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Overexpression of the transcriptional activators Mxr1 and Mit1 enhances lactic acid production on methanol in *Komagataella phaffii*

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ABSTRACT

A bio-based production of chemical building blocks from renewable, sustainable and non-food substrates is one key element to fight climate crisis. Lactic acid, one such chemical building block is currently produced from first generation feedstocks such as glucose and sucrose, both requiring land and water resources. In this study we aimed for lactic acid production from methanol by utilizing *Komagataella phaffii* as a production platform. Methanol, a single carbon source has potential as a sustainable substrate as technology allows (electro)chemical hydrogenation of CO_2 for methanol production. Here we show that expression of the *Lactiplantibacillus plantarum* derived lactate dehydrogenase leads to L-lactic acid production in *Komagataella phaffii*, however, production resulted in low titers and cells subsequently consumed lactic acid again. Gene expression analysis of the methanol-utilizing genes *AOX1*, *FDH1* and *DAS2* showed that the presence of lactic acid downregulates transcription of the aforementioned genes, thereby repressing the methanol-utilizing pathway. For activation of the methanol-utilizing pathway in the presence of lactic acid, we constructed strains deficient in transcriptional repressors Nrg1, Mig1-1, and Mig1-2 as well as strains with overrepresentation of transcriptional activators Mxr1 and Mit1. While loss of transcriptional repressors had no significant impact on lactic acid production, overexpression of both transcriptional activators, *MXR1* and *MIT1*, increased lactic acid titers from 4 g L^{-1} to 17 g L^{-1} in bioreactor cultivations.

1. Introduction

Lactic acid is one of the few large-scale chemicals that is nowadays almost entirely produced by microbial fermentation, with bacteria or engineered yeasts serving as the primary production platform (Abdel-Rahman et al., 2013). It is a key chemical in various industries, including food, chemical, cosmetic, and pharmaceuticals, serving multiple functions such as a preservative, repellent, moisturizer, pH regulator or neutralizer (Abedi and Hashemi, 2020). However, lactic acid has also gained a lot of momentum as a building block to produce poly-lactic acid (PLA), a biodegradable bio-based aliphatic polyester that can replace fossil-based polymers for certain applications (Balasubramanian et al., 2024). Forecasts indicate a robust growth trajectory for the lactic acid market, projecting an anticipated annual expansion rate of 8%, potentially reaching 6.9 billion USD by 2030 (Grandviewresearch, 2024). Commercially, lactic acid bacteria are the main producers of lactic acid on an industrial scale, converting pentoses and hexoses reaching titers of up to 150 g L^{-1}. However, lactic acid bacteria often rely on complex media requirements and exhibit a low pH sensitivity, which leads to costly downstream processes (Ghaffar et al., 2014; Abedi and Hashemi, 2020; Kim et al., 2022). Certain fungi, such as *Rhizopus* sp., are also used in industry and metabolize renewable carbon resources, particularly starch, to produce lactic acid with their advantageous amylolytic properties, achieving titers of 90 g L^{-1} (Wee et al., 2006). Yeasts are more tolerant to low pH-values and genetic engineering enabled lactic acid production in yeasts of different genera such as *Saccharomyces cerevisiae*, *Candida* spp., *Kluyveromyces lactis*, *Torulaspora delbrueckii*, *Pichia (Scheffersomyces) stipitis and Zygosaccaromyces bailii* (Abedi and Hashemi, 2020). While glucose, sucrose, maltose, molasses, and wheat bran are commonly used as preferred substrates for lactic acid production (Abedi and Hashemi, 2020), concerns about their sustainability due to competition with food and feed production (Gasparatos et al., 2013) have led researchers to explore alternative carbon resources, including lignocellulosic biomass (Yankov, 2022), glycerol (Tamires Moreira Melo et al., 2020), and single carbon (C1) substrates (Yamada et al., 2019; Wefelmeier et al., 2023), with methanol emerging as a particularly promising candidate. The potential to produce methanol from CO_2 through (electro)chemical hydrogenation offers an essentially limitless

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carbon supply, fostering both economic viability and social responsibility (Li et al., 2022; Ewis et al., 2023; Izadi et al., 2024). Methylotrophic yeasts represent an attractive option as a production platform, given their natural ability to utilize methanol as a carbon source (Chistoserdova et al., 2009). The first step in methanol utilization is catalyzed by the alcohol oxidase Aox, which oxidizes methanol to formaldehyde, while the harmful by-product hydrogen peroxide is converted into oxygen and water through the activity of catalase (Cat) (Ellis et al., 1985; Cregg et al., 1989). Further, formaldehyde can be either metabolized in the assimilatory pathway for biomass generation via the dihydroxyacetone synthase Das, or used for energy generation in the dissimilation pathway by two dehydrogenase reactions (Fld, Fdh) (Hartner and Glieder, 2006; Rußmayer et al., 2015). Notably, the expression of *AOX1* is intricately controlled by the carbon source, being suppressed by glucose, ethanol, and glycerol, while induced by methanol (Hartner and Glieder, 2006). This catabolite repression is a regulatory mechanism to ensure the timely adequate expression of genes required for the utilization of a secondary carbon source only in the absence of the preferred substrate. This regulatory mechanism is partly managed at a transcriptional level including pathway-specific transcription factors (Prielhofer et al., 2015). Several of those transcription factors, i.e., Mxr1, Prm1, Mit1, Mig1-1, Mig1-2, and Nrg1 were identified to regulate the transcription of *AOXI* and other genes of the MUT pathway, but also represent interfaces to other metabolic pathways. Mxr1 is a global regulator of multiple pathways in *K. phaffii* and plays a crucial role in activating methanol utilization and peroxisome biogenesis genes but is also involved in amino acid biosynthesis and ethanol metabolism (Lin-Cereghino et al., 2006; Sahu and Rangarajan, 2016a; Gupta et al., 2021; Inoue et al., 2022). While Mxr1 operates mainly during carbon derepression, two other transcription factors, Mit1 and Prm1, positively regulate P_{AOX1} in response to MeOH induction (Wang et al., 2016b). Each of the three transcription factors binds to distinct sites on the P_{AOX1} in a cascade-like fashion, without directly interacting with one another (Wang et al., 2016b). Nrg1, a transcriptional repressor of the MUT pathway functions mainly in glucose and glycerol repression and competes with Mxr1 for the same binding motif at P_{AOX1}. Mig1-1 and Mig1-2 are characterized as catabolite repressors of the MUT pathway (Wang et al., 2016a, 2017; Shi et al., 2018), however, the precise mechanism governing their regulation remains to be fully elucidated.

In this study, we demonstrate that the production of L-lactic acid in *K. phaffii* using methanol as a carbon source results in repression of methanol-utilizing genes, suggesting a role of lactic acid as a catabolite repressor of the MUT pathway. Expression of the *Lactiplantibacillus plantarum* derived L-lactate dehydrogenase controlled by the methanol inducible P_{AOX1} promotor enabled L-lactic acid formation up to 4 g L^{-1} in MeOH-fed batch cultivations, however, cells subsequently metabolized lactic acid as a carbon source in the presence of MeOH. By targeting transcriptional regulation of the MUT pathway, we were able to unlock a pathway to enhance lactic acid production up to 17 g L^{-1} in fed-batch cultivations (Fig. 1).

2. Materials and methods

Strain generation. *Komagataella phaffii* wild type CBS7435 (Centraalbureau voor Schimmelcultures, NL) was used as a host for engineering the lactic acid-producing strains (Kurtzman, 2009). For lactic acid production the L-lactate dehydrogenase from *Lactiplantibacillus plantarum* was chosen, codon optimized for *K. phaffii* and expressed under the methanol inducible promoter P_{AOX1} and targeted to the *RGI2* locus (Figure S1 A). The deletion of the L-lactate cytochrome-c oxidoreductase *CYB2* (PP735_Chr4-0730) was performed using CRISPR-Cas9 and a donor DNA (dDNA) template containing the homologous 5′- and 3′- UTR regions of *CYB2* as described by Baumschabl et al. (2022) (Figure S1 B). Overexpression of the *K. phaffii JEN1* (PP7435_Chr3-0773) gene encoding for a monocarboxylate/proton

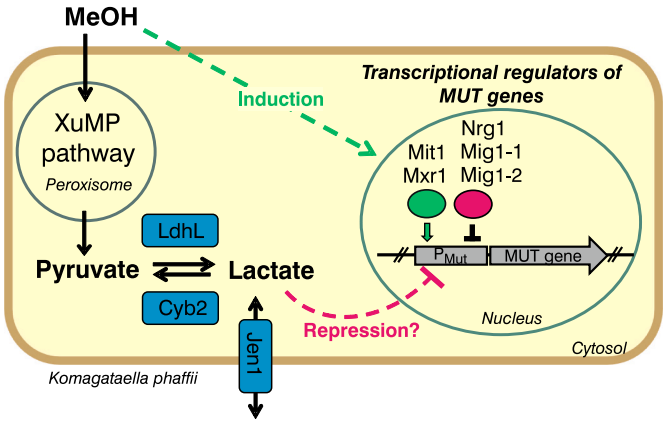

Fig. 1. Metabolic pathway from methanol to lactic acid in *K. phaffii* with targeted genes in focus. While MeOH leads to an induction of the MUT pathway, production of lactic acid represses methanol-utilizing genes. By investigating transcriptional regulators with activating (Mxr1, Mit1) and repressing functions (Nrg1, Mig1-1 and Mig1-2), it was possible to improve lactic acid titers markedly. XuMP: xylulose monophosphate pathway, LdhL: L-lactate dehydrogenase, Cyb2: cytochrome *b*2 (L-lactate cytochrome-c oxidoreductase), Jen1: monocarboxylate/proton symporter of the plasma membrane, Mxr1: methanol expression regulator 1, Mit1: methanol-induced transcription factor 1, Nrg1: negative regulator of glucose-repressed genes, Mig1-1/1–2: multicopy inhibitor of *GAL* gene expression.

symporter was engineered with the P_{PDC1} promotor and directed to the *GUT1* locus (Figure S1 C). Similar to the *CYB2* knock out, deletion strains of the transcriptional repressors *NRG1* (PP7435_Ch3-0228), *MIG1-1* (PP7435_Chr4-0661) and *MIG1-2* (PP7435_Chr1-1325) were generated (Fig. S2). Overexpression of the global regulator *MXR1* (PP7435_Chr4-0490) was engineered under a strong constitutive promoter P_{GAP} to ensure consistent and stable level of *MXR1*, while *MIT1* (PP7435_Chr3-0971) was engineered with the strong methanol inducible promoter P_{FDH1} as this transcription factor is specifically activated by methanol induction (Fig. S3). All plasmids were constructed using the Golden Gate Assembly (GGA) and the CRISPR-Cas9 systems as described (Prielhofer et al., 2017; Gassler et al., 2019). Primers were designed using the Qiagen CLC Genomics Workbench and ordered at the Integrated DNA technologies (IDT) company and are listed in Table S1. The full sequences of the transformed dDNA elements used for *K. phaffii* transformation are listed in Table S2.

K. phaffii transformations were performed as described by (Gasser et al., 2013). Briefly, prior to transformation donor DNA (dDNA) was amplified by PCR using Q5® High-Fidelity DNA Polymerase from the respective plasmids. Generated amplicons were subsequently purified with the JENA Bioscience PCR purification kit (PP-201L). Transformation of *K. phaffii* was performed by electroporation (BioRad Gene Pulser, 2000 V, 25 mF and 4 mS) using 5–10 μg dDNA and 0.8 μg of the respective CRISPi plasmids. Transformed cells were regenerated for 1.5–3 h in YPD medium and then plated on selective YPD plates containing the appropriate antibiotics (G418, hygromycin or nourseothricin). After 48 h at 28 °C, randomly selected transformants were streaked on selective YPD plates and incubated for another 48 h at 28 °C. Integration of the dDNA into the correct locus or absence of deleted genes was verified by diagnostic PCR. All generated strains are listed in Table 1.

Shake flask cultivations of engineered strains. First screenings of potential producer strains were done in small shake flask cultivations (Gasser et al., 2013). Here a two-phase screening protocol was used. For biomass production, strains were inoculated in 15 mL YPG (yeast extract 10 g L^{-1}, soy peptone 20 g L^{-1}, glycerol 20 g L^{-1}) and incubated overnight at 25 °C and 180 rpm. Cells from the preculture were subsequently used for inoculation of the main culture with a starting OD_{600} of 4. For that, the cells were cultivated in 200 mL wide neck flasks in

Table 1
K. phaffii strains used in this study.

Short name	Genotype	References
Parent strain	CBS7435	Kurtzman (2009)
ldhL	*RGI2*::P_{AOX1}-*ldhL*	This study
ldhL cyb2Δ	*RGI2*::P_{AOX1}-*ldhL cyb2Δ*	This study
ldhL JEN1	*RGI2*::P_{AOX1}-*ldhL* + *GUT1*::P_{PDC1}-*JEN1*	This study
ldhL cyb2Δ nrg1Δ	*RGI2*::P_{AOX1}-*ldhL cyb2Δ* + *nrg1Δ*	This study
ldhL cyb2Δ mig1-1Δ	*RGI2*::P_{AOX1}-*ldhL cyb2Δ* + *mig1-1Δ*	This study
ldhL cyb2Δ mig1-2Δ	*RGI2*::P_{AOX1}-*ldhL cyb2Δ* + *mig1-2Δ*	This study
ldhL cyb2Δ MIT1	*RGI2*::P_{AOX1}-*ldhL cyb2Δ* + *GUT1*::P_{FDH1}-*MIT1*	This study
ldhL cyb2Δ MXR1	*RGI2*::P_{AOX1}-*ldhL cyb2Δ* + *ENO*::P_{GAP}-*MXR1*	This study
ldhL cyb2Δ MXR1+MIT1	*RGI2*::P_{AOX1}-*ldhL cyb2Δ* + *ENO*::P_{GAP}-*MXR1*+ *GUT1*::P_{FDH1}-*MIT1*	This study

Table 2
Equations used for calculation of fermentation parameters.

Parameter	Equation
μ [h^{-1}]	$\frac{\ln\left(\frac{X_1}{X_0}\right)}{t_1 - t_0}$
q_P [$g{\cdot}g^{-1}{\cdot}h^{-1}$]	$\frac{P_2 - P_1}{(X_1 + 0.67 * (X_2 - X_1)) * (t2 - t1)}$
q_S [$g{\cdot}g^{-1}{\cdot}h^{-1}$]	$\frac{S_2 - S_1}{(X_1 + 0.67 * (X_2 - X_1)) * (t2 - t1)}$
STY [$g{\cdot}L^{-1}{\cdot}h^{-1}$]	$\frac{P_2 - P_1}{(t2 - t1) * V}$
$Y_{P/S}$ [$g{\cdot}g^{-1}$]	$\frac{P_2 - P_1}{S_2 - S_1}$
$Y_{P/X}$ [$g{\cdot}g^{-1}$]	$\frac{P_2 - P_1}{(X_1 + 0.67 * (X_2 - X_1))}$
$Y_{X/S}$ [$g{\cdot}g^{-1}$]	$\frac{X_2 - X_1}{S_2 - S_1}$

phosphate buffered Yeast Nitrogen Base (YNB; 3.4 g L^{-1}, pH 6, supplemented with 10 g L^{-1} $(NH_4)_2SO_4$ as the nitrogen source) at 25 °C and 180 rpm. Cells were induced with 0.5% MeOH (v/v) overnight and were then continuously fed twice a day with MeOH for approximately 70 h. Depending on the screening, cultures were either fed with 1, 2, or 3 % MeOH (v/v.). Cell growth (OD_{600}) was monitored throughout the cultivation and extracellular metabolite concentrations (methanol and lactic acid) were measured depending on the producer strain by high-performance liquid chromatography (HPLC) at certain time points. Samples for RNA and protein extraction were taken at specific time points during the cultivation. For analyzing the effect of lactate on gene expression, 10 g L^{-1} of sodium lactate was added to the cultures and incubated for 2 h prior sampling for RNA extraction.

HPLC Measurements. A Biorad Aminex HPX-87H HPLC column (300 × 7.8 mm) was used for the HPLC measurements. H_2SO_4 at a concentration of 4 mmol L^{-1} was used as the mobile phase, with a 0.6 mL min^{-1} flow rate at 60 °C. Lactic acid and methanol concentrations were measured with a refraction index detector (RID-10A, Shimadzu). Before HPLC measurement, the supernatant of each sample was mixed with H_2SO_4 (c = 40 mmol L^{-1}) resulting in a final concentration of 4 mM. Samples were vortexed and centrifuged at full speed (16,100 g) for 5 min at room temperature. After centrifugation, they were filtered using a 0.22 μm filter into the vials for the HPLC analysis. For identification and quantification, a standard solution for lactic acid and methanol was prepared and measured. Under these conditions, methanol and lactic acid showed a retention time of 20.2 and 14.2 min, respectively.

Fed batch cultivation. Fed batch cultivations were carried out in 1.4 L DASGIP reactors (Eppendorf bioreactors SR0700ODLS) A preculture was prepared as inoculum. For that 1 mL of a working cell bank from the respective strain was used to inoculate 100 mL YPD and incubated at 28 °C, 180 rpm overnight. An OD of 1 was used for inoculation of the batch medium (13.5 mL L^{-1} H_3PO_4 (85%), 0.5 g L^{-1} $CaCl_2{\cdot}2H_20$, 7.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 9 g L^{-1} K_2SO_4, 2 g L^{-1} KOH, 33 g L^{-1} glycerol, 4.4 g L^{-1} citric acid monohydrate, 0.25 g L^{-1} NaCl, 4.35 mL biotin (0.2 g L^{-1}), 4.35 mL PTM0, 0.1 mL L^{-1} Glanapon2000) with 25% NH_3 as the nitrogen source (19 mL). The pH of 5.5 was stabilized using 25% NH_3 during the cultivation period. The cultivations were performed either at 25 °C or 30 °C. Dissolved oxygen concentration was controlled by adjusting the stirrer speed and inlet gas flow whereby 200 rpm and 6 sL h^{-1} were the minimal setpoints. After the glycerol batch, an end biomass of 20 g L^{-1} was reached based on a yield on glycerol of 0.5. To induce the MeOH genes, a limited glycerol feed (4 mL h^{-1}) was performed for 4 h after the batch culture, ending with 40 g L^{-1} biomass. Then a limited MeOH feed was applied for the rest of the cultivation (2.6 g L^{-1} h^{-1}). Samples were taken continuously throughout the experiment for OD_{600}, dry cell weight (DCW), HPLC and RNA analysis. Specific growth rates and productivity were calculated according to following equations listed in Table 2.

Modification to the Fed-Batch cultivation. To limit biomass production in the bioreactor cultivations, the fed-batch procedure was modified and the base for pH maintenance was switched to 5 M KOH, instead of 25% NH_3. For this fermentation, a MeOH feed rate of 2.4 g L^{-1} h^{-1} was applied.

RNA extraction and quantitative gene expression analysis. For determination of the gene expression, RNA was extracted from pellets collected either from shake flask or bioreactor cultivations. Total RNA was isolated using the TRI Reagent® (Ambion) as described by the manufactures protocol. Remaining genomic DNA was removed with the DNA-free™-kit (Invitrogen) following the suppliers' manuals. The absence of genomic DNA was further verified with PCR using primers that bind in the *ACT1* gene (Table S1). RNA quality, purity and concentration were further checked by gel electrophoresis as well as spectrophotometric analysis using NanoDrop 2000 (Thermo Scientific). Finally, cDNA was synthesized with the Biozym cDNA Synthesis Kit according to the manufacturer's instructions. For that, oligo d(T)23 VN (NEB) primers were used instead of the provided. For the qRT-PCR, 2x qPCR S'Green BlueMix (Biozym Blue S'Green qPCR Kit), appropriate primers (Table S1), cDNA and water were mixed according to the manufacturer's recommendations. All samples were tested in technical triplicates. Measurements were performed using a real-time PCR cycler (Rotor Gene, Qiagen) and the Rotor Gene software, with results analyzed using the Comparative Quantitation (QC) method. Efficiency of primers was previously analyzed and kept between 90 and 110 %. Relative expression levels were calculated using the ΔΔCt method (Pfaffl, 2001). Experiments were performed in biological and technical duplicates. Levels of mRNA were related to the constitutively expressed reference gene *ACT1* (PP7435_Chr3-0993) and transcript levels of the engineered strains were compared to the respective parent strain transcript levels. Primer sequences are listed in Table S1.

PI staining. To measure the viability of fed-batch yeast cultures, samples were diluted in PBS (0.24 g L^{-1} KH_2PO_4, 1.8 g L^{-1} $Na_2HPO_4 \cdot 2H_2O$, 0.2 g L^{-1} KCl, 8.0 g L^{-1} NaCl) to an OD_{600} of 0.1–0.4 and stained with a propidium iodide (PI) stock solution to final concentrations of 2.0 M. PI acts as a DNA-intercalating fluorescence dye that permeates cells with compromised cell membranes thereby indicating cell death. When PI binds to DNA it leads to a strong increase in red fluorescence (excitation/emission at 480/630 nm). Samples were analyzed on a CytoFlex S Flow Analyser 4L 13 (Color, Beckman Coulter), where the percentage of viable cells was estimated based on PI negativity.

3. Results

3.1. *K. phaffii* consumes lactic acid when methanol is used as a carbon source

For the production of L-lactic acid, pyruvate undergoes reduction to lactic acid catalyzed by the cytosolic enzyme L-lactate dehydrogenase

(LdhL). To facilitate lactic acid production in *K. phaffii*, we introduced the LdhL from *Lactiplantibacillus plantarum* under the control of the methanol-inducible P_{AOX1} promoter (from now on referred to as *ldhL* strain). Expression of this gene alone was sufficient for generating L-lactic acid in shake flask cultivations utilizing MeOH as the sole carbon source. However, initial titers were low, yielding only 0.5 g L^{-1} within the first 24 h (Fig. 2 A). Inconveniently the cells consumed lactic acid again throughout further cultivation, ending with titers below 0.1 g L^{-1} after 72 h (Fig. 2 A).

To improve lactic acid productivity, we deleted *CYB2*, a L-lactate cytochrome *c* oxidoreductase. Previously, it was shown that deletion of *CYB2* prevents lactic acid consumption in *Saccharomyces cerevisiae* and the synthetic autotrophic lactic acid-producing *K. phaffii* strain (Ookubo et al., 2008; Baumschabl et al., 2022). In the *ldhL* strain absence of *CYB2* initially increased lactic acid titers, reaching a maximum of 0.65 g L^{-1} after 24 h. However, it failed to prevent the cells from re-consuming the product, ultimately resulting in titers of 0.3 g L^{-1} (Fig. 2 A). Since the LdhL enzyme operates in both directions, lactic acid can be effectively converted back into pyruvate, creating an additional carbon source for the cell and consequently decreasing the product titers. This is evidenced by the strain's capability to thrive on lactic acid alone as a carbon source. (Fig. 2 B). In this instance, despite a markedly reduced growth rate compared to the CBS7435 and the *ldhL* strain, the *ldhL cyb2*Δ strain still grew on lactic acid (Fig. 2 B). While both the CBS7435 and the *ldhL* strains utilized 11–12 mg g^{-1} h^{-1}, the *ldhL cyb2*Δ strain metabolized 3 mg g^{-1} h^{-1} of lactate (Table S3).

Further engineering attempts to boost lactic acid productivity included the expression of the *JEN1* homolog encoding for a monocarboxylate transporter. In *S. cerevisiae* expression of Sc*JEN1* leads to higher external lactic acid concentrations when cultivated on glucose (Pacheco et al., 2012). Similar results have been reported on glycerol in engineered *K. phaffii* expressing either *ScJEN1* or its identified homolog *KpJEN1*, indicating that the homologous transporter improves L-lactic acid production (de Lima et al., 2016). To investigate the effect of the transporter in methanol cultures, we expressed *KpJEN1* under the regulation of the P_{PDC1} promotor. The resulting strains showed the same growth and lactic acid profile as the *ldhL* strain; hence no improvement was observed (Fig. S4).

3.2. Bioreactor cultivation of ldhL cyb2Δ results in low titers and high biomass formation on methanol

Unlike shake flasks, bioreactor cultivations offer better control over crucial process variables such as oxygen supply, pH, and MeOH feed. To explore whether lactate consumption also occurs on a larger scale, we assessed lactic acid production of the *ldhL cyb2*Δ strain in 1.4 L DASGIP bioreactor systems (Eppendorf bioreactors SR0700ODLS). As process temperature, we tested both 25 °C and 30 °C and used 25% NH_3 as a base to stabilize the pH at 5.5. Dissolved oxygen concentration was maintained at 30% by adjusting the stirrer speed, oxygen concentration, and airflow rate of the inlet gas. The minimal setpoints were 200 rpm, 21 %, and 9.5 sL h^{-1} for the respective parameters. After an initial batch phase on glycerol, a biomass of 20 g L^{-1} was reached. Further, we applied a limited glycerol feed for 4 h to activate the respective MeOH utilizing genes (Chang et al., 2018). Finally, a MeOH feed phase was initiated for 86 h, yielding 4 g L^{-1} of lactic acid, which resulted in a space-time yield (STY) of approximately 50 mg L^{-1} h^{-1} (Fig. 3). Although less lactate was consumed than produced, low titers (4 g L^{-1}) and high biomass (130 g L^{-1}) were the result. No difference was observed in biomass accumulation and lactic acid titers at 25 °C and 30 °C. Detailed fermentation parameters are summarized in Table 3.

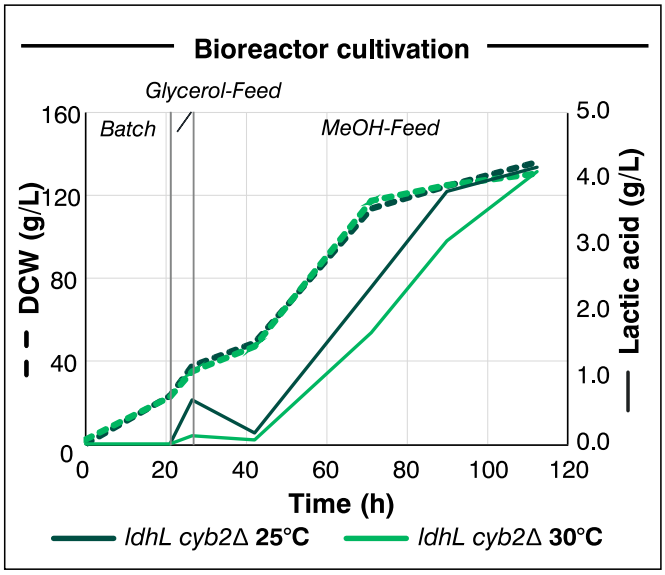

Fig. 3. Performance of the *ldhL cyb2*Δ strain in a MeOH fed-batch cultivation. Clones were previously tested in shake flask cultivations and then transferred to bioreactor cultivations. Lactic acid production was induced by starting the MeOH feed after 26 h of process time. Several samples were taken during the cultivation to determine biomass formation and lactic acid concentrations in the supernatant. Two different process temperatures i.e., 25 °C and 30 °C were tested to analyze if lactate acid productivity shows any differences.

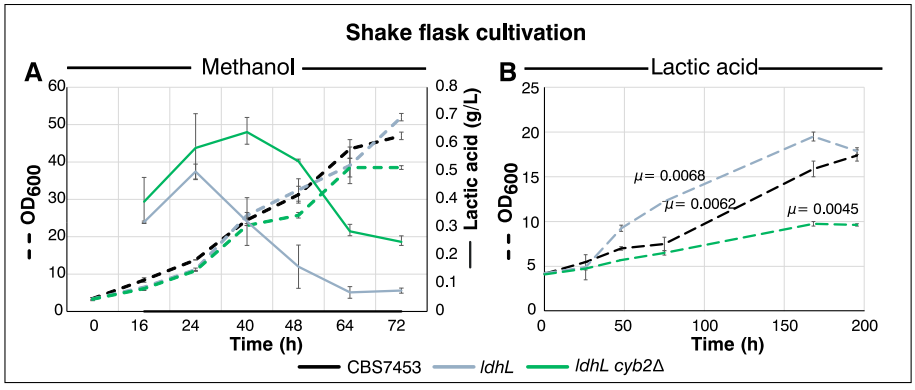

Fig. 2. Loss of Cyb2 results in higher lactic acid titers but does not prevent consumption. (A) Growth and lactic acid profile are shown for CBS7435, *ldhL* and *ldhL cyb2*Δ in shake flask screenings using methanol as the carbon source. Time axis corresponds to the cultivation time. (B) Growth profile is shown for respective strains on lactic acid as the sole carbon source. Experiments were performed in biological duplicates. Mean values and standard deviations are shown.

Table 3
Fermentation parameters.

Strain	Temperature	μ_{YDM} [h^{-1}]	$Y_{P/X}$ [g g^{-1}]	$Y_{P/S}$ [g g^{-1}]	STY [g L^{-1} h^{-1}]
ldhL cyb2Δ	25 °C	0.020	0.010	0.014	0.050
	30 °C	0.020	0.010	0.013	0.050
ldhL cyb2Δ	30 °C	0.029	0.007	0.006	0.020
ldhL cyb2Δ OE:*MXR1*		0.030	0.014	0.013	0.020
ldhL cyb2Δ OE: *MXR1+MIT1*		0.030	0.005	0.007	0.000
ldhL cyb2Δ OE: *MXR1+MIT1 – MeOH accumulation*		0.000	0.110	0.116	0.180
ldhL cyb2Δ OE: *MXR1+MIT1*	30 °C	0.003	0.029	0.081	0.110
ldhL cyb2Δ		0.006	0.004	0.000	0.025

3.3. The presence of lactic acid represses MeOH-utilizing genes

To investigate if lactic acid affects the MUT pathway, we performed another shake flask cultivation in which the wild-type strain CBS7435 was grown and fed continuously with 1% MeOH (v/v). After 55 h, 10 g L^{-1} of sodium lactate was added and served as a second carbon source. In both phases, samples for RNA were taken and analyzed for changes in gene expression of the MeOH utilizing genes *AOX1*, *FDH1,* and *DAS2*. Cells grown solely in lactate served as a negative control. Notably, the strain did not show any altered growth behavior when lactate was added as a second carbon source. HPLC analysis of MeOH and lactate indicated that both carbon sources were metabolized by the cells with no obvious change in the MeOH consumption rate (Fig. 4 A). However, the addition of lactate resulted in a downregulation of all investigated genes. While *AOX1*, *FDH1* and *DAS2* were active during the MeOH feed phase, the addition of lactate reduced the expression of the respective genes to 15–20% of the level when compared to the expression in pure MeOH conditions. As expected, no expression of the MeOH-utilizing genes was detected when solely grown on lactate (Fig. 4 B). This experiment was repeated using lower concentrations of lactic acid, ranging from 0 to 5 g L^{-1}, to mimic the levels produced by the producer strain in the previous shake flask cultivations (Fig. 2) and bioreactor cultivation (Fig. 3). A similar trend was observed as with the previous 10 g L^{-1} concentration, but with a weaker repression of the *AOX1*, *FDH1*, and *DAS2*. While the addition of 0.5 and 1 g L^{-1} lactic acid had only little effect on gene expression, addition of 5 g L^{-1} lactic acid reduced the gene expression

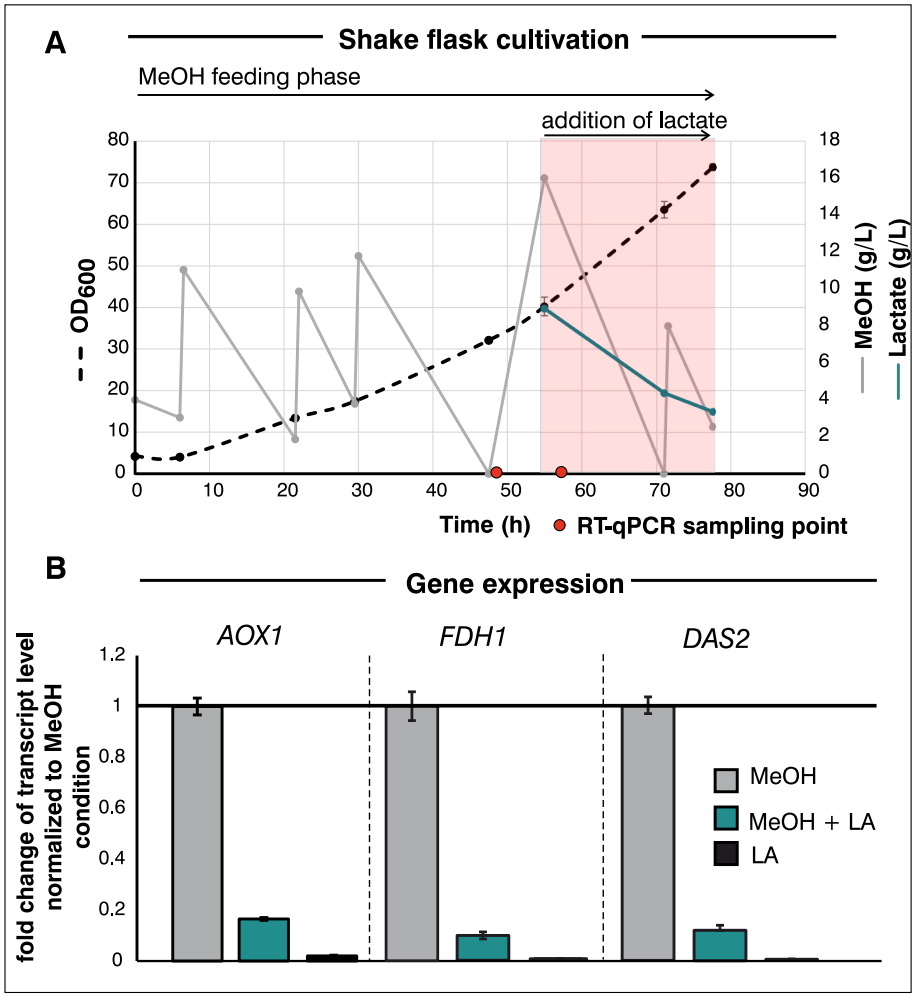

Fig. 4. Effect of MeOH and lactic acid on growth and gene expression of CBS7435. (A) Wild-type CBS7435 cells were grown in YNB and fed continuously with 1% MeOH. After 49 h samples were taken to analyze the gene expression during the MeOH phase. Notably, the cells were fed with MeOH 1 h before to ensure that MeOH is present in the cultures. Subsequently, 10 g L^{-1} sodium lactate (LA) were added to the cultures (55 h) and after 2 h samples for gene expression analysis were taken. Sampling points for RT-qPCR are indicated as red dots on the x-axis. Experiments were performed in duplicates. Mean values and standard deviations are shown. (B) *AOX1*, *FDH1* and *DAS2* were analyzed with RT-qPCR from prior reverse transcribed cDNA. While all of the MeOH utilizing genes are expressed in the presence of MeOH, addition of lactate lead to repression. As a negative control cells cultivated in lactate solely showed no expression of *AOX1*, *FDH1* and *DAS2*. Mean values and standard deviations are shown.

levels to 41–48% (Fig. S5).

3.4. Overexpression of transcription factors leads to higher lactic acid productivity

The MUT pathway undergoes rigorous regulation across multiple layers to guarantee its activation only under specific environmental conditions, i.e., when methanol is present as a carbon source. Transcription factors ensure the timely expression of the respective genes and several of such transcription factors are known for *K. phaffii* (Park et al., 1999; Sahu and Rangarajan, 2016a; Wang et al., 2016b; Shi et al., 2018; Cámara et al., 2019). Additionally, some of them are involved in catabolite repression, thus repressing P_{AOX1} in the presence of glucose or glycerol such as the repressors Nrg1, Mig1-1 and Mig1-2. To analyze if (i) an overrepresentation or loss of transcription factors leads to higher activation of the MUT pathway during lactic acid production and (ii) to see if lactic acid acts as a catabolite repressor, similar to glucose and glycerol, we constructed strains lacking the transcriptional repressors Nrg1, Mig1-1, and Mig1-2, alongside strains overexpressing the transcriptional activators Mxr1 and Mit1 in the *ldhL cyb2*Δ background. The strains were tested in shake flask screenings in YNB with MeOH as the sole carbon source and screened for their growth phenotype and lactic acid production (Fig. 5 A/B). Noteworthy, deletion of the repressors *NRG1*, *MIG1-1*, and *MIG1-2* did not lead to any significant changes in biomass formation or product titers. Contrary to the repressor deficient strains, overexpression strains of *MXR1* and *MXR1*+*MIT1* affected growth and lactic acid production more severely. In detail, overexpression of *MXR1* resulted in a final decrease of OD_{600} reaching only 39 % of parent level, while additional overexpression of *MIT1* further decreased on methanol to 26% when compared to the parent OD_{600} level (Fig. 5 A). Noteworthy, both strains *ldhL cyb2*Δ OE:*MXR1* and *ldhL cyb2*ΔOE *MXR1*+*MIT1* produced higher lactic acid titers, showing an increase of 183 % and 400 % when compared to the parent (Fig. 5 B). To determine if lactic acid affects the expression of *AOX1* in a similar way as shown previously for the CBS7435 wild type strain (Fig. 4), we repeated the shake flask screening using MeOH for the first 48 h as the sole carbon source with subsequent addition of sodium lactate. We observed similar growth phenotypes as before with *ldhL cyb2*ΔOE *MXR1*+*MIT1* showing the most pronounced severity (Fig. 5 C). Interestingly, after the addition of sodium lactate, lactate consumption was decreased in *ldhl cyb2*ΔOE *MXR1* and stagnated in *ldhl cyb2*ΔOE *MXR1*+*MIT1,* while the other transcriptionally engineered strains consumed lactate in a parent-like manner (Fig. 5 D). Analysis of MUT gene expression revealed that the addition of lactate reduced *AOX1* and *FDH1* expression to 50% in the *ldhL cyb2*Δ strain. Loss of the transcriptional repressors Nrg1, Mig1-1 and Mig1-2 showed in general a lower *AOX1* expression under MeOH conditions, however no repression when lactate was added. Surprisingly, *AOX1* expression decreased in *ldhL cyb2*ΔOE:*MIT1,* while parent levels were achieved for *ldhL cyb2*ΔOE:*MXR1* and *ldhL cyb2*ΔOE:*MXR1*+*MIT1* in the presence of MeOH. Addition of lactate did not result in a repression in those strains, contrary, a higher *AOX1* expression was observed for *ldhL cyb2*ΔOE:*MXR1*. Similar results were obtained for *FDH1* expression. Here, presence of lactate reduces expression to 33% in the parent strain *ldhL cyb2*Δ. However, in most of the repressor deficient strains *FDH1* expression was lower when lactate was added. Only the overexpression of *MXR1* and *MXR1*+*MIT1* exhibited similar or higher expression levels as in the MeOH phase. *DAS2* expression showed similar levels for all of the strains in both conditions, thus lactate addition did not affect *DAS2* expression markedly in those strains. Similarly, *ldhL cyb2*ΔOE:*MXR1* showed higher expression after lactate addition (Fig. 5 E). In general, the gene expression data of the biological duplicates showed high standard deviations, suggesting that even in similar experimental conditions, loss or overrepresentation of transcription factors results in significant biological variability within the samples.

3.5. Upscaling of transcriptional activator strains resulted in higher lactic acid concentrations in bioreactor cultivations

Based on the results of the small-scale screening we investigated the best producer strains (*ldhL cyb2*Δ OE: *MXR1* and *ldhL cyb2*Δ OE: *MXR1*+*MIT1)* for their productivity and compared it with the parent strain, *ldhL cyb2*Δ, in a bioreactor cultivation (Fig. 6 A/B and Table 3). The same settings were applied as described previously and a limited MeOH feed of 3.8 g L^{-1} h^{-1} was chosen. All strains accumulated up to 140 g L^{-1} biomass after 90 h process time (Fig. 6 A). The highest titers of lactic acid were produced after 40 h with 5.5 g L^{-1} by the *ldhL cyb2*Δ OE: *MXR1*+*MIT1*, followed by 3.8 g L^{-1} of the *ldhL cyb2*Δ OE:*MXR1* strain. The parent strains *ldhL cyb2*Δ only produced 1.1 g L^{-1} (Fig. 6 B). However, all strains metabolized lactic acid throughout the process with titers ending between 1 and 2 g L^{-1}. Unexpected technical issues lead to excessive methanol accumulation in one of the bioreactors containing the *ldhL cyb2*Δ OE:*MXR1*+*MIT1* (shown by the dashed line, Fig. 6A–B). Here, the MeOH concentrations reached 120 g L^{-1} at the end of the fermentation and severely affected growth and viability of this strain (Fig. S6). In detail, the strain only formed up to 40 g L^{-1} biomass (Fig. 6 A) and viability dropped to 5 percent (Fig. S6). Nevertheless, under these conditions lactic acid titers of up to 12.8 g L^{-1} were achieved, suggesting that a higher MeOH feed can be beneficial for the process (Fig. 6 B). This incident prompted us to test if a higher MeOH feed is beneficial for lactic acid production and thus, we performed shake flask cultivations using either a 2 % or 3 % (v/v) MeOH feed (Fig. 6C–F). In brief, strains were cultured in YNB and after an adaptation period to 0.5 % MeOH overnight, the cells were fed twice daily with either 2 % or 3 % MeOH (v/v). At 2 %, the OD_{600} of *ldhL cyb2*Δ OE:*MXR1* was markedly reduced to 60 %, while *ldhL cyb2*Δ OE:*MXR1*+*MIT1* showed a reduction of 38% when compared to the parent strain at the end of the cultivation (Fig. 6 C). However, both, *ldhL cyb2*Δ OE:*MXR1* and *ldhL cyb2*Δ OE: *MXR1*+*MIT1* produced 1.4 g L^{-1} and 3.1 g L^{-1} lactic acid, respectively, while the parent strain only yielded 0.4 g L^{-1} (Fig. 6 D). The MeOH concentrations ranged from 10 to 20 g L^{-1}, resulting in an actual MeOH feed of 1.25–2.5%. When supplied with a 3% MeOH feed, both transcriptional overexpression strains showed a similar reduction in OD_{600}, that is 56% for *ldhL cyb2*Δ OE:*MXR1* and 31% for *ldhL cyb2*Δ OE: *MXR1*+*MIT1* when compared to parent strain. The parent strain reached a similar OD_{600} when compared to the 2% MeOH feed (Fig. 6 E). In that scenario the *ldhL cyb2*Δ OE:*MXR1* produced 1.9 g L^{-1} lactic acid, whereas *ldhL cyb2*Δ OE:*MXR1*+*MIT1* yielded 1.8 g L^{-1} (Fig. 6 F), suggesting that for the latter strain 3% of MeOH are no longer advantageous for LA production. The MeOH concentrations fluctuated between 20 and 40 g L^{-1}, translating to an actual MeOH feed of 2.5–5% (Fig. 6 E).

3.6. Elevated MeOH levels enhance lactic acid production in bioreactor cultures of transcriptionally overexpressed strains

Overexpression of both transcription factors *MXR1* and *MIT1* in the *ldhL cyb2*Δ background achieved the best lactic acid titers in the previous fed-batch and shake flask cultivations when MeOH concentrations ranged between 1.5 and 2.5 % (Fig. 6). To verify these results, we performed another bioreactor cultivation and compared the *ldhL cyb2*Δ OE: *MXR1*+*MIT1* with the *ldhL cyb2*Δ strain. To mitigate excessive biomass accumulation, as observed in prior experiments we restricted growth by nitrogen limitation and exchanged the base for pH maintenance from 25% NH_3 to 5M KOH. The MeOH feed was set to 2.4 g L^{-1} h^{-1}. As expected the *ldhL cyb2*Δ OE:*MXR1*+*MIT1* strain showed a drastic reduction in biomass formation in comparison to the parent strain, reaching only 44 g L^{-1} instead of 85 g L^{-1}. As for the lactic acid concentrations, peak titers were achieved after 92 h reaching 17 g L^{-1} while the parent strain only yielded 2 g L^{-1}, indicating an 8.5-fold improvement (Fig. 7 A and Table 3). Notably, viability of both strains remained high during the bioreactor cultivation and never dropped below 85% (Fig. S7). To analyze the cells' biomass and lactic acid production over a longer time

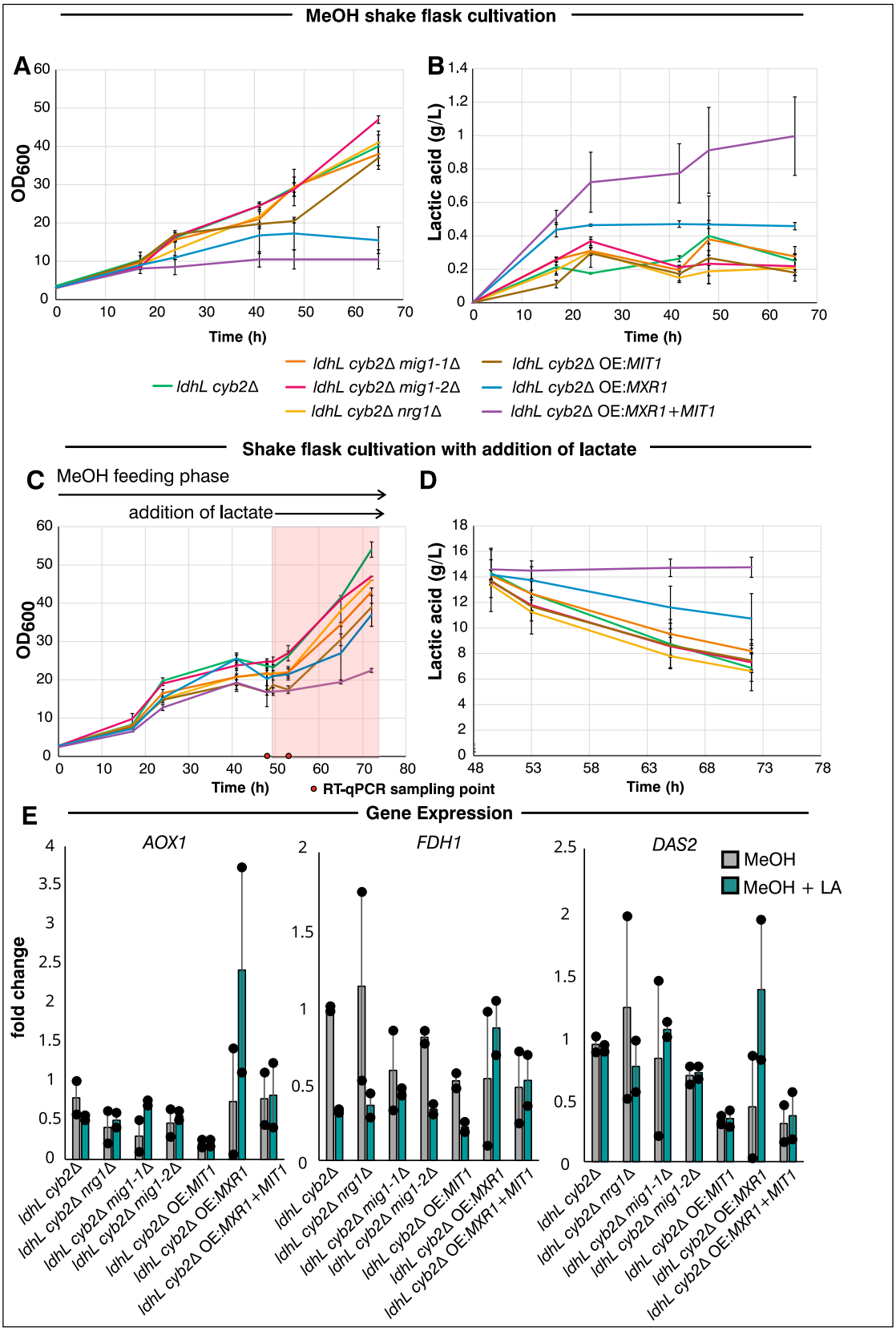

Fig. 5. Engineering of transcription factors leads to elevated lactic acid titers. (A) Growth and (B) lactic acid profile is shown for engineered strains in methanol shake flask cultivations. (C) Growth profile of engineered strains is shown when sodium lactate is added to MeOH shake flask cultivations. RT-qPCR sampling points are indicated as red dots on the x-axis. (D) Consumption of lactic acid is monitored. Time axis corresponds to cultivation time. Experiments were performed in biological duplicates (E) *AOX1*, *FDH1* and *DAS2* were analyzed with RT-qPCR from prior reverse transcribed cDNA. Experiments were done in biological duplicates. Mean values, standard deviations and individual datapoints are shown.

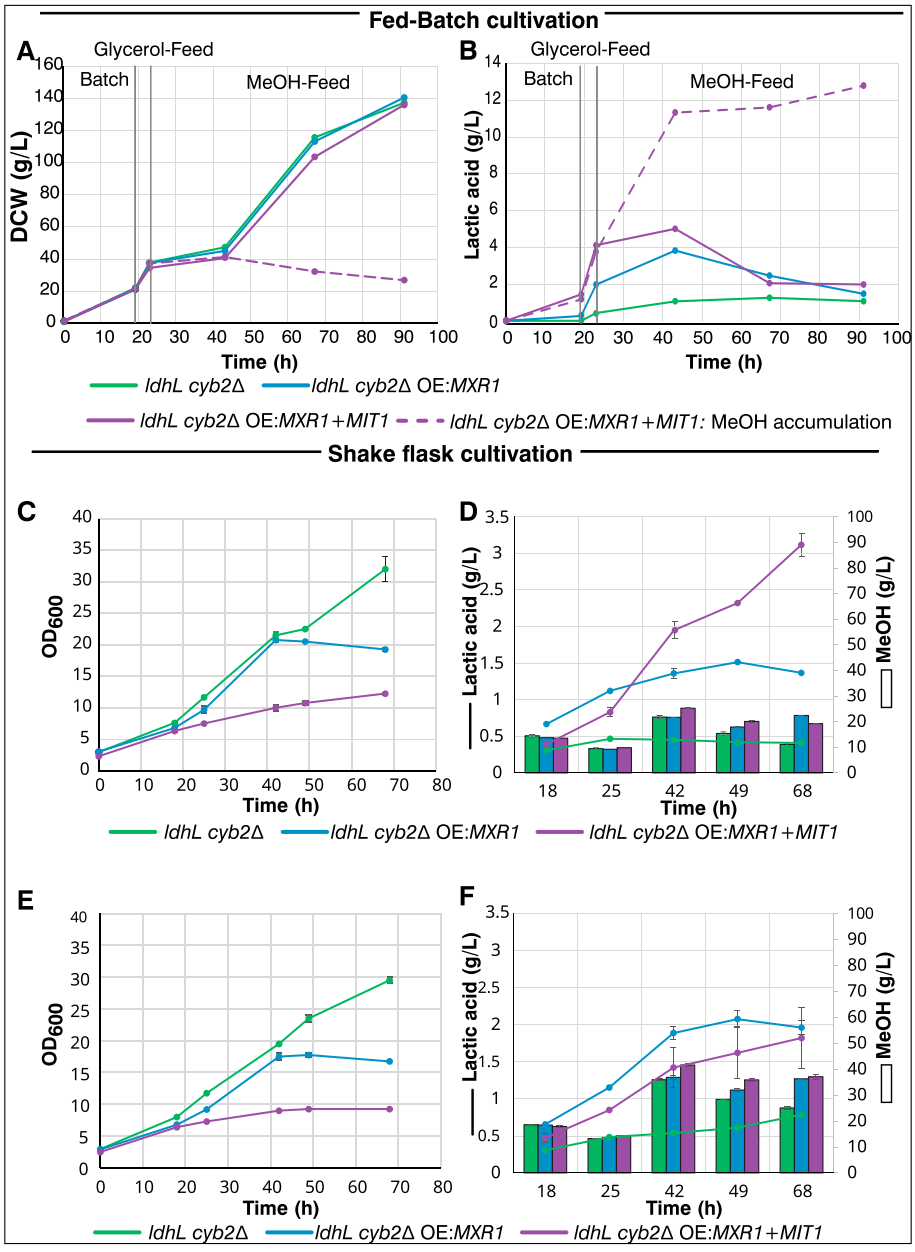

Fig. 6. The effect of increased MeOH concentrations on lactic acid production. (A) Growth and (B) lactic acid profiles of bioreactor cultivation comparing the parent strain (*ldhL cyb2*Δ) with the transcriptional overexpression strains *ldhL cyb2*Δ OE:*MXR*1 and *ldhL cyb2*Δ OE:*MXR1*+*MIT1*. Growth and lactic acid titers were analyzed in shake flask cultivations with (C–D) 2 % MeOH feed and (E–F) 3% MeOH feed. (C–F) Experiments were performed in biological duplicates. Mean values and standard deviations are shown.

period, we extended the experiment to 140 h instead of the previous 100 h. Intriguingly, between 92 and 140 h the *ldhL cyb2*Δ OE:*MXR1*+*MIT1* strain resumed lactate metabolism, causing titers to drop to 15 g L^{-1}. Gene expression analysis of the MeOH-utilizing genes *AOX1* and *FHD1* indicated a 15 to 4-fold higher activity in the *ldhL cyb2*Δ OE:*MXR1*+*MIT1* strain respectively when compared to the parent strain (Fig. 7 B). However, at 115 and 140 h gene activity decreased to the level observed in the parent strain, potentially correlating with the lactate consumption phenotype during this period.

The overexpression of *MXR1* and *MIT1* had a slightly different impact on *DAS2*. At the early fermentation stage, *DAS2* was twofold higher expressed but soon dropped below the parents' level. *DAS2* encodes a dihydroxyacetone synthase responsible for converting formaldehyde to glyceraldehyde 3-phosphate, which is subsequently incorporated into the central carbon metabolism to generate biomass (Hartner and Glieder, 2006; Rußmayer et al., 2015). Thus it might be plausible that the reduced biomass formation in *ldhL cyb2*Δ OE:*MXR1*+*MIT1* correlates with the decreased activity of *DAS2*. The gene expression of *MXR1* was markedly increased in *ldhL cyb2*Δ OE:*MXR1*+*MIT1* during the whole process time, whereas expression of *MIT1* showed a strong increase (up to 40-fold) at the early stage and declined to 10-fold at the end of the process. Nevertheless, the expression was constantly higher than in the parent strain (Fig. 7 B). These differences might emerge from the different promotors used. *MXR1* overexpression was operated by the constitutive P_{GAP} promotor while expression of *MIT1* was regulated via the methanol-inducible P_{FDH1}.

4. Discussion

First studies already demonstrated lactic acid production from MeOH

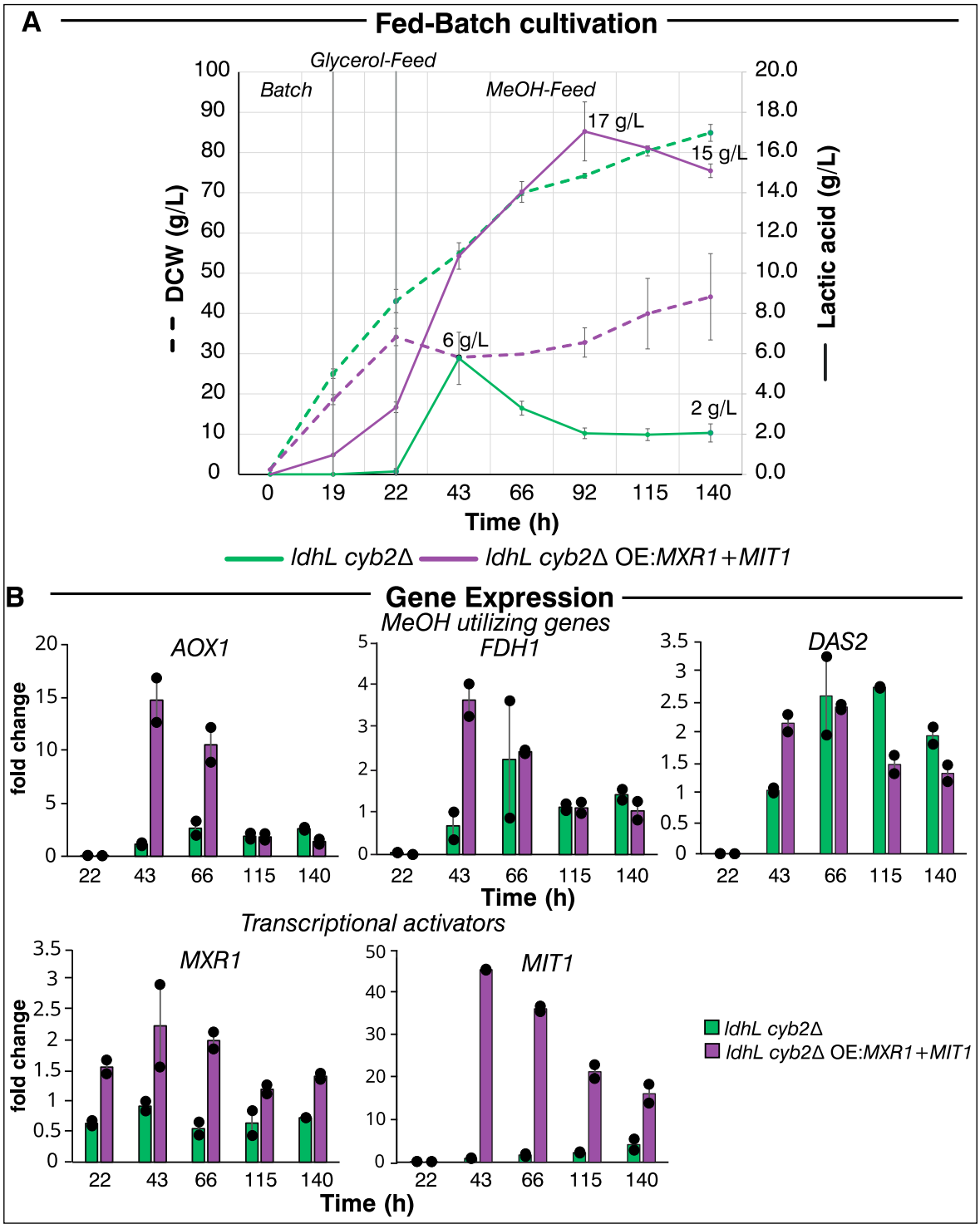

Fig. 7. Process optimization results in high lactic acid titers of *ldhL cyb2*Δ OE:*MXR1*+*MIT1* in bioreactor cultivation. (A) Growth and lactic acid profile were significantly altered in transcriptional overexpression strain. (B) Gene expression analysis of *AOX1, FDH1, DAS2, MXR1* and *MIT1*. For the determination of transcript levels, RNA was extracted and transcribed into cDNA prior to RT-qPCR. The *ldhL cyb2*Δ expression after 43 h was arbitrarily set as 1 for one biological duplicate. Experiments were performed in biological duplicates. Mean values, standard deviations and individual datapoints are shown.

by *Komagataella phaffii* and *Ogataea polymorpha*, reaching D- and L-lactic acid titers of 3.48 g L^{-1} and 3.8 g L^{-1}, respectively (Yamada et al., 2019; Wefelmeier et al., 2023). Also, in this study, we could show that the insertion of LdhL from *L. plantarum* is sufficient to produce L-lactic acid in a similar range (4 g L^{-1}) from MeOH in *K. phaffii.* However, with greater significance, our findings revealed that the presence of lactic acid represses the MUT pathway, consequently leading to the consumption of the product under these conditions.

K. phaffii is recognized for its ability to utilize various carbon sources such as glucose, glycerol, methanol, L-rhamnose, ethanol, succinic acid, lactic acid, acetic acid, mannitol, and sorbitol (Ata et al., 2021). Typically, glucose stands out as the preferred carbon source in yeast, exerting a suppressive effect on the molecular processes related to the utilization of alternative carbon sources (Kayikci and Nielsen, 2015). This

suppression extends to the MUT pathway, specifically targeting P_{AOX1} when glucose, glycerol, or ethanol are available (Hartner and Glieder, 2006). The regulatory cascade orchestrating this repression encompasses multiple factors. In the context of glucose repression, hexose transceptors play a pivotal role, exemplified by Gcr1 in *O. polymorpha* and Hxt in *K. phaffii* (Stasyk et al., 2004; Krasovska et al., 2007; Zhang et al., 2010). Additionally, transcription factors such as Mig1-1, Mig1-2, Nrg1, and Mit1 have been targeted in this regulatory network to engineer methanol-free expression systems (Wang et al., 2017). But transcription factors represent only one regulatory layer in metabolism. Investigations into ethanol repression highlight the significance of downstream products such as acetate and acetyl-CoA in mediating the repression of the MUT pathway (Sahu and Rangarajan, 2016b; Ohsawa et al., 2018). Remarkably, while organic acids have been recognized as mediators of repression in other microbial systems, their role in yeast repression mechanisms remains relatively unexplored. Notably, in rhizobia, C4 organic acids have been implicated in the repression of sugar utilization, with succinic acid serving as a prominent mediator of repression (Iyer et al., 2016). Whether lactic acid acts as a catabolite repressor on its own, or downstream products mimic a potential catabolite repressor in *K. phaffii* has yet to be elucidated and cannot be answered at this point.

Mxr1 and Adr1 are key transcriptional regulators in *K. phaffii* and *S. cerevisiae*, respectively, involved in controlling gene expression in response to specific carbon sources. While Mxr1 predominantly regulates the MUT pathway in *K. phaffii*, Adr1 is known for its role in ethanol, glycerol, and fatty acid metabolism in *S. cerevisiae* (Turcotte et al., 2010). Interestingly, Adr1 also regulates lactate utilization in *S. cerevisiae* by controlling the expression of genes such as *CYB2* and *JEN1* (Young et al., 2003). Although Mxr1 and Adr1 have diverged evolutionarily to adapt to the specific metabolic demands and ecological niches, it is reasonable to speculate that Mxr1 and Adr1 may have similar regulatory roles in their respective species. Both transcription factors are involved in the regulation of genes related to energy and carbon metabolism, and they likely respond to similar metabolic signals. Promoter analysis based on transcription factor binding sites via MatInspector indicated that the *CYB2* promoter harbors a putative binding site for Mxr1 in *K. phaffii* (Figure S8 A). According to (Prielhofer et al., 2015), transcriptomic microarray data of CBS7435 in different cultivation types suggest that indeed *CYB2* is higher expressed in MeOH-limited fed batch than in glucose-limited fed batch cultivations, implying that the presence of MeOH might not only induce MeOH-relevant genes but affects *CYB2* expression as well (Figure S8 B). In contrast, *JEN1* expression is highest under glucose-limited conditions and less expressed in MeOH-limited conditions. If Mxr1 truly binds to *CYB2* or *JEN1* promoters under MeOH conditions needs experimental validation. In our study, we could show that deletion of *CYB2* initially enhances lactic acid production however did not abolish the re-consumption, while overexpressing *JEN1* had no significant impact on lactic acid production.

With the overexpression of both, *MXR1* and *MIT1*, we were able to enhance *AOX1* and *FDH1* expression during lactic acid formation, which led consequently to improved titers in the range of 17 g L^{-1}. Similar strategies to enhance the efficiency of the MUT pathway in the presence of other carbon sources such as glucose and glycerol by deploying transcription factors has been validated by other studies (Chang et al., 2018; Vogl et al., 2018; Haghighi Poodeh et al., 2022). The most prominent target is increasing the efficiency of P_{AOX1} either via overexpression of transcription factors *MXR1*, *MIT1* or *PRM1* (Vogl et al., 2018; Haghighi Poodeh et al., 2022) or by creating a positive feedback loop of Mxr1 to increase protein production in methanol free systems (Chang et al., 2018). In our fermentation experiments, overexpression of *MIT1* and *MXR1* lead initially to a higher expression of *AOX1* and *FDH1* but weakened over time and reached the parent level at the end of the bioreactor cultivation, coinciding with metabolizing lactic acid again as a carbon source. This indicates also that overexpression of *MXR1* and *MIT1* are not sufficient to keep the MUT pathway efficiently active for a longer time period (>140 h). Adaptive laboratory evolution (ALE) might be a promising next step to improve the efficiency of the MUT pathway under high MeOH and lactic acid concentrations and thus overcome repression. However, as lactic acid production *per se* is not beneficial for the cell itself, ALE might compromise production. In general, more studies are needed to fully elucidate the regulatory circuits of the MUT pathway and identify additional factors or approaches that can sustain long-term efficiency in methanol metabolism and lactic acid production.

5. Conclusion

In this study, we demonstrated that the production of lactic acid is not straightforward when using MeOH as a carbon source in *K. phaffii*, as potential catabolite repression from the product itself impacts the methanol utilization pathway. By enhancing the efficiency of the methanol utilization pathway through the simultaneous overexpression of the transcriptional activators *MXR1* and *MIT1*, along with optimizing methanol feeding, we were able to increase titers from 4 g L^{-1} to 17 g L^{-1}. Further research is warranted to explore the potential involvement of organic acids or metabolic downstream products as repressors in regulatory pathways and to elucidate the intricate regulatory mechanisms governing carbon utilization in *K. phaffii*.

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CRediT authorship contribution statement

Simone Bachleitner: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Manja Mølgaard Severinsen:** Methodology, Investigation, Conceptualization. **Gregor Lutz:** Investigation. **Diethard Mattanovich:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data that supports the findings of this study are included in this article and the suppl files. Source data of figures and tables are available at figshare.com (doi 10.6084/m9.figshare.26181083)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2024.07.013.

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