of erythritol increased from 0.28 g/L/h for the A101 strain to 0.41 g/L/h for the modified strain.

Conclusions

Overexpression of the gene *YALI0B07117g* resulted in increased production of erythritol in the yeast *Y. lipolytica*. Disruption of the metabolic pathway by deletion of the gene results in higher production titers of mannitol and arabitol. Application of the research may prove positive for shortening the cultivation time due to the increased consumption rate of the substrate combined with increased parameters of erythritol synthesis.

Background

The increase of sugar and fat consumption has led to a drastic rise in numbers of overweight and obese individuals. Obesity increases the risk of diabetes, cardiovascular diseases and certain types of cancer [1, 2]. To avoid the health issues resulting from excessive sugar consumption, substitutes of sugar were implemented. Polyols, or sugar alcohols, are food additives that occur naturally in nature, especially fruit and vegetables (Martău et al., 2020; Rzechonek et al., 2018). The application of polyols as sweeteners became possible due to their industrial production. However, the constantly rising demand for polyols necessitates further improvement in the yield on an industrial scale.

Erythritol is a four-carbon polyol that can be found in fruits, such as pears, grapes or melon, where it acts as a carbon store, while many microorganisms produce erythritol under high osmotic pressure as an
osmoprotectant [4]. It is characterized by stability at high temperatures and over a wide range of pH [5]. The popularity of erythritol is based on its low caloric value (0-0.2 kcal g\(^{-1}\)) in comparison to sucrose (4 kcal g\(^{-1}\)) while reaching approximately 60-80% of the sweetness level of sucrose [6], the low impact on insulin release due to its chemical structure (Wölnerhanssen et al., 2016), therefore being safe for diabetics (Noda K, Nakayama K, 1994), and antibacterial properties in relation to creation of dental plaque [9]. Initial reports suggested that erythritol is not metabolized in the human body but the study by Hootman et al. indicates that a fraction of erythritol intake may be processed to erythronate. Moreover, the study revealed that erythritol can be synthesized by human cells via the pentose phosphate pathway (PPP) [10]. Industrial scale production of erythritol relies on fermentation of Moniliella spp. [11] or Candida magnoliae [12] from glucose (Rzechonek et al., 2018).

The oleaginous yeast Yarrowia lipolytica is a microorganism that is well studied in relation to lipid synthesis [13–15], polyol production [16, 17] and citric acid production (Papanikolaou et al., 2008; Rymowicz et al., 2010; Rzechonek et al., 2018). Y. lipolytica possesses the ability to utilize crude glycerol, containing many contaminants, including heavy metals, methanol and salts [21], resulting in a low market price of this carbon source. Despite many years of research, the synthesis pathway for erythritol and other polyols (arabitol and mannitol) is still partially unknown, while a few of the enzymes involved in the process have been cast in doubt. A recent study identified an erythrose reductase (YER) YALI0B16192g [22] and its role in erythritol synthesis. Additionally, 8 homologs of ER were characterized. Similarly the d-mannitol dehydrogenase gene (YALI0B16192g) was reported by Wang et al. (2020).

Erythritol metabolism remains an actively studied research area, while erythritol production by Y. lipolytica faces problems such as low yield and production of byproducts. Interestingly, the yeast Y. lipolytica is capable of erythritol synthesis from raw glycerol (Rymowicz and Rywińska, 2009) as well as its utilization [25] when the main source of carbon is depleted. Therefore this microorganism could potentially become a model in erythritol metabolism in eukaryotic cells. Further research could resolve the issues and lead to an increase of erythritol synthesis and simultaneous decrease of by-products from the media.

The aim of this study was to analyze the impact of the YER homolog YALI0B07117g as a potential way to improve the production of polyols from glycerol, as a low-cost carbon source, by the yeast Y. lipolytica. Next, we analyzed the influence of erythrose reductase YALI0B07117g on polyol metabolisms. Deletion of YALI0B07117g resulted in significantly lower erythritol synthesis and the carbon flux was redirected into mannitol production. Moreover, overexpression of the gene resulted in enhanced erythritol yield and productivity.

Results And Discussion

The yeast Y. lipolytica is able to synthesize erythritol from glycerol, as a response to high osmotic stress [4]. It was shown that the last step of the erythritol synthesis pathway is reduction of d-erythrose to erythritol (Figure 1). This reaction is carried out by an enzyme belonging to the aldo/keto reductase family. Erythrose reductase is an enzyme responsible for catalyzing the conversion of erythrose to erythritol, with NADPH as an electron donor [26] following the dephosphorylation of erythrose-4-
phosphate. Erythrose reductase in *Y. lipolytica* was characterized by similarity to known enzymes from different organisms such as *Candida magnoliae* and *Moniliella megachiliensis*. The analysis of protein sequences indicated that YALI0F018590p shows 41% and 44% similarity between the ER in aforementioned organisms respectively. The study proposed this protein as the main ER in *Y. lipolytica*. The reported protein has been described with 8 potential homologs, including YALI0B07117p [22, 27].

In silico analysis of potential homologs of YIER has shown similarity between the proteins and their correlation in the phylogenetic tree. To date, three potential YIER homologs have been analyzed: YALI0F18590g [22], YALI0C013508g and YALI0D07634g [27]. The latter two are being investigated due to their dependency on NADPH as a cofactor for erythrose reductase activity. The deletion of YALI0C013508g and YALI0D07634g did not result in significant decrease of erythritol production, leading the authors to speculate that a different protein is responsible for carrying out the reduction of erythrose to erythritol [27]. From among the previously described homologs of YIER YALI0B07117p was selected for further analysis due to the discrepancy in the N-terminal end. YALI0B07117p is the only one of the group to have a 28 amino acid sequence preceding the highly similar sequences with aldo-keto reductase (AKR) domains (Figure 2). The role of this sequence remains unknown, but it probably does not possess any significance in erythritol synthesis. The domain and motifs search in silico yielded no results to suggest the potential role of this sequence. All of the homologs of ER possess highly conserved regions, one of them being the aldo-keto reductase region, which is responsible for the potential role as erythrose reductase in *Y. lipolytica*. The average size of the protein varies between 309 and 313 amino acids, with two exceptions. YALI0F18590p is made up of 323 amino acids, while YALI0B07117p is composed of 337 amino acids. The most distinguishing part of YALI0B07117p is the N-terminal end, where the 28 amino acid chain is located. It does not appear in any of the other homological proteins. In all of the sequences, the conserved regions are highly visible. The differences between amino acids usually do not affect their properties.

In this study the role of YALI0B07117p was analyzed in the aspect of a possible role in erythritol synthesis. To determine the effects of this protein on the metabolic pathway resulting in erythritol production the obtained strains were grown in Erythritol Synthesis Medium [28]. For further information about the role of YALI0B07117g a knock-out mutant was obtained; the strain was named AJD ΔB07117. Shake flask experiments were performed to determine the influence of deletion of YALI0B07117g on erythritol synthesis. As shown in Figure 3, the strain AJD ΔB07117 was characterized by slower glycerol consumption compared to the control A101 strain. Initially, the knock-out strain showed no glycerol utilization on the first day of the experiment. After 72 h of the experiment the WT strain exhausted nearly all the glycerol from the medium (Q_S = 1.52 g/L/h), while the modified strain used 90% of the supplemented carbon source (Q_S = 1.26 g/L/h). Erythritol synthesis in both strains remained at similar levels thought the entire experiments, with the wild type strain producing 30.02 g/L (Y_{ERY} = 0.275), whereas the AJD ΔB07117 strain produced 28.22 g/L after 72h (Y_{ERY} = 0.31). The final concentration of erythritol reached 29.53 g/L for AJD ΔB07117 after 96 h. The wild type strain used erythritol as a carbon source on the last day of the experiment after depletion of glycerol, resulting in a
decrease of the erythritol level to 15.16 g/L. In addition to erythritol and glycerol, the content of by-products of erythritol synthesis were tested. Interestingly, the AJD ΔB07117 strain produced roughly 3 times more mannitol and 2 times more arabitol in 72 h than the control strain, before the wild type strain started to utilize these polyols (Table 1). It can be speculated that disruption of the erythritol synthesis pathway caused the carbon flux into mannitol and arabitol synthesis. The probable cause of this phenomenon is maintenance of oxide-reductase homeostasis. However, the actual mechanism of carbon flux in polyol synthesis remains unknown.

Next, to confirm the impact YALI0B07117g on erythritol synthesis the gene was overexpressed under the hybrid promoter UAS1_B16 [29]. Additionally, YALI0B07117g was overexpressed in a knockout mutant, resulting in the complementary strain AJD-c-B07117.

First, the obtained strains AJD pAD-B07117 and AJD-c-B07117 were analyzed by the microplate reader Bioscreen C test, to determine the influence of genetic modifications on their growth. The A101 strain was used as a control. The growth was measured on two carbon sources, glycerol (YNB + 5% (w/v) glycerol) (Figure 4) and glucose (YPD and YNB + 5% (w/v) glucose; data not shown). The growth curves for each experiment show no significant difference between modified and wild type strains on either medium, but the growth of the AJD-c-B07117 strain on glycerol was initially impaired. During the first 24 hours of the experiment the complementary strain continued to have a slower growth rate but eventually all of the strains grew to a similar OD factor at the end of 48 h. The WT strain and AJD pAD-B07117 show similar growth on glycerol as a sole carbon source; therefore it can be used as a control of erythritol production by the overexpression strain in shake flask and batch culture experiments, where glycerol is the main carbon source.

Overexpression of YALI0B07117g in Y. lipolytica resulted in increased production of erythritol in shake flask experiments, as shown in Figure 3, compared to the control A101 strain. Additionally, the production of erythritol synthesis by-products decreased compared to both the WT strain and the knock-out mutant (Table 1). These results suggested the potential for increase of erythritol synthesis with glycerol as the main carbon source. Interestingly, the strain AJD pAD-B07117 showed a similar profile of glycerol consumption to the knockout mutant. To determine the impact of YALI0B07117g on erythritol synthesis the AJD pADB07117 strain was used for batch experiments to appropriately manage the culture conditions, mainly the pH, which has a significant influence on erythritol synthesis. The procedures were carried out as described before in Erythritol Synthesis Medium.

The strain AJD pAD-B07117 is characterized by a significant increase in erythritol synthesis compared to the control strain. The highest yield of erythritol was observed after 72 hours. $Y_{\text{ERY}}$: AJD pAD-B07117 $Y_{\text{ERY}} = 0.4$ g/g; A101 $Y_{\text{ERY}} = 0.32$ g/g. The yield of erythritol increased by 25% for the AJD pAD-B07117 strain. The productivity of erythritol ($Q_{\text{ERY/GLY}}$) reached 0.41 g/L/h for the modified strain and 0.28 g/L/h for the WT strain. That is equal to a 46% increase in erythritol productivity. Both strains then used erythritol as a carbon source, resulting in a decrease of the total amount of erythritol at the end of the experiment, as shown in Figure 5.
In contrast to AJD ΔB07117, the AJD pAD-B07117 strain utilized glycerol more efficiently than the control strain. There was no initial impairment in glycerol consumption compared to the knock-out mutant. During the first day of the experiment, both strains used similar amounts of glycerol, whereas after 48 h the amount of glycerol in the modified strain dropped below 60 g/L, while in the A101 strain it was more than 15 g/L higher. As a result, the Qs at this point in the experiment reached 1.95 g/L/h in the modified strain, compared to 1.52 g/L/h for the WT strain. This feature, alongside increased erythritol production, can shorten the batch fermentation time. During the experiment, the biomass production was analyzed. The results indicate that the biomass production for the modified strain was lower than the control; the biomass reached 17.6 g/L for the AJD pAD-B07117 strain, and 22.2 g/L for A101 at the peak of erythritol synthesis. That observation correlates with the overall specific consumption rate (qs). The average calculated qs for A101 is 5.83 g/g/L, while for the modified strain it is 8.49 g/g/L after 72 hours of the experiment. Moreover, the overexpression of YALI0B07117g resulted in a decrease of mannitol and arabitol production compared to the AJD-ΔB07117 (Table 2) strain, indicating its role in control of carbon flux towards erythritol synthesis.

The analysis of polyol production by the strain AJD pAD-B07117 indicated its role as an ER. Not only is the lack of YALI0B07117g responsible for the lower erythritol synthesis, but also it increases the production of byproducts of erythritol synthesis in the form of arabitol and mannitol, altering the carbon flux of glycerol utilization. Additionally, the increase of erythritol synthesis by 25% in the AJD pAD-B07117 strain compared to the wild type strain in correlation with higher efficiency of substrate consumption shows the potential for using this strain in erythritol synthesis on a larger scale. Increased consumption of glycerol by the strain AJD pAD-B07117 can shorten the cultivation time, resulting in more efficient erythritol synthesis. The higher glycerol uptake in the modified strain resulted in depletion of the carbon source within 72 hours.

Genetic modifications of genes involved in glycerol assimilation, mainly YALI0F00484g encoding glycerol kinase (GK) and YALI0B02948g encoding glycerol-3-P dehydrogenase (GDH), were reported to improve glycerol uptake in Y. lipolytica [26]. Here, the overexpression of the erythrose reductase gene improved the glycerol consumption significantly. Obtaining a strain with overexpression of all of the mentioned genes could have potential in high scale batch cultivations and as a result reduce the costs of erythritol synthesis. Further improvement of glycerol consumption may require the implementation of fed-batch cultures to supplement the carbon source during the cultivation.

The AJD pAD-B07117 strain reached the yield of erythritol production of 0.4 while using glycerol as a main carbon source. A study by Cheng et al. (2018) focused on erythrose reductase identification analyzed the erythritol production in mutants overexpressing YALI0D07634g and YALI0C13508g. The strains were characterized by the yield of erythritol of 0.58 and 0.59 respectively, but the culture was conducted with glucose as the main carbon source. Additionally, the initial concentration of glucose in the medium was set at 300 g/L. Similar yield of erythritol (0.44) was obtained in the analysis of YALI0F18590g, where glycerol was used as a carbon source [22]. The research by Janek et al. (2017), Cheng et al. (2018) and the analysis of YALI0B07117g focused on endogenous targets to improve
erythritol synthesis. An additional advantage of *YALI0B07117g* is the usage of glycerol as a carbon source instead of glucose, which is more valuable commercially.

The strain AJD pAD-B07117 was analyzed as a potential means to improve commercial production of erythritol, but it is worth noting that the knock-out mutant of *YALI0B07117g* produces relatively large amounts of other polyols. The discrepancy between the mannitol and arabitol production in the overexpression and knock-out mutant in comparison with the WT strain indicates the significance of the role of *YALI0B07117g* in synthesis of these polyols. The characterization of genes responsible for mannitol synthesis by *Y. lipolytica* [23] from glycerol is necessary not only to overcome the problem of purification of erythritol due to the byproducts in the medium but also to decipher the mannitol synthesis pathway. The strain AJD ΔB07117 may be used to analyze the genes involved in mannitol synthesis and become a potential asset in future research to obtain a *Y. lipolytica* strain able to produce mannitol from glycerol with high values of yield and productivity. Future research should focus on crude glycerol as a carbon source for further improvement in enhancing erythritol production. However, the lack of a significant decrease of erythritol synthesis in the AJD ΔB07117 strain indicates the potential for another gene as the main erythrose reductase in the yeast *Y. lipolytica*. The high percentage of homology between all of the ER homologs may cause a problem in establishing which one of these proteins is responsible for erythritol synthesis in general. Genetic modifications resulting in alteration of erythrose reductase genes may cause the increase of expression levels of other genes in this pathway, as a response to osmotic stress, masking the potential influence of ER gene knock-outs.

**Conclusions**

The research indicates the role of the gene *YALI0B07117g* in erythritol synthesis. Despite the increase of erythritol synthesis parameters such as yield and titer compared to the A101 strain, one of the most valuable feature of the obtained strain AJD pAD-B07117 is a higher specific consumption rate resulting in shortening of the cultivation time. Deletion of *YALI0B07117g* results in the increased production of mannitol and arabitol, what can help in investigating the genes responsible for metabolic pathway of polyols. Further research should focus on other ER homologs to determine the main enzyme responsible for the last step in erythritol synthesis.

**Method**

*Strains, media and culture conditions*

Strains used in this study are shown in Table 3. All of the strains belong to the Department of Biotechnology and Food Microbiology at Wroclaw University of Environmental and Life Sciences, Poland.

*Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. Supplementation of ampicillin (100 mg/L) was necessary to screen transformants on media plates.
The *Y. lipolytica* strains were grown at 28°C in YPD medium containing 1% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) glucose. YPD medium was used for the preparation of inoculation for bioreactor studies. During shake flask experiments the yeast cultures were grown in 0.25 L baffled flasks containing 0.03 L of medium on a rotary shaker. The parameters were set at 28°C and 200 rpm. Erythritol synthesis was carried out in Erythritol Synthesis Medium containing 100 g/L of glycerol as described by Mirończuk et al. (2017).

**Cloning and transformation protocols**

All of the restriction enzymes, Phusion high-fidelity DNA polymerase and T4 DNA ligase were purchased from Thermo Scientific (USA). The reactions followed standard protocols as described by manufacturers. The Plasmid Mini Kit, Gel Out extraction kit and Genomic Mini AX Yeast Spin kit were obtained from A&A Biotechnology (Poland). Isolation of plasmid DNA, DNA from gel purification and gDNA extraction followed protocols supplied by the manufacturer.

*E. coli* transformation followed the standard chemical protocol with selective medium containing ampicillin to plate *Y. lipolytica* strains, which were transformed with overexpression cassettes or a deletion cassette by the lithium acetate method. Transformations resulted in strains AJD ΔB07117g and AJD pAD-B07117g. Additionally the strain AJD ΔB07117 was transformed with an overexpression cassette resulting in the strain AJD-c-B07117g.

**Gene disruption**

To disrupt *YALI0B07117g* the disruption cassette was obtained. First, the upstream region was amplified with primers Up-B07117-F-HindIII and Up-B07117-R-Sall, resulting in a PCR product of 1039 bp. The upstream region of the gene was cloned to the plasmid pUC-Ura. Both the region and plasmid were digested with HindIII and Sall enzymes. This resulted in a pUpB07117 vector. Next, the 1088 bp downstream region was amplified using the primers Down-B07117-F-Notl and Down-B07117-R-SacII. The PCR product was then cloned into the pUpB07117 vector and digested with Notl and SacII enzymes, resulting in the pΔB07117 vector. The obtained vector was used as a template for PCR reaction with primers Up-B07117-F-HindIII and Down-B07117-R-SacII, resulting in a 3533 linear deletion cassette. Following the PCR reaction, the product was extracted by the Gel Out extraction kit. *Y. lipolytica* strain AJD was transformed, resulting in AJD ΔB07117. PCR reaction was performed to verify the proper integration. All of the primers used in this study are listed in Table 4.

**Construction of overexpression vectors**

*YALI0B07117g* was amplified from *Y. lipolytica* gDNA with primers B07117-F-SgsI and B07117-R-Nhel, resulting in a 1034 bp PCR product. It was then digested with Fast Digest restriction enzymes SgsI and Nhel and cloned into the pAD UAS1B<sub>16</sub>-TEF promoter vector. The obtained plasmid pAD-B07117 was digested with the MssI enzyme to create a linear overexpression cassette with *Y. lipolytica* rDNA.
sequences for targeted integrations. *Y. lipolytica* strains AJDD and AJDD Δ*B07117* were used for lithium acetate transformations resulting in strains AJDD pAD-*B07117* and AJDD c-*B07117* respectively.

**Bioscreen C analysis**

The strains were grown for 72 hours in 0.1 L Erlenmeyer flasks with the working volume of 0.01 L of YNB medium with ammonium sulfate supplemented with 2% (w/v) glucose, on a rotary shaker at 28°C and 200 rpm. 1 ml of each culture was then transferred for an additional 48 h culture in YNB medium with glucose to ensure that the yeast cells were not able to accumulate nutrients. The analysis was performed in 100-well plates in 200 µL of YPD medium, YNB medium supplemented with 5% (w/v) glycerol, 5% (w/v) glucose or 5% (w/v) erythritol. The cells were washed with Milli-Q water and inoculated to an OD<sub>600</sub> of 0.1 in each well. Quintuple experiments were performed with the Bioscreen C system (Oy Growth Curves Ab Ltd., Finland). Experiment parameters were set at 28°C under continuous agitation. Growth was monitored by measuring the optical density (OD) at 420-560 nm every 30 minutes for 72 hours.

**Bioreactor studies**

To perform bioreactor studies the yeast strains were grown in YPD medium for 72 h in 0.3 L Erlenmeyer flasks (0.1 L working volume) on a shaker at 28°C and 200 rpm. The cultivations were performed using Erythritol Synthesis Medium containing 150 g/L of glycerol as described before (Mirończuk et al. 2017). An inoculum of 0.2 L was introduced to 1.8 L of the medium in a 5-L jar bioreactor (Biostat B Plus, Germany) resulting in a working volume of 2 L. The batch cultures were grown at 28°C with the stir speed set to 800 rpm. The aeration was set to 0.8 L/min. The pH was established at 3.0 and monitored automatically and adjusted to a set value by addition of NaOH (40% w/v).

**Analytical methods**

Samples from shake flask experiments (300 µL) were centrifuged at 4°C for 15 min at 15000 rpm. The supernatant was then transferred to new Eppendorf tubes. Samples from batch culture experiments were centrifuged at 4°C for 5 min at 6000 rpm. 1 ml of the supernatant was transferred to an Eppendorf tube for HPLC analysis, while the biomass was determined via filtration on 0.45 µm pore membranes and drying at 120°C. The concentration of the metabolites (erythritol, mannitol, arabitol and citric acid) as well as a carbon source (glycerol) was determined by HPLC analysis using a HyperRez Carbohydrate H+ Column (CarbH+) (Thermo Scientific), coupled to a UV (λ = 210 nm) (Dionex, USA) and a refractive index detector (Shodex, Japan). The column was eluted with 25 mM trifluoroacetic acid at 65°C and a flow rate of 600 µL/min.

**Calculation of fermentation parameters**

To control the pH in batch studies NaOH was required. This resulted in dilution of the medium. To avoid a calculation mistake, the added amount of NaOH had to be taken into consideration. Therefore two parameters were calculated: mass yield of erythritol (Y<sub>ERY</sub>) and volumetric erythritol productivity.
(Q_{\text{ERY/GLY}}), expressed in g/L/h of total produced erythritol. Due to glycerol being used as a carbon source, Y_{\text{ERY}} was expressed in the amount of produced erythritol from used glycerol (g/g). The calculations followed the formula:

\[ Y_{\text{ERY}} = \frac{P}{S}. \quad Q_{\text{ERY/GLY}} = \frac{P}{V \cdot t}. \]

P stands for the amount of erythritol in the medium (g); S is the amount of consumed glycerol (g), V is the volume of the medium after addition of NaOH; t is the time of fermentation when the samples were collected.

**Declarations**

**Funding**

This work was financially supported by the National Science Centre, Poland, project UMO-2018/31/B/NZ9/01025.

**Availability of supporting data**

The authors promise the availability of supporting data.

**Ethical approval and consent to participate**

Not applicable.

**Competing interest**

The authors declare that they have no competing interests.

**Consent for publication**

The authors have consented for publication.

**Authors’ contributions**

MS constructed the plasmids and the strains, analyzed the data, and wrote the manuscript. DAR participated in planning the experiment; AD participated in bioreactor experiments and planned the experiments, AMM planned the experiments, analyzed the data and revised the manuscript.

**Authors’ details**

Department of Biotechnology and Food Microbiology, Faculty of Biotechnology and Food Science, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland

**References**
1. Martău GA, Coman V, Vodnar DC. Recent advances in the biotechnological production of erythritol and mannitol. Crit Rev Biotechnol. 2020;40:608–22. doi:10.1080/07388551.2020.1751057.

2. Hruby A, Manson JE, Qi L, Malik VS, Rimm EB, Sun Q, et al. Determinants and Consequences of Obesity. 2016;106.

3. Rzechonek DA, Dobrowolski A, Rymowicz W, Mirończuk AM. Recent advances in biological production of erythritol. Crit Rev Biotechnol. 2018;38:620–33. doi:10.1080/07388551.2017.1380598.

4. Rzechonek DA, Szczepańczyk M, Wang G, Borodina I, Mirończuk AM. Hog-independent osmoprotection by erythritol in yeast yarrowia lipolytica. Genes (Basel). 2020;11:1–15.

5. Grembecka M. Sugar alcohols — their role in the modern world of sweeteners: a review. Eur Food Res Technol. 2015;:1–14. doi:10.1007/s00217-015-2437-7.

6. Mirończuk AM, Biegalska A, Zugaj K, Rzechonek DA, Dobrowolski A. A role of a newly identified isomerase from Yarrowia lipolytica in erythritol catabolism. Front Microbiol. 2018;9 MAY:1–12.

7. Wölnerhanssen, Bettina K; Cajacob, Lucian; Keller Nino; Doody, Alison; Rehfeld, Jens F; Drewe, Juergen; Peterli, Ralph; Beglinger, Christoph; Meyer-Gerspach AC. Gut hormone secretion, gastric emptying, and glycemic responses to erythritol and xylitol in lean and obese subjects. Am J Physiol - Endocrinol Metab. 2016;11:1053–61.

8. Noda K, Nakayama K OT. Serum glucose and insulin levels and erythritol balance after oral administration of erythritol in healthy subjects. Eur J Clin Nutr. 1994;48:286–92.

9. Cock P De, Mäkinen K, Honkala E, Saag M, Kennepohl E, Eapen A. Erythritol Is More Effective Than Xylitol and Sorbitol in Managing Oral Health Endpoints. 2016;2016.

10. Hootman KC, Trezzi J, Kraemer L, Burwell LS, Dong X, Guertin KA, et al. Erythritol is a pentose-phosphate pathway metabolite and associated with adiposity gain in young adults. 2017;:1–8.

11. Lin S, Wen C, Wang P, Huang J, Wei C, Chang J, et al. High-level production of erythritol by mutants of osmophilic Moniliella sp. Process Biochem. 2010;45:973–9. doi:10.1016/j.procbio.2010.03.003.

12. Ryu Y, Park CY, Park JB, Kim SY, Seo J-H. Optimization of erythritol production by Candida magnoliae in fed-batch culture. 2000;:100–3.

13. Papanikolaou S, Aggelis G. Modeling lipid accumulation and degradation in Yarrowia lipolytica cultivated on industrial fats. Curr Microbiol. 2003;46:398–402.

14. Papanikolaou S, Aggelis G. Lipid production by Yarrowia lipolytica growing on industrial glycerol in a single-stage continuous culture. 2002;82 August 2001:43–9.
15. Lazar Z, Liu N, Stephanopoulos G. Holistic Approaches in Lipid Production by Yarrowia lipolytica. Trends Biotechnol. 2018;xx:1–14. doi:10.1016/j.tibtech.2018.06.007.

16. Rakicka M, Biegalska A, Rymowicz W, Dobrowolski A, Mirończuk AM. Polyol production from waste materials by genetically modified Yarrowia lipolytica. Bioresour Technol. 2017;243:393–9.

17. Mirończuk AM, Kosiorowska KE, Biegalska A, Rakicka-Pustułka M, Szczepańczyk M, Dobrowolski A. Heterologous overexpression of bacterial hemoglobin VHb improves erythritol biosynthesis by yeast Yarrowia lipolytica. Microb Cell Fact. 2019;18:1–8. doi:10.1186/s12934-019-1231-9.

18. Rymowicz W, Fatykhova AR, Kamzolova S V., Rywińska A, Morgunov IG. Citric acid production from glycerol-containing waste of biodiesel industry by Yarrowia lipolytica in batch, repeated batch, and cell recycle regimes. Appl Microbiol Biotechnol. 2010;87:971–9.

19. Rzechonek DA, Dobrowolski A, Rymowicz W, Mirończuk AM. Aseptic production of citric and isocitric acid from crude glycerol by genetically modified Yarrowia lipolytica. Bioresour Technol. 2018. doi:10.1016/j.biortech.2018.09.118.

20. Papanikolaou S, Galiotou-panayotou M, Fakas S. Citric acid production by Yarrowia lipolytica cultivated on olive-mill wastewater-based media. 2008;99:2419–28.

21. Dobrowolski A, Mituła P, Rymowicz W, Mirończuk AM. Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast Yarrowia lipolytica. Bioresour Technol. 2016;207:237–43.

22. Janek T, Dobrowolski A, Biegalska A, Mirończuk AM. Characterization of erythrose reductase from Yarrowia lipolytica and its influence on erythritol synthesis. Microb Cell Fact. 2017;16:1–13.

23. Wang N, Chi P, Zou Y, Xu Y, Xu S, Bilal M, et al. Metabolic engineering of Yarrowia lipolytica for thermostability and enhanced erythritol productivity. Biotechnol Biofuels. 2020;13:1–20. doi:10.1186/s13068-020-01815-8.

24. Rymowicz W, Rywińska A. High-yield production of erythritol from raw glycerol in fed-batch cultures of Yarrowia lipolytica. 2009;377–80.

25. Rzechonek DA, Neuvéglise C, Devillers H, Rymowicz W, Mirończuk AM. EUF1-A newly identified gene involved in erythritol utilization in Yarrowia lipolytica. Sci Rep. 2017;7:1–8.

26. Mirończuk AM, Rzechonek DA, Biegalska A, Rakicka M. Biotechnology for Biofuels A novel strain of Yarrowia lipolytica as a platform for value-added product synthesis from glycerol. Biotechnol Biofuels. 2016;1–12.

27. Cheng H, Wang S, Bilal M, Ge X, Zhang C, Fickers P, et al. Identification, characterization of two NADPH-dependent erythrose reductases in the yeast Yarrowia lipolytica and improvement of erythritol
productivity using metabolic engineering. Microb Cell Fact. 2018;17:1–12. doi:10.1186/s12934-018-0982-z.

28. Mirończuk AM, Biegalska A, Dobrowolski A. Functional overexpression of genes involved in erythritol synthesis in the yeast Yarrowia lipolytica. Biotechnol Biofuels. 2017;10:1–12.

29. Blazeck J, Hill A, Jamoussi M, Pan A, Miller J, Alper HS. Metabolic engineering of Yarrowia lipolytica for itaconic acid production. Metab Eng. 2015;32:66–73. doi:10.1016/j.ymben.2015.09.005.

30. Hanahan D. DNA cloning: a practical approach. McLean: IRL Press; 1985.

31. Wojtatowicz M, Rymowicz W. Effect of inoculum on kinetics and yield of citric acids production on glucose by Yarrowia lipolytica A-101. Acta Alimentaria Polonica (Poland). 1991;41(2):137–43.

32. Mirończuk AM, Rakicka M, Biegalska A, Rymowicz W, Dobrowolski A. A two-stage fermentation process of erythritol production by yeast Y. lipolytica from molasses and glycerol. Bioresour Technol. 2015;198:445–55.

Tables

Table 1. Erythritol, by-product synthesis and glycerol utilization by the Y. lipolytica strains in shake-flasks experiment.
| Strain         | Time [h] | Glycerol [g/L] | Erythritol [g/L] | Mannitol [g/L] | Arabitol [g/L] | Citric acid [g/L] |
|---------------|----------|----------------|------------------|---------------|----------------|------------------|
| A101          | 24       | 87.74±3.78     | 0.35± 0.09       | 0             | 0.01±0.01      | 1.18±0.34        |
| AJD ΔB07117   |          | 107.15±3.2     | 0.27± 0.15       | 0             | 0              | 0.10±0.08        |
| AJD pAD B07117|          | 107.4±1.0      | 0.35± 0.11       | 0             | 0              | 0                |
| A101          | 48       | 38.16±1.86     | 16.54±0.96       | 1.02±0.16     | 1.61±0.03      | 5.70±0.59        |
| AJD ΔB07117   |          | 57.61±7.17     | 15.03±1.92       | 4.61±1.18     | 2.10±0.46      | 12.04±2.08       |
| AJD pAD B07117|          | 65.1±6.39      | 11.0 ± 2.4       | 0.13±0.11     | 0.15±0.13      | 8.08±1.20        |
| A101          | 72       | 0.1±0.18       | 30.02±1.39       | 2.43±0.20     | 2.48±0.44      | 5.85±0.82        |
| AJD ΔB07117   |          | 12.5±4.73      | 28.22±0.78       | 7.48±1.49     | 5.07±0.44      | 17.12±3.83       |
| AJD pAD B07117|          | 21.78±6.23     | 30.60±3.14       | 1.07±0.18     | 0.75±0.17      | 9.79±1.60        |
| A101          | 96       | 0              | 15.16±1.20       | 0.95±0.03     | 1.30±0.16      | 7.16±0.67        |
| AJD ΔB07117   |          | 0              | 29.52±1.64       | 8.01±1.10     | 6.10±0.22      | 18.08±2.87       |
| AJD pAD B07117|          | 0              | 39.79±1.60       | 1.00±0.07     | 1.71±0.03      | 9.80±1.32        |

Table 2. Erythritol, by-product synthesis and glycerol utilization by *Y. lipolytica* strains in bioreactor experiments.
| Strain       | Time [h] | Glycerol [g/L] | Erythritol [g/L] | Mannitol [g/L] | Arabitol [g/L] | Citric acid [g/L] |
|-------------|----------|----------------|------------------|---------------|---------------|------------------|
| A101        | 24       | 127.92±7.26    | 5.68±0.84        | 0.20±0.09     | 0.13±0.05     | 0.84±0.30        |
| AJD pAD-B07117 | 127.25±3.46 | 7.37±1.62    | 0.05±0.09        | 0.04±0.06     | 0.55±0.21     |
| A101        | 48       | 77.49±5.62     | 23.50±2.27       | 1.59±0.12     | 0.64±0.12     | 2.58±0.01        |
| AJD pAD-B07117 | 59.05±2.84   | 33.99±3.45    | 2.12±0.48        | 0.70±0.06     | 6.20±2.75     |
| A101        | 72       | 21.28±3.05     | 41.15±4.97       | 3.74±0.09     | 1.06±0.11     | 4.57±1.69        |
| AJD pAD-B07117 | 3.42±0.18 | 59.83±4.86    | 4.72±0.29        | 1.19±0.13     | 12.97±8.03    |
| A101        | 96       | 0.20±0.35      | 37.57±7.59       | 2.09±0.74     | 0.63±0.14     | 9.99±3.69        |
| AJD pAD-B07117 | 0       | 45.78±5.76     | 4.05±0.21        | 1.04±0.07     | 18.60±7.29    |

**Table 3. Strains used in this study.**

| Strain       | Genotype or plasmid                                                                 | Source |
|-------------|-----------------------------------------------------------------------------------|--------|
| *E. coli*   |                                                                                   |        |
| DH5α        | F− endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17(rK-mK+), λ− | [30]   |
| DH5α        | pAD                                                                               | [28]   |
| DH5α        | pΔB07117                                                                          | This study |
| DH5α        | pADB07117g                                                                        | This study |
| *Y. lipolytica* |                                                                                   |        |
| A101        | Wild type                                                                         | [31]   |
| AJD         | MATA, A101: ura3-302                                                              | [32]   |
| AJD ΔB07117 | MATA, A101: ura3-302 ΔB07117                                                     | This study |
| AJD ΔB07117 | MATA, A101: ura3-302, ΔB07117 pAD-B07117                                         | This study |
| AJD pADB07117 | MATA, A101: ura3-302, pAD-B07117                                               | This study |

**Table 4. Primers used in this study.**
| Primer                        | Sequence (5' -> 3')                                      |
|-------------------------------|---------------------------------------------------------|
| YALI0B07117_up_F_HindIII      | CGTAAGCTTTTACACTCCGCACAAAC                              |
| YALI0B07117_up_R_SalI         | CGTGTCGACCGTCTTGCTCGGATTC                               |
| YALI0B07117_down_F_NotI       | TCAGCGGCGGCAGCTTGGTGAACCATATTT                         |
| YALI0B07117_down_R_SacII      | TATCCGCAGGCCCTCCAGACGAGTAATAC                          |
| Test-YALI0B07117_F            | CCCGTTTTATTTGACCTCTTTACAGC                             |
| Test-YALI0B7117_R             | CGGAACTTTCTTCTGCTCCATCTGAC                             |
| URA_col_F                     | GGTACTGGTGCTTGACAGTG                                   |
| URA_col_R                     | CTCGAGCTAAGTCTCCACAAG                                  |
| YALI0B07117_F_AscI            | ATAGGCGCGCCATGTTCGGGTCAGTATAAAC                        |
| YALI0B07117_R_NheI            | GCAGCTAGCTTAGCGAAGTCAAGCGGTCAATGCGGGAAT                |
| TEF_F                         | GTCAACTCACACCCGAAATC                                   |
| YALI0B07117_R_NheI            | GCAGCTAGCTTAGCGAAGTCAAGCGGTCAATGCGGGAAT                |

**Figures**
Figure 1

Overview of erythritol synthesis from glycerol in yeast Y. lipolytica. Erythrose reductase (ER) shown in red, other enzymes in blue. Green arrows indicate involvement in the pentose phosphate pathway.

Figure 2

N-terminal fragments protein sequence alignment (T-COFFEE, Version 11.00).
Figure 3

Glycerol utilization (bar chart) and erythritol production (line chart) by yeast strains A101 (gray), AJD ΔB07117 (black) and AJD pAD-B07117g (light gray) in shake flask experiments using Erythritol Synthesis Medium. Standard deviation added to the chart
Figure 4

Bioscreen analysis of strains created in this study. A101 strain (gray) was used as a control. AJD pAD-B07117 (black) showed similar growth to control, while AJD-c-B07117 (light grey) displayed initially impaired growth.
Figure 5

Erythritol synthesis (line chart) and glycerol utilization (bar chart) in batch reactors in Erythritol Synthesis Medium for A101 strain (gray) and AJD pAD-B07117 strain (black).