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DOI
10.1016/j.ymben.2023.09.010

Publication date
2023

Document Version
Final published version

Published in
Metabolic Engineering

Metabolic Engineering 80 (2023) 151–162

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Co-cultivation of *Saccharomyces cerevisiae* strains combines advantages of different metabolic engineering strategies for improved ethanol yield

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ARTICLE INFO

Keywords:
Redox-engineering
Synthetic microbial consortia
Acetate reduction
PRK-RuBisCO
Bioethanol
Co-cultures

ABSTRACT

Glycerol is the major organic byproduct of industrial ethanol production with the yeast *Saccharomyces cerevisiae*. Improved ethanol yields have been achieved with engineered *S. cerevisiae* strains in which heterologous pathways replace glycerol formation as the predominant mechanism for anaerobic re-oxidation of surplus NADH generated in biosynthetic reactions. Functional expression of heterologous phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) genes enables yeast cells to couple a net oxidation of NADH to the conversion of glucose to ethanol. In another strategy, NADH-dependent reduction of exogenous acetate to ethanol is enabled by introduction of a heterologous acetylating acetaldehyde dehydrogenase (A-ALD). This study explores potential advantages of co-cultivating engineered PRK-RuBisCO-based and A-ALD-based strains in anaerobic bioreactor batch cultures. Co-cultivation of these strains, which in monocultures showed reduced glycerol yields and improved ethanol yields, strongly reduced the formation of acetaldehyde and acetate, two byproducts that were formed in anaerobic monocultures of a PRK-RuBisCO-based strain. In addition, co-cultures on medium with low acetate-to-glucose ratios that mimicked those in industrial feedstocks completely removed acetate from the medium. Kinetics of co-cultivation processes and glycerol production could be optimized by tuning the relative inoculum sizes of the two strains. Co-cultivation of a PRK-RuBisCO strain with a *Δgpd1 Δgpd2* A-ALD strain, which was unable to grow in the absence of acetate and evolved for faster anaerobic growth in acetate-supplemented batch cultures, further reduced glycerol formation but led to extended fermentation times. These results demonstrate the potential of using defined consortia of engineered *S. cerevisiae* strains for high-yield, minimal-waste ethanol production.

1. Introduction

Saccharomyces cerevisiae is extensively used for industrial production of ethanol, the largest-volume product of industrial biotechnology. Large-scale yeast-based ethanol production predominantly occurs in the United States of America and Brazil, using hydrolyzed corn starch or cane sugar, respectively, as main feedstocks (Renewable Fuels Association). Process configurations that are typically used for conversion of these two feedstocks are usually different. Brazilian sugarcane-based ethanol production processes generally involve yeast biomass recycling, which leads to low average specific growth rates. Since conditions are not aseptic, population dynamics can occur during long-term operation (Della-Bianca and Gombert, 2013). While the low average specific growth rate reduces loss of feedstock to yeast biomass formation, the lack of asepsis complicates introduction of genetically engineered yeast strains (Della-Bianca et al., 2013; Basso et al., 2011). In contrast, ethanol production from hydrolyzed corn starch is typically operated as a batch process, in which each new fermentation run is started with fresh pre-cultures of defined and often genetically engineered yeast strains provided by specialist companies (Ingledew and Lin, 2011; Jacobus et al., 2021). In these corn-starch-based batch processes, growth is fast until ethanol accumulates to inhibitory concentrations and/or nutrients are depleted (Ingram and Buttke, 1985; Russell et al., 2003). Since the concepts that will be discussed in this paper are related to growth-coupled byproduct formation by engineered yeast strains, we will discuss them in the context of corn-based ethanol production.

Anaerobic fermentation of glucose by *S. cerevisiae* starts by its oxidation to pyruvate via the Embden-Meyerhof glycolysis, yielding ATP

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https://doi.org/10.1016/j.ymben.2023.09.010
Received 9 June 2023; Received in revised form 14 September 2023; Accepted 15 September 2023
Available online 24 September 2023

and NADH (Embden et al., 1933; Cori, 1983). Reduction of pyruvate by pyruvate decarboxylase and NAD^+-dependent alcohol dehydrogenase re-oxidizes the NADH formed in glycolysis and completes the conversion of sugars into ethanol and carbon dioxide (de Smidt et al., 2008). During ethanol production with *S. cerevisiae* in corn-starch-based fermentation processes, product yields of up to 89% of the theoretical maximum can be reached (Brown and Wright, 2009), with yeast biomass and glycerol as the major organic byproducts (Nissen et al., 2000a, 2000b). In *S. cerevisiae*, formation of these byproducts is coupled via redox-cofactor balances because biomass synthesis leads to a net reduction of NAD^+ to NADH. In anaerobic cultures, this 'surplus' NADH can neither be re-oxidized by mitochondrial respiration nor by the redox-cofactor-balanced pathway for ethanol production. Instead, anaerobic yeast cultures rely on the NADH-dependent reduction of the glycolytic intermediate dihydroxyacetone-phosphate to glycerol-3-phosphate (Bakker et al., 2001; Verduyn et al., 1990). This reaction is catalyzed by Gpd1 and Gpd2 (Eriksson et al., 1995; Albertyn et al., 1994) and followed by the hydrolysis of glycerol-3-phosphate to glycerol and phosphate by Gpp1 and Gpp2 (Norbeck et al., 1996). As approximately 4% of the potential ethanol yield in industrial processes is estimated to be lost to glycerol (Nissen et al., 2000b), multiple metabolic engineering strategies have focused on reducing glycerol formation by engineering of yeast redox metabolism (van Aalst et al., 2022a).

Expression in *S. cerevisiae* of a *Spinacia oleracea PRK* gene encoding phosphoribulokinase (PRK) and *Thiobacillus denitrificans cbbM* encoding ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), along with *Escherichia coli* genes encoding the chaperonins GroES and GroEL, enables a bypass of glycolysis (Guadalupe-Medina et al., 2013; Papapetridis et al., 2018). When ribulose-5-phosphate is generated via the native non-oxidative pentose pathway, this bypass, based on introduction of the two abovementioned Calvin-cycle enzymes, enables redox-cofactor neutral formation of 3-phosphoglycerate from sugars (Fig. 1). This concept has been implemented and optimized to construct fast-growing, low-glycerol-producing *S. cerevisiae* strains with an up to 10% higher ethanol yield on glucose in fast-growing anaerobic laboratory cultures (0.29 h^{-1}; (Papapetridis et al., 2018)). Due to the coupling of glycerol formation and biomass formation, the predicted impact of this metabolic engineering strategy on ethanol yield strongly correlates with specific growth rate (van Aalst et al., 2022a).

In industrial corn-starch-based alcoholic fermentation, an initial phase of fast growth is followed by a deceleration phase caused by ethanol inhibition and/or depletion of non-sugar nutrients (Ingledew et al., 2017). A recent study showed that, in slow-growing anaerobic chemostat cultures (0.05 h^{-1}), a PRK-RuBisCO strain produced up to 80-fold more acetaldehyde and 30-fold more acetate than a reference strain (van Aalst et al., 2023a). This production of acetaldehyde and acetate was attributed to an *in vivo* overcapacity of the key enzymes of the PRK-RuBisCO bypass. Reduction of the copy number of the expression cassette for RuBisCO led to lower acetaldehyde and acetate production in slow-growing cultures and a corresponding increase in ethanol yield (van Aalst et al., 2023a). The production of acetaldehyde and acetate was further decreased by reducing PRK activity by lowering protein abundance by a C-terminal extension of PRK, or by expressing the spinach *PRK* gene from the growth-rate-dependent *ANB1* promoter (van Aalst et al., 2023a; Mehta et al., 1990). However, the resulting strains still showed trade-offs in performance at low and high specific

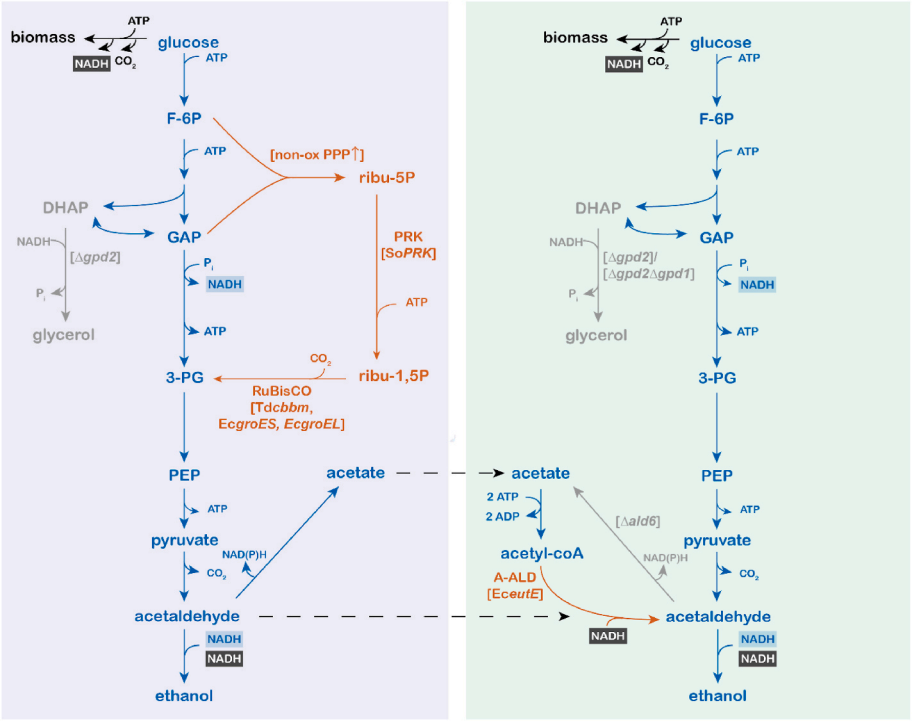

Fig. 1. Simplified schematic representation of ethanol and biomass formation from glucose and acetate by two engineered strains of *Saccharomyces cerevisiae* by heterologous expression of genes encoding the enzymes phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and acetylating acetaldehyde dehydrogenase (A-ALD). Genetic modifications are indicated between square brackets. Blue: native reactions including the redox-neutral conversion of glucose to ethanol via glycolysis and alcoholic fermentation, the NAD(P)H-dependent conversion of acetaldehyde to acetate via acetaldehyde dehydrogenase and the conversion of acetate into acetyl-coA via acetyl-coA synthetase. Black: biosynthetic reactions with a net input of ATP and a net production of CO_2 and NADH. Orange: heterologous reduction pathways for NADH recycling either via a non-oxidative bypass of glycolysis via PRK-RuBisCO (left) or via acetyl-coA reduction via A-ALD (right) using exogenous acetate or acetate and acetaldehyde produced by the PRK-RuBisCO strain. Grey: native pathways which are (partially) impaired by a knock-out of a gene required in this route; glycerol reduction pathway and acetaldehyde dehydrogenase pathway. Heterologous genes encode the following enzymes: Ec*eutE*, *E. coli* A-ALD; So*PRK*, *S. oleracea* PRK; Td*cbbM*, *T. denitrificans* RuBisCO; Ec*groEL* and Ec*groES*, *E. coli* GroEL and GroES, respectively. Abbreviations indicate the following metabolites: F–6P, fructose-6-phosphate; GAP, glyceraldehyde-3-P; DHAP, dihydroxyacetone phosphate, 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; ribu-5P, ribuolose-5-phosphate; ribu-1,5 P, ribulose-1,5-bisphosphate.

growth rates, which illustrated the challenges involved in tuning the activity of engineered pathways under dynamic conditions.

An alternative redox-engineering strategy is based on the expression of a heterologous gene encoding acetylating acetaldehyde dehydrogenase (A-ALD). Together with native acetyl-CoA synthetase and alcohol dehydrogenase, A-ALD can catalyse the NADH-dependent reduction of exogenous acetate to ethanol (Guadalupe-Medina et al., 2010). In the presence of acetate, anaerobic cultures of engineered A-ALD-expressing strains carrying a deletion in *GPD2*, which encodes one of the two *S. cerevisiae* isoenzymes of glycerol-3-phosphate dehydrogenase, show reduced glycerol yields and improved ethanol yields on glucose (Papapetridis et al., 2017). When *GPD1* and *GPD2* are both deleted, A-ALD strains can even grow anaerobically in the presence of acetate without producing glycerol (Guadalupe-Medina et al., 2010; Papapetridis et al., 2016). However, since glycerol plays a key role in osmotolerance of *S. cerevisiae* (Ansell et al., 1997; Blomberg and Adler, 1992; Nevoigt and Stahl, 1997), such strains are highly osmosensitive and metabolic and evolutionary engineering studies have only partially restored their osmotolerance (Guadalupe-Medina et al., 2014; Guo et al., 2011). Complete elimination of glycerol formation is therefore not compatible with industrial process conditions.

The presence of acetate in corn-starch hydrolysates, at concentrations of up to 20 mmol L^{-1}, is generally attributed to bacterial contamination, in particular by lactic acid bacteria (Russell et al., 2003; Rasmussen et al., 2015; Phibro Animal Health Corporation; van Zuyl et al., 2017). This concentration of acetate is, however, insufficient to reach the same ethanol yield improvement with acetate-reducing strains as with PRK-RuBisCO strains (van Aalst et al., 2023b).

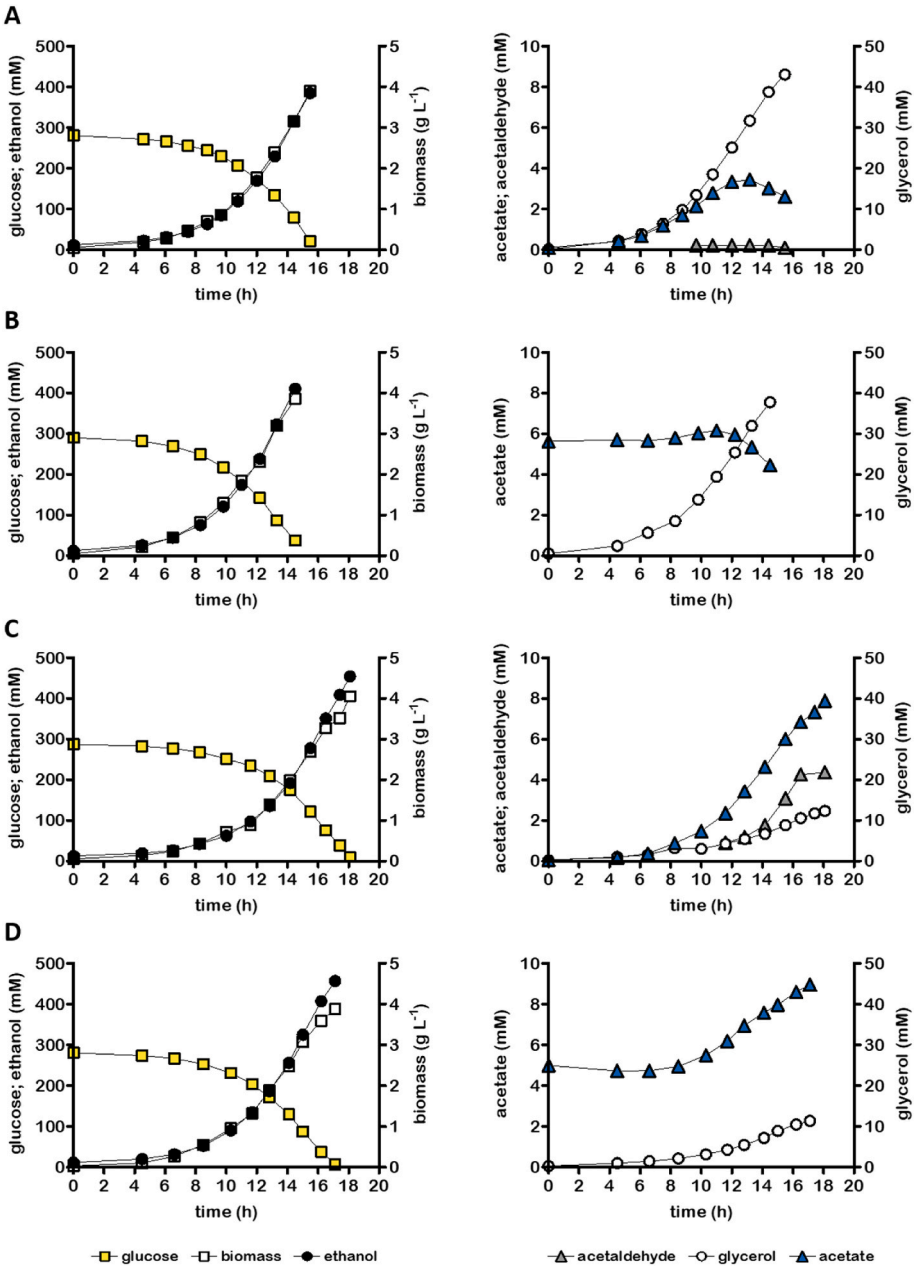

Fig. 2. Growth, glucose consumption and product formation of anaerobic bioreactor batch cultures of individual *S. cerevisiae* strains, grown on SM with 50 g L^{-1} glucose (panels A and C) or on SM with 50 g L^{-1} glucose and 5 mmol L^{-1} acetate (panels B and D). Panels show data for *S. cerevisiae* strains IME324 (reference strain, A and B) and IMX2736 (*Δgpd2* non-ox PPP↑ *PRK* 2x *cbbm groES/groEL*, C and D). Non-ox PPP↑ indicates integration of the overexpression cassettes for *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1* and *TKL2*. Representative cultures of independent duplicate experiments are shown, the corresponding replicate of each culture is shown in Fig. S2. Data on strain IME324 on SM with 50 g L^{-1} glucose and 5 mmol L^{-1} acetate were taken from (van Aalst et al., 2023b).

Defined microbial consortia are already used as starter cultures in food fermentation (Buckenhüskes, 1993) and there is a growing interest in the potential advantages of this approach in industrial biotechnology (Roell et al., 2019; Zhou et al., 2015; Shong et al., 2012). Co-cultivation of PRK-RuBisCO and A-ALD-based strains may, in theory, prevent accumulation of acetaldehyde and acetate generated by PRK-RuBisCO strains while limiting reduced fermentation rates and increased glycerol yields of A-ALD-based strains upon depletion of acetate in growth media (Fig. 1). To evaluate this strategy, the present study explores co-cultivation of PRK-RuBisCO and A-ALD-based strains in anaerobic batch cultures on glucose, grown in the presence and absence of acetate. In addition to an A-ALD-containing strain in which only *GPD2* is deleted and which can therefore still grow in the absence of exogenous acetaldehyde or acetate, co-cultures with an A-ALD strain lacking both glycerol-3-phosphate dehydrogenase isoenzymes are investigated.

2. Results

2.1. Acetaldehyde and acetate as byproducts of PRK-RuBisCO-containing *S. cerevisiae* strains

In previous studies, growth and product formation by PRK-RuBisCO-containing *S. cerevisiae* strains were studied in anaerobic bioreactor batch cultures on 20 g L^{-1} glucose, in which fast exponential growth continues until glucose is almost completely consumed (Papapetridis et al., 2018; van Aalst et al., 2023a). To capture some of the dynamics in large-scale processes, anaerobic bioreactor batch cultures of the reference strain IME324 and the PRK-RuBisCO-based strain IMX2736 (*Δgpd2* non-ox PPP↑ *PRK* 2x *cbbm groES groEL*; non-ox PPP↑ indicates integration of the overexpression cassettes for the non-oxidative pentose phosphate pathway genes *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1* and *TKL2*; (van Aalst et al., 2023a)) were grown on synthetic medium with 50 g L^{-1} glucose. In these cultures, both strains showed an initial exponential growth phase with a specific growth rate of 0.3 h^{-1}, after which the growth rate gradually declined to approximately 0.15 h^{-1} (Fig. 2, Fig. S1). These growth dynamics mimicked those in industrial fermentation processes for ethanol production from corn starch hydrolysates (Russell et al., 2003; Ingledew et al., 2017) and may reflect ethanol inhibition. Due to these changing growth rates, stoichiometries of glucose consumption and product formation could not be assumed constant throughout batch cultivation. Overall stoichiometries were therefore calculated from measurements on the first and final two time points of cultivation experiments.

Consistent with previous studies on PRK-RuBisCO-based *S. cerevisiae* strains, the glycerol yield on glucose of strain IMX2736, grown anaerobically on 50 g L^{-1} glucose, was 73% lower than that of the reference strain, while its ethanol yield on glucose was 7% higher ($p = 0.037$, Table 1). In these cultures of strain IMX2736, yields of the byproducts acetate and acetaldehyde on glucose were 0.030 and 0.018 mol (mol glucose)$^{-1}$, respectively (Table 1). This acetaldehyde yield was two-fold higher than reported for anaerobic cultures of strain IMX2736 on 20 g L^{-1} glucose, in which the specific growth rate remained 0.3 h^{-1} throughout batch cultivation (van Aalst et al., 2023a). Since acetaldehyde production by anaerobic chemostat cultures of PRK-RuBisCO-based strains increases at low specific growth rates (van Aalst et al., 2023a), the higher acetaldehyde yield of batch cultures grown on 50 g L^{-1} glucose was attributed to their declining specific growth rate.

In earlier batch experiments with PRK-RuBisCO-based *S. cerevisiae* strains grown on 20 g L^{-1} glucose, biomass yields on glucose were approximately 5% higher than in cultures of reference strains (Papapetridis et al., 2018; van Aalst et al., 2023a). Those observations were consistent with lower ATP costs for NADH regeneration via the RuBisCO bypass than via the native glycerol pathway (van Aalst et al., 2022a). In cultures grown on 50 g L^{-1} glucose, biomass yields of strains IME324 and IMX2736 were not significantly different. Absence of a higher biomass yield of strain IMX2736 in these cultures may be related to the accumulation of 4.1 ± 0.4 mmol L^{-1} acetaldehyde and 7.8 ± 0.1 mmol L^{-1} acetate in the culture broth. Combined, formation of these metabolites already accounted for a loss of ca. 6 mmol L^{-1} glucose, while loss of acetaldehyde via the gas phase (Guan et al., 2012), toxicity effects of acetaldehyde and weak-acid uncoupling by acetate may affect biomass yield even further (Narendranath et al., 2001; Stanley et al., 1993; Stanley and Pamment, 1993).

Table 1
Key physiological parameters of anaerobic bioreactor batch cultures of *S. cerevisiae* strains IME324 (reference), IMX2736 (*Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm*, *groES, groEL*), IMX2503 (*Δgpd2 Δald6 eutE*) and IMS1247 *(Δgpd1 Δgpd2 Δald6 eutE*, evolved). Non-ox PPP↑ indicates integration of the overexpression cassettes for *RPE1, TKL1, TAL1, NQM1, RKI1 and TKL2*. Cultures were grown on synthetic medium with 50 g L^{-1} of glucose, with or without addition of 5 mM acetate, at pH 5 and at 30 °C and sparged with a 90:10 mixture of N_2 and CO_2. Y indicates yield, subscript x denotes biomass. Acetate and acetaldehyde concentrations indicate values in the culture broth, measured at the end of the cultivation experiments. Negative acetate yields indicate net acetate consumption and were calculated from data derived from sampling points before acetate depletion. Yields were calculated using the average of the first two and last two sampling points. Degree-of-reduction balances (Roels, 1980) were used to verify data consistency. Values represent averages ± mean deviations of measurements on independent duplicate cultures for each combination of strain and medium. n.d., not determined.

Strain name Relevant genotype	IME324 reference		IMX2736 *Δgpd2 prk* 2x *cbbm*		IMX2503 *Δgpd2 Δald6 eutE*	IMS1247 *Δgpd1 Δgpd2 Δald6 eutE*
Initial acetate concentration	0 mM	5 mM[a]	0 mM	5 mM	5 mM[a]	5 mM
$Y_{biomass/glucose}$ (g_x g^{-1})	0.084 ± 0.000	0.084 ± 0.000	0.082 ± 0.005	0.081 ± 0.001	0.085 ± 0.000	0.100 ± 0.001
$Y_{ethanol/glucose}$ (mol mol^{-1})	1.49 ± 0.02	1.55 ± 0.01	1.60 ± 0.00	1.64 ± 0.01	1.58 ± 0.00	1.78 ± 0.02
$Y_{acetaldehyde/glucose}$ (mol mol^{-1})	<0.001	n.d.	0.018 ± 0.001	n.d.	n.d.	n.d.
$Y_{glycerol/glucose}$ (mol mol^{-1})	0.169 ± 0.006	0.150 ± 0.000	0.046 ± 0.002	0.041 ± 0.001	0.125 ± 0.000	<0.001
$Y_{acetate/glucose}$ (mol mol^{-1})	0.012 ± 0.002	−0.004 ± 0.002	0.030 ± 0.002	0.017 ± 0.002	−0.064 ± 0.003	−0.100 ± 0.004
mmol glycerol per g_x	11.2 ± 0.3	9.9 ± 0.0	3.1 ± 0.0	2.8 ± 0.1	8.2 ± 0.0	0.1 ± 0.0
Final concentration acetaldehyde (mM)	0.1 ± 0.0	n.d.	4.1 ± 0.4	n.d.	n.d.	n.d.
Final concentration acetate (mM)	2.8 ± 0.3	4.4 ± 0.1	7.8 ± 0.1	9.3 ± 0.5	<0.01	<0.01
Electron recoveries	99–101	99–99	98–98	99–100	99–99	101–102

[a] Data taken from (van Aalst et al., 2023b).

2.2. Anaerobic co-cultivation of PRK-RuBisCO-based and A-ALD-based *S. cerevisiae* strains on acetate-containing medium

First-generation feedstocks for ethanol production such as corn mash can contain up to 20 mmol L^{-1} acetate (Russell et al., 2003; Rasmussen et al., 2015; Phibro Animal Health Corporation) while glucose concentrations can reach 300 g L^{-1} (Kumar et al., 2020; Secches et al., 2022). To mimic these acetate-to-glucose ratios, anaerobic bioreactor batch cultures were grown on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate (Table 1, Fig. 2). Under these conditions, the reference strain IME324 consumed approximately 1.6 mmol L^{-1} acetate (Table 1), probably reflecting its conversion to the biosynthetic precursor molecule acetyl-Coenzyme A (Flikweert et al., 1999). In contrast, the PRK-RuBisCO-containing strain IMX2736 produced 2.8 mmol L^{-1} acetate. In these acetate-supplemented anaerobic cultures, the PRK-RuBisCO strain showed a 2.4% higher ethanol yield than in cultures grown without acetate supplementation ($p = 0.011$, Table 1), which may reflect an increased ATP demand caused by mild weak-acid uncoupling by acetate (Narendranath et al., 2001).

Introduction of a heterologous acetylating acetaldehyde dehydrogenase (A-ALD) enables anaerobic *S. cerevisiae* cultures to re-oxidize 'surplus' NADH from biosynthetic reactions by NADH-dependent reduction of exogenous acetate to ethanol (Guadalupe-Medina et al., 2010; van Aalst et al., 2023b). In anaerobic batch cultures on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate, the A-ALD-containing strain IMX2503 (*Δgpd2 Δald6 eutE*; (van Aalst et al., 2023b)), had already converted all acetate after 12 h, when 67% of the glucose was still available (Fig. 4A). Moreover, the rate of acetate consumption already declined before this time point (Fig. 4A). This deceleration is likely to reflect Monod kinetics for acetate consumption, possibly influenced by the high K_m of Acs2 (ca. 8.8 mM; (de Jong-Gubbels et al., 1997)). Consequently, over the whole process, glycerol formation via Gpd1 was the predominant mechanism for reoxidizing 'surplus' NADH in strain IMX2503 and its glycerol yield on glucose of was only 17% lower than that of the reference strain IME324 ($p < 0.001$, Table 1).

The PRK-RuBisCO strain IMX2736 (*Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm, groES, groEL*; (van Aalst et al., 2023a)) does not depend on acetate for glycerol-independent NADH cofactor balancing and its byproducts acetate and acetaldehyde could potentially be used as electron acceptors for the A-ALD-strain IMX2503 (Fig. 1). We therefore investigated whether co-cultivation of these two strains could combine low-glycerol fermentation with complete conversion of acetate. Two unique single-nucleotide polymorphisms (SNPs) in strain IMX2736, on Chromosome 11 (location 331,347) and Chromosome 15 (location 912,014), allowed for estimation of the cell ratio of strains IMX2503 and IMX2736 in co-cultures by counting reads containing and lacking these SNPs in whole-genome sequence data (Fig. 3).

When strains IMX2503 (A-ALD) and IMX2736 (PRK-RuBisCO) were inoculated at a ratio of 1.4:1 (IMX2503:IMX2736) in medium containing 50 g L^{-1} glucose and 5 mmol L^{-1} acetate, acetate was completely consumed when, after 14 h, ca. 50% of the glucose was still unused (Fig. 4B). When the initial abundance of the A-ALD-based strain was decreased by changing the inoculum ratio to 0.8:1 (IMX2503:IMX2736), complete consumption of glucose and acetate almost coincided (Fig. 4C). As a consequence, acetate limitation of A-ALD strain was delayed and the glycerol yield of the co-culture on glucose was 20% lower than at the higher inoculum ratio ($p < 0.001$, Table 2). Glycerol yields in co-cultures grown at inoculum ratios of 0.8 and 1.4 were 49% and 34% lower than in corresponding monocultures of the reference strain IME324 (Tables 1 and 2). Ethanol yields of the consortium cultures with an inoculum ratio of 0.8 were not significantly different from that of a monoculture of the PRK-RuBisCO strain IMX2736 and 6% higher than that of monocultures of the reference strain IME324 ($p = 0.015$, Tables 1 and 2). Biomass yields on glucose of these co-cultures were 6.7% higher than those of a monoculture of the PRK-RuBisCO strain IMX2736 grown on the same medium ($p = 0.002$, Tables 1 and 2). The higher biomass yield of the consortium cultures is likely to reflect consumption of acetaldehyde and acetate by the A-ALD strain.

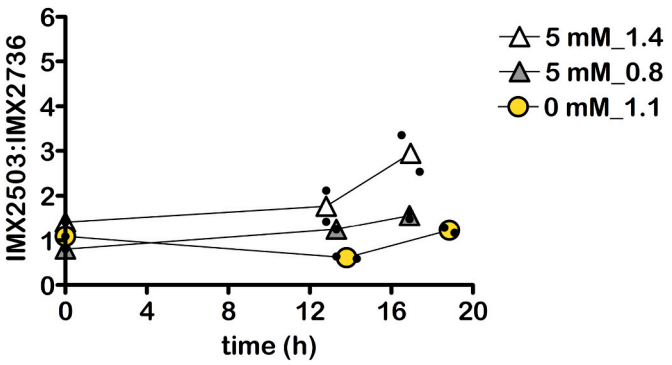

Fig. 3. Ratio of *S. cerevisiae* strains IMX2503 (*Δgpd2 Δald6 eutE*) relative to IMX2736 (*Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm*, *groES, groEL*) in anaerobic bioreactor batch co-cultures on synthetic medium containing 50 g L^{-1} glucose, with or without the addition of 5 mM acetate (indicated in the Figure as either 5 mM or 0 mM). Ratio was calculated based on whole genome sequencing. Non-ox PPP↑ indicates the integration of the overexpression cassettes for *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1* and *TKL2*. Values represent means and individual values of measurements on independent batch duplicate cultures. Cultures were inoculated at ratios of strain IMX2503 relative to strain IMX2736 of 1.4, 0.8 or 1.1 as indicated in the Figure.

2.3. Anaerobic co-cultivation of A-ALD-based and RuBisCO-based strains on glucose

In anaerobic batch cultures grown on glucose, the PRK-RuBisCO-based strain IMX2736 produced acetaldehyde and acetate as byproducts (Table 1, Fig. 2). To investigate whether co-cultivation with an A-ALD-based strain could reduce or eliminate this undesirable byproduct formation, anaerobic batch cultures of strains IMX2736 and IMX2503 were grown on glucose (50 g L^{-1}) as sole carbon source. In these cultures, which were grown with an inoculum ratio of 1.1:1 (IMX2503:IMX2736), strain IMX2503 (*Δgpd2 Δald6 eutE*) can only use acetate and acetaldehyde generated by strain IMX2736. Yields of acetate and acetaldehyde were 84% and 72% lower, respectively, than in corresponding monocultures of the RuBisCO-based strain IMX2736 (Tables 1 and 2). Co-cultivation did not extend the fermentation time relative to monocultures of strain IMX2736, while the ethanol yield of the consortium was 1.5% higher than that of monocultures of strain IMX2736 and 8.8% higher than that of monocultures of the reference strain IME324 ($p = 0.026$ and $p = 0.034$, respectively, Figs. 2 and 4, Tables 1 and 2). The glycerol yield of these co-cultures was 58% lower than that of monocultures of the reference strain IME324, but 55% higher than that of monocultures of the PRK-RuBisCO-containing strain IMX2736 (Figs. 2 and 4, Tables 1 and 2).

In contrast to co-cultures of strains IMX2736 and IMX2503 on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate, the co-culture on 50 g L^{-1} glucose still produced some acetate and acetaldehyde (Table 2, Fig. 3). A dependency of the A-ALD strain IMX2503 on acetate for fast growth (Nissen et al., 2000b; van Aalst et al., 2023b) was reflected by a decrease of its relative abundance in the mixed culture during the first phase of batch cultivation on 50 g L^{-1} glucose (Fig. 3). Such a decrease was not observed in co-cultures of these strains that, with a similar inoculum ratio of the two strains, were grown on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate (Fig. 3).

2.4. Additional deletion of GPD1 in A-ALD-based strain prevents glycerol production in the absence of acetate

The A-ALD expressing strain IMX2503 (*Δgpd2 Δald6 eutE*) retains a functional *GPD1* gene and therefore, albeit slower than the reference

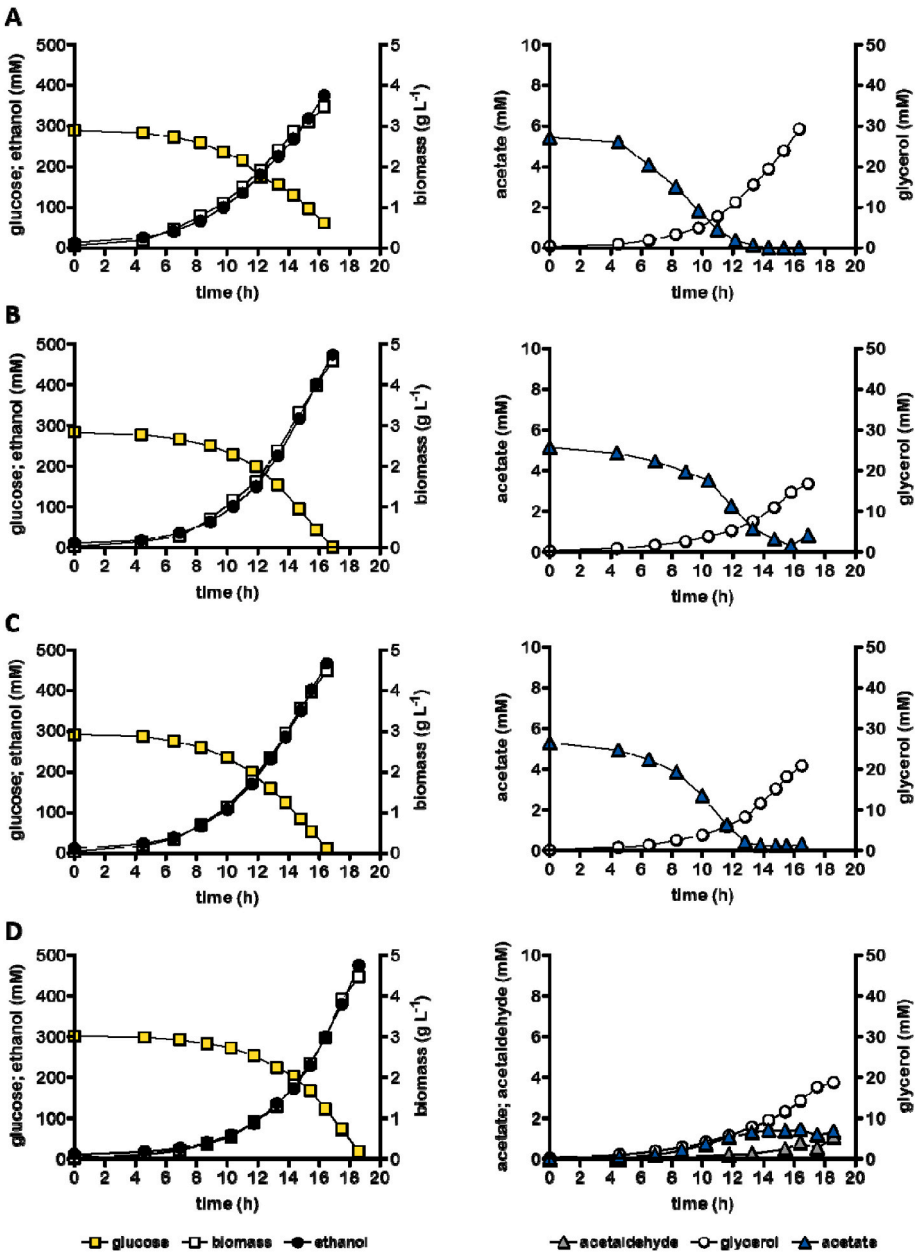

Fig. 4. Growth, glucose consumption and product formation of anaerobic bioreactor batch cultures of *S. cerevisiae* strain IMX2503 (*Δgpd2 Δald6 eutE*) (A) and co-cultures of IMX2503 and IMX2736 *(Δgpd2, non-ox PPP↑,* PRK, *2x cbbm, groES, groEL)*. Non-ox PPP↑ indicates integration of the overexpression cassettes for *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1* and *TKL2*. Cultures were grown on synthetic medium containing 50 g L^{-1} glucose (panel D) or 50 g L^{-1} glucose and 5 mmol L^{-1} acetate (panels A–C) and were inoculated at a ratio of 1.42 ± 0.19 (B), 0.80 ± 0.11 (C) or 1.09 ± 0.02 (D) (inoculum ratio was estimated based on whole genome sequencing). Representative cultures of independent duplicate experiments are shown, the corresponding replicate of each culture is shown in Fig. S3. Data on monocultures of strain IMX2503 were taken from (van Aalst et al., 2023b).

strain (Nissen et al., 2000b; van Aalst et al., 2023b) can grow in the absence of acetate. Therefore, when the inoculum of co-cultures with the RuBisCO strain IMX2736 contained a high fraction of strain IMX2503 and acetate was consumed before glucose was exhausted, the co-culture displayed a higher glycerol yield (Fig. 4; Table 2). Ideally, depletion of acetate should not lead to enhanced glycerol formation, since this goes at the expense of ethanol yield. Avoiding this requires that the A-ALD strain stops growing when acetate is depleted. We therefore constructed the A-ALD strain IMX2744 (*Δgpd1 Δgpd2 Δald6 eutE*) whose anaerobic growth, due to the elimination of both glycerol-3-phosphate-dehydrogenase isoenzymes, depended on external supply of acetate or acetaldehyde. We decided to not also delete *GPD1* in strain IMX2736 because of the role of glycerol in osmotolerance of *S. cerevisiae* (Blomberg and Adler, 1992; Nevoigt and Stahl, 1997; Holst et al., 2000). In this way, glycerol can still be produced in co-cultures of strains IMS1247 and IMX2736.

As reported for previously constructed *Δgpd1 Δgpd2 eutE* strains (Papapetridis et al., 2016), strain IMX2744 showed a suboptimal specific growth rate in anaerobic cultures grown on glucose and acetate (Fig. S4). A faster-growing single-cell isolate, IMS1247, was obtained by adaptive laboratory evolution of strain IMX2744 (initial specific growth rate of ca. 0.20 h^{-1}) in sequential batch reactors on 50 g glucose L^{-1} and 17 mmol L^{-1} acetate (Fig. S4).

In anaerobic batch cultures on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate, the evolved strain IMS1247 still grew slower than strain IMX2503 (*Δgpd2 Δald6 eutE*) (Figs. 5A and 4A, initial specific growth

Table 2
Key physiological parameters of anaerobic bioreactor batch co-cultures of *S. cerevisiae* strains IMX2736 (*Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm*, *groES, groEL*) and IMX2503 (*Δgpd2 Δald6 eutE*). Non-ox PPP↑ indicates integration of the overexpression cassettes for *RPE1, TKL1, TAL1, NQM1, RKI1 and TKL2*. Inoculum ratios of the two strains were calculated by genome sequencing. Cultures were grown on synthetic medium with 50 g L^{-1} glucose, with or without addition of 5 mM acetate. Y indicates yield, subscript x denotes biomass. Acetate and acetaldehyde concentrations indicate values in the culture broth, measured at the end of the cultivation experiments. Yields were calculated using the average of the first two and last two sampling points. Negative acetate yields indicate net acetate consumption and were calculated from data derived from sampling points before acetate depletion. Degree-of-reduction balances (Roels, 1980) were used to verify data consistency. Values represent averages ± mean deviations of measurements on independent duplicate cultures for each combination of strain and medium. n.d., not determined.

Strain name Relevant genotype	IMX2736 & IMX2503 *Δgpd2 prk* 2x *cbbm* & *Δgpd2 Δald6 eutE*		
Initial acetate concentration	0 mM	5 mM	5 mM
Inoculum ratio IMX2503: IMX2736	1.1 ± 0.0	1.4 ± 0.2	0.8 ± 0.1
$Y_{biomass/glucose}$ (g_x g^{-1})	0.090 ± 0.000	0.088 ± 0.001	0.090 ± 0.000
$Y_{ethanol/glucose}$ (mol mol $^{-1}$)	1.62 ± 0.00	1.63 ± 0.01	1.63 ± 0.01
$Y_{acetaldehyde/glucose}$ (mol mol $^{-1}$)	0.004 ± 0.001	n.d.	n.d.
$Y_{glycerol/glucose}$ (mol mol $^{-1}$)	0.070 ± 0.002	0.073 ± 0.002	0.058 ± 0.000
$Y_{acetate/glucose}$ (mol mol $^{-1}$)	0.007 ± 0.003	−0.045 ± 0.000	−0.030 ± 0.004
mmol glycerol per g_x	4.3 ± 0.1	4.6 ± 0.1	3.6 ± 0.0
Final acetaldehyde concentration (mM)	1.2 ± 0.1	n.d.	n.d.
Final acetate concentration (mM)	1.5 ± 0.23	0.3 ± 0.1	0.7 ± 0.2
Electron recoveries	99–100	99–99	98–101

rates ca. 0.32 h^{-1} and 0.27 h^{-1}, respectively). After 19 h, when acetate had been completely consumed, anaerobic cultures of strain IMS1247 had only consumed a quarter of the glucose initially present in the culture (Fig. 5A). However, in contrast to strain IMX2503, strain IMS1247 did not produce any glycerol (Figs. 4A and 5A).

Whole-genome sequencing of the evolved strain IMS1247 revealed single-nucleotide mutations in *HXK2*, *GIS3* and in the intergenic region in front of *GUT1* (Table S2). The SNP on Chr. 7 location 30,770 (*HXK2*) in IMS1247 was used in addition to the two previously described unique SNPs in strain IMX2736. These mutations were used to estimate the ratio of strains IMS1247 and IMX2736 in co-cultures (Fig. 6).

To compensate for the slow growth of strain IMS1247, initial co-cultivation experiments with strain IMX2736 on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate were grown with an inoculum ratio of 5.5:1 (IMS1247:IMX2736). In these cultures, acetate was completely consumed after 16 h, when only half of the glucose had been consumed (Fig. 5B). Complete consumption of glucose occurred after 20 h, which was 3 h later than in monocultures of strain IMX2736 on the same medium (Fig. 2D). This slower conversion was anticipated due to the lower inoculum density of strain IMX2736 and the dependency of strain IMS1247 on exogenous acetate or acetaldehyde.

When the inoculum ratio of the two strains was changed to 1:1 (IMS1247:IMX2736), approximately 1 mmol L^{-1} acetate was left in the culture when glucose was exhausted (Fig. 5C). As a consequence, growth arrest of IMS1247 was prevented and the overall fermentation time was close to that of monocultures of PRK-RuBisCO strain IMX2736 on the same medium (Fig. 6). Glycerol yields in co-cultures of strains IMS1247 and IMX2736 grown on acetate-supplemented medium at inoculum ratios of 5.5 and 1.0 were 77% and 82% lower, respectively, than in corresponding monocultures of the reference strain IME324 and 22% and 37% lower than the monocultures of PRK-RuBisCO strain IMX2736. Moreover, ethanol yields were 8.3% and 7.1% higher, respectively, for co-cultures grown at inoculum ratios of 5.5 and 1.0, than for monocultures of the reference strain IME324 ($p = 0.002$ and $p = 0.014$, respectively, Tables 1 and 3).

During anaerobic co-cultivation on 50 g L^{-1} glucose as sole carbon source, growth of the glycerol-negative A-ALD strain IMS1247 was anticipated to depend on supply of acetate and acetaldehyde by the PRK-RuBisCO-based strain IMX2736. Acetate and acetaldehyde yields of the co-cultures of strains IMS1247 and the PRK-RuBisCO IMX2736 on 50 g L^{-1} glucose, inoculated at a ratio of 1.3:1 (IMS1247:IMX2736), were 47% and 61% lower, respectively, than those of a monocultures of strain IMX2736 on 50 g L^{-1} and their ethanol yield was 2.7% higher ($p = 0.046$, Tables 1 and 3). These byproduct yields were 2.3- and 1.7-fold higher, respectively than observed in co-cultures of strain IMX2736 and strain IMX2503 (*Δgpd2 Δald6 eutE*). The latter observation probably reflects the population dynamics of the co-cultures of strains IMS1247 and IMX2736 on glucose as sole carbon source, which showed a 50% decrease of the relative abundance of strain IMS1247 during fermentation. In contrast, in co-cultures on 50 g L^{-1} glucose and 5 mmol L^{-1} acetate of the two strains with an inoculation ratio of 1, strain IMS1247 represented approximately half of the population throughout the fermentation (Fig. 6).

3. Discussion

Advantages of microbial interactions are well described for multi-species natural microbial ecosystems and microbial consortia applied in food fermentation processes (Lindemann et al., 2016; Bachmann et al., 2015). Previous laboratory studies on the use of consortia of different engineered *S. cerevisiae* strains for ethanol production focused on conversion of polysaccharides or sugar mixtures by consortia of engineered 'specialist' strains (Tabañag et al., 2018; Verhoeven et al., 2018). The present study focused on efficient conversion of glucose to ethanol, both in the absence and presence of low concentrations of acetate in growth media. Its results show that consortia of engineered PRK-RuBisCO-based and A-ALD-based *S. cerevisiae* strains enable higher ethanol yields than obtained with a non-engineered reference strain, while reducing net formation of byproducts originating from PRK-RuBisCO-based *S. cerevisiae* and removing acetate from growth media.

Introduction of PRK and RuBisCO, combined with modifications in the central metabolism of *S. cerevisiae*, enables improved ethanol yields in fast-growing anaerobic cultures (Papapetridis et al., 2018). However, during slower anaerobic growth in continuous cultures (van Aalst et al., 2023b), and in batch cultures on 50 g L^{-1} glucose (Fig. 2 CD), PRK-RuBisCO strains optimized for fast growth generate acetaldehyde and acetate as byproducts. In a recent study, PRK-RuBisCO strains into which an A-ALD pathway had been introduced showed inferior acetate reduction relative to a strain that only expressed the A-ALD pathway (van Aalst et al., 2023b). A lack of *in vivo* reductive A-ALD activity in 'dual pathway' strains was attributed to the impact of acetaldehyde and a low $NADH/NAD^+$ ratio, both generated by activity of the PRK-RuBisCO bypass, on the reversible A-ALD reaction ($\Delta G^{0'} = 17.6$ kJ mol^{-1} for the reductive reaction (Beber et al., 2022),). Further engineering to adapt *in vivo* activity of PRK and RuBisCO in dual-pathway strains to NADH availability in dynamic industrial cultures would require introduction of dynamic regulation circuits (van Aalst et al., 2023b). Our results show that, alternatively, interference of the two pathways can be mitigated by their compartmentation in separate co-cultivated strains. Co-cultivation of the PRK-RuBisCO strain IMX2736 with the A-ALD strain IMX2503 (*Δgpd2 Δald6 eutE*) enabled high ethanol yields in glucose-grown cultures (Tables 2 and 3). Moreover, at an inoculation ratio of 1.1, volumetric productivities of these co-cultures were not significantly different from those of monocultures of the PRK-RuBisCO strain (Table S1), while acetate and acetaldehyde production was strongly reduced (Figs. 4D and 5D). Minimizing acetaldehyde production is not only relevant for improving ethanol yield but

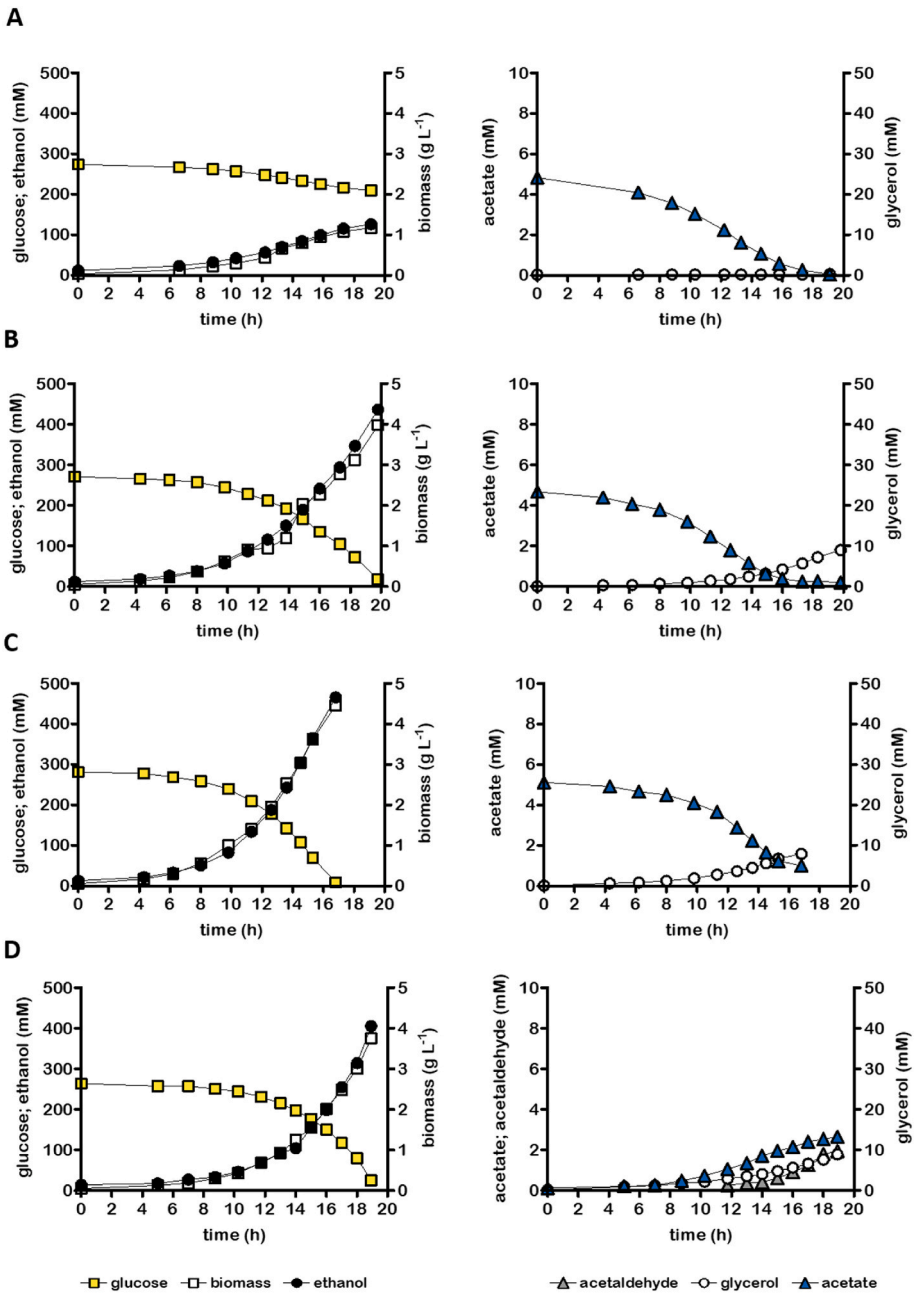

Fig. 5. Growth, glucose consumption and product formation of anaerobic bioreactor batch cultures of *S. cerevisiae* strain IMS1247 (*Δgpd1 Δgpd2 Δald6 eutE*, evolved) (**A**) and co-cultures of IMS1247 and IMX2736 (*Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm*, *groES, groEL*). Non-ox PPP↑ indicates integration of overexpression cassettes for *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1* and *TKL2*. Cultures were grown on synthetic medium containing 50 g L^{-1} glucose (panel **D**) or 50 g L^{-1} glucose and 5 mmol L^{-1} acetate (panels **A**–**C**) and were inoculated at a ratio of 5.5 ± 1.3 (**B**), 1.0 ± 0.2 (**C**) or 1.3 ± 0.4 (**D**). Representative cultures of independent duplicate experiments are shown, the corresponding replicate of each culture is shown in Fig. S4.

also to prevent its toxicity to yeast cells (Stanley et al., 1993; Stanley and Pamment, 1993) and also in view of environmental and health issues (Batista and Meirelles, 2009; Cohen et al., 2021).

Consortia of the PRK-RuBisCO strain IMX2736 with the A-ALD strain IMX2503 (*Δgpd2 Δald6 eutE*) or IMS1247 (*Δgpd1 Δgpd2 Δald6 eutE*) removed essentially all acetate from media with acetate-to-glucose ratios similar to those in feedstocks for first-generation bioethanol production (Fig. 4 BC, Fig. 5 BC). Complete removal of acetate during fermentation does not only contribute to increased ethanol yields, but also prevents its recycling into subsequent fermentation runs and, potentially its accumulation to inhibitory levels, as a result of recycling thin stillage and evaporator condensate (Ingledew et al., 2003). When inoculation ratios of the PRK-RuBisCO and A-ALD strains were optimized to avoid premature depletion of acetate, volumetric productivities of co-cultures in acetate-containing media were not significantly different from those in monocultures of the PRK-RuBisCO strain (Table S1). Ideally, low-glycerol growth and vigorous fermentation by A-ALD-based strains should continue even when acetate availability in the medium becomes growth limiting. In addition to the option of supplying small amounts of acetate during fermentation, this goal may be pursued by further strain engineering to only allow for a strongly constrained rate of glycerol production. Alternatively, *S. cerevisiae* strains may be used in which A-ALD reduces acetyl-CoA synthesized from glucose via engineered pathways that generates fewer than 2 mol of NADH per mol of acetyl-CoA. Such strategies can be based on introduction of a heterologous pyruvate-formate lyase (PFL) (de Bont and

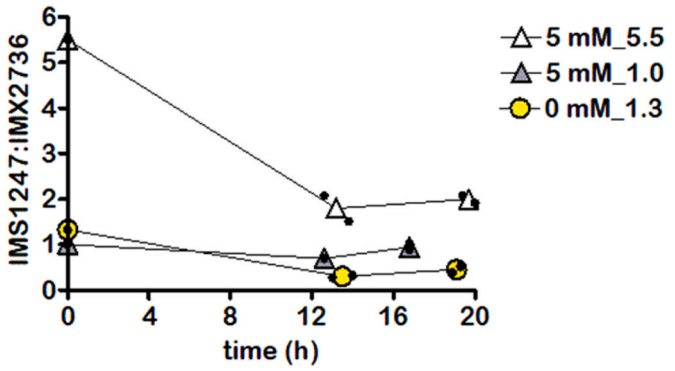

Fig. 6. Ratio of *S. cerevisiae* IMS1247 *(Δgpd1 Δgpd2 Δald6 eutE*, evolved) relative to strain IMX2736 *(Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm*, *groES, groEL)* in anaerobic bioreactor batch co-cultures on synthetic medium containing 50 g L^{-1} glucose with or without the addition of 5 mM acetate (indicated in the Figure as either 5 mM or 0 mM). Ratio was calculated based on whole genome sequencing. Non-ox PPP↑ indicates the integration of the overexpression cassettes for *RPE1*, *TKL1*, *TAL1*, *NQM1*, *RKI1* and *TKL2*. Values represent means and individual values of measurements on independent batch duplicate cultures. Cultures were inoculated at ratios of strain IMX1247 relative to strain IMX2736 of 5.5, 1.0 or 1.3 as indicated in the Figure.

Table 3
Key physiological parameters of anaerobic bioreactor batch co-cultures of *S. cerevisiae* strains IMX2736 *(Δgpd2*, non-ox PPP↑, *PRK*, 2x *cbbm*, *groES, groEL)* and IMS1247 *(Δgpd1 Δgpd2 Δald6 eutE*, evolved). Non-ox PPP↑ indicates integration of the overexpression cassettes for *RPE1, TKL1, TAL1, NQM1, RKI1 and TKL2*. Inoculum ratios of the two strains were calculated by genome sequencing. Cultures were grown on synthetic medium with 50 g L^{-1} glucose, with or without addition of 5 mM acetate. Y indicates yield, subscript x denotes biomass. Acetate and acetaldehyde concentrations indicate values in the culture broth, measured at the end of the cultivation experiments. Yields were calculated using the average of the first two and last two sampling points. Negative acetate yields indicate net acetate consumption and were calculated from data derived from sampling points before acetate depletion. Degree-of-reduction balances (Roels, 1980) were used to verify data consistency. Values represent averages ± mean deviations of measurements on independent duplicate cultures for each combination of strain and medium. n.d., not determined.

Strain name Relevant genotype	IMX2736 & IMS1247 *Δgpd2 prk* 2x *cbbm* & *Δgpd1 Δgpd2 Δald6 eutE*		
Initial acetate concentration	0 mM	5 mM	5 mM
Inoculum ratio IMS1247: IMX2736	1.33 ± 0.36	5.51 ± 1.26	1.00 ± 0.15
$Y_{biomass/glucose}$ (g_x g^{-1})	0.088 ± 0.000	0.088 ± 0.002	0.091 ± 0.000
$Y_{ethanol/glucose}$ (mol mol^{-1})	1.64 ± 0.01	1.67 ± 0.01	1.66 ± 0.00
$Y_{acetaldehyde/glucose}$ (mol mol^{-1})	0.009 ± 0.000	n.d.	n.d.
$Y_{glycerol/glucose}$ (mol mol^{-1})	0.037 ± 0.000	0.035 ± 0.000	0.029 ± 0.000
$Y_{acetate/glucose}$ (mol mol^{-1})	0.012 ± 0.000	−0.049 ± 0.002	−0.019 ± 0.000
mmol glycerol per g_x	2.4 ± 0.1	2.2 ± 0.0	1.8 ± 0.0
Final concentration acetaldehyde (mM)	2.1 ± 0.2	n.d.	n.d.
Final concentration acetate (mM)	2.9 ± 0.3	0.2 ± 0.0	0.9 ± 0.1
Electron recoveries	98–99	99–100	98–99

Teunissen, 2012; Argyros et al., 2015), or of a heterologous phosphoketolase (PK) and phosphotransacetylase (PTA) (Meadows et al., 2016; Bergman et al., 2016; Andrei and Munos, 2017).

In contrast to sugar-cane-based ethanol production processes in Brazil, in which yeast biomass is continually recycled and extensive population dynamics can occur during extended fermentation runs (Della-Bianca et al., 2013; Basso et al., 2011), corn-based ethanol production is typically operated as batch fermentation process preceded by a standardized seed culture train (Ingledew and Lin, 2011). This process configuration facilitates use of genetically engineered yeast strains. However, when exclusively relying on metabolic engineering of monocultures, adaptation of yeast strains to fluctuations in feedstock composition and/or changes in process configuration may be costly and time consuming. Use of co-cultures may enable faster and more cost-effective optimization of ethanol yield and productivity via formulation of mixtures of already available strains (Figs. 4 and 5). For example, ethanol plants dealing with higher acetic acid levels, for example due to contamination with lactic acid bacteria, might increase the fraction of acetate-reducing yeast in strain blends. A recent report, which does not disclose details on the strains involved, indicates that blends of engineered yeast strains are already applied at full industrial scale for corn-based ethanol production (IFF, 2022).

4. Materials and methods

4.1. Strains, media and maintenance

The *S. cerevisiae* strains used in this study (Table 5) were derived from the CEN. PK lineage (Entian and Kötter, 2007; Nijkamp et al., 2012). Synthetic medium (SM), containing 3.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4{\cdot}7H_2O$, 5.0 g L^{-1} $(NH_4)_2SO_4$, trace elements and vitamins, was prepared as described previously (Verduyn et al., 1992). Shake-flask cultures were grown on SM supplemented with 20 g L^{-1} glucose (SMD) and bioreactor cultures on SM with 50 g L^{-1} glucose. Anaerobic growth media were supplemented with ergosterol (10 mg L^{-1}) and Tween 80 (420 mg L^{-1}) (Mooiman et al., 2021). Anaerobic cultures were grown on SMD in which the KH_2PO_4 concentration was raised to 14.4 g L^{-1} for extra pH buffering. Complex medium (YPD) contained 10 g L^{-1} Bacto yeast extract (Thermo Fisher Scientific, Waltham MA), 20 g L^{-1} Bacto peptone (Thermo Fisher Scientific) and 20 g L^{-1} glucose. To select for presence of an acetamidase marker cassette (Solis-Escalante et al., 2013), $(NH_4)_2SO_4$ was replaced by 6.6 g L^{-1} K_2SO_4 and 0.6 g L^{-1} filter-sterilized acetamide. Where indicated, pure acetic acid solution (≥99.8%, Honeywell, Charlotte NC) was added to media at a concentration of 0.30 g L^{-1} or 1.0 g L^{-1}. *Escherichia coli* XL1-Blue cultures were grown on lysogeny broth (LB) (Bertani, 2004) containing 10 g L^{-1} tryptone (Brunschwig Chemie B.V., Amsterdam, The Netherlands), 5.0 g L^{-1} yeast extract and 10 g L^{-1} NaCl. Where relevant, LB was supplemented with 100 mg L^{-1} ampicillin (Merck, Darmstadt, Germany). *E. coli* strains were grown overnight at 37 °C in 15-mL tubes containing 5 mL LB, shaken at 200 rpm in an Innova 4000 Incubator (Eppendorf AG, Hamburg, Germany). Solid media were prepared by adding 20 g L^{-1} agar (Becton Dickinson, Breda, The Netherlands) prior to heat sterilization. *S. cerevisiae* plate cultures were incubated at 30 °C until colonies appeared (1 to 5 days), while *E. coli* plates were incubated overnight at 37 °C. Frozen stock cultures were prepared by freezing samples from fully grown batch cultures at −80 °C after addition of 30% (v/v) glycerol.

4.2. Plasmid construction

Cas9 target sequences in *GPD1* and *GPD2* were identified as described previously (Mans et al., 2015). To construct plasmid pUDR203 (Table 4), a linear backbone fragment of pROS11 was first PCR amplified with primer 5793 (overview of primers used in this study can be found in Table S3). Subsequently, DNA fragments encoding *GPD1*-targeting and *GPD2*-targeting gRNA cassettes were PCR-amplified using primers 6965/6966. Phusion high-fidelity DNA Polymerase (Thermo Fisher) was used as specified by the manufacturer. Plasmid-backbone and insert fragments were isolated from gels with the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine CA). DNA concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher) at a wavelength of 260 nm. Plasmid assembly was performed by in vitro

Table 4
Plasmids used in this study.

Plasmid	Characteristics	Reference
pROS11	2 μm ori, *AmdS*, gRNA-*CAN1*.Y gRNA-*ADE2*.Y	Mans et al. (2015)
pUDR103	2 μm ori, *KIURA3*, gRNA-*SGA1*.Y	Papapetridis et al. (2018)
pUDR203	2 μm ori, *AmdS*, gRNA.*GPD1*.Y gRNA.*GPD2*.Y	This work
pUDR774	2 μm ori, *KIURA3*, gRNA.*GPD2*.Y gRNA.*GPD2*.Y	van Aalst et al. (2023b)

Table 5
S. cerevisiae strains used in this study. *Kl* denotes *Kluyveromyces lactis*.

Strain name	Relevant genotype	Parental strain	origin
CEN.PK113-5D	*MATa ura3-52*	–	Entian and Kötter (2007)
IMX581	*MATa ura3-52 can1::cas9-natNT2*	CEN.PK113-5D	Mans et al. (2015)
IME324	*MATa ura3-52 can1::cas9-natNT2* p426-TEF (empty)	IMX581	Papapetridis et al. (2018)
IMX2503	*MATa ura3-52 can1::cas9 natNT2 ALD6::*p*TDH3-eutE gpd2Δ* pUDR774 (*KlURA3*)	IMX581	van Aalst et al. (2023b)
IMX2744	*MATa ura3-52 can1::cas9 natNT2 ALD6::*p*TDH3-eutE gpd2Δ gpd1Δ* pUDR774 (*KlURA3*)	IMX2503	This study
IMS1247	Strain IMX2744 evolved for faster anaerobic growth on 50 g L^{-1} glucose and 1 g L^{-1} acetic acid	IMX2744	This study
IMX2736	*MATa ura3-52 can1::cas9 natNT2 gpd2::* (p*TDH3-RPE1,* p*PGK1-TKL1,* p*TEF1-TAL1,* p*PGI1-NQM1,* p*TPI1-RKI1,* p*PYK1-TKL2*) *sga1::* (*pDAN1-PRK,* p*TDH3-cbbm* (2 copies) *pTPI1-groES*, *pTEF1-groEL*) pUDR103 (*KlURA3*)	–	van Aalst et al. (2023a)

Gibson Assembly using a HiFi DNA Assembly master mix (New England Biolabs, Ipswich, MA), downscaled to 5 μL reaction volumes. 1 μL of the reaction mixture was used to transform *E. coli* XL-1 Blue cells with a heat-shock protocol (Froger and Hall, 2007). Plasmid pUDR203 was isolated from *E. coli* XL-I Blue cells with the Sigma GenElute Plasmid Miniprep Kit (Sigma-Aldrich) as specified by the manufacturer.

4.3. Genome editing

A dsDNA-repair fragment for deletion of *GDP1* was obtained by mixing primers 6969/6970 in a 1:1 M ratio. This mixture was heated to 95 °C for 5 min and allowed to cool to room temperature. *S. cerevisiae* IMX2744 was constructed by co-transforming strain IMX2503 with gRNA-plasmid pUDR203 and the *GPD1* repair fragment, using the lithium-acetate method (Gietz and Woods, 2001). Transformants were selected on SM supplemented with 20 g L^{-1} glucose (SMD) and acetamide (Solis-Escalante et al., 2013), after which correct deletion of *GPD1* was checked by diagnostic colony PCR with DreamTaq polymerase (Thermo Fisher). pUDR203 was removed by growing non-selectively on SMD, while pUDR774 was retained to support uracil prototrophy. A correct transformant was restreaked thrice on SMD and stored at −80 °C.

4.4. Anaerobic shake-flask cultivation

Anaerobic shake-flask cultures of single-colony isolates from sequential-batch-reactor (SBR) evolution experiments with *S. cerevisiae* IMX2744 were grown at 30 °C in 50-mL round-bottom shake-flasks containing 30 mL extra buffered SM supplemented with vitamins, 50 g L^{-1} glucose, 1 g L^{-1} acetic acid and Tween 80/ergosterol. Seven single-colony isolates from each reactor isolated after 38 repeated-batch cycles, along with four isolates from reactor I and seven from reactor II isolated after 63 cycles, were analysed for their specific growth rates. Of these 25 single colony isolates, IMS1247 was selected with the fastest growth rate (0.27 h^{-1}), which was isolated from reactor I after 63 cycles. Shake flasks were placed on an IKA KS 260 basic shaker (Dijkstra Verenigde BV, Lelystad, The Netherlands, 200 rpm) in a Bactron anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR) under an atmosphere of 5% (v/v) H_2, 6% (v/v) CO_2 and 89% (v/v) N_2 (Mooiman et al., 2021).

4.5. Bioreactor cultivation

Anaerobic bioreactor batch and sequential-batch cultures were grown at 30 °C in 2-L bioreactors (Applikon, Delft, The Netherlands). Culture pH was maintained at 5.0 by automatic addition of 2 M KOH. Bioreactor cultures were grown on SM, supplemented with glucose (50 g L^{-1}), acetic acid (0.3 g L^{-1} or 1 g L^{-1} as indicated), Tween 80 (420 mg L^{-1}) and ergosterol (10 mg L^{-1}), and antifoam C (0.2 g L^{-1}) (Sigma-Aldrich). Bioreactor cultures were operated at a working volume of 1 L and sparged at 0.5 L min^{-1} with an N_2/CO_2 (90/10%) gas mixture, except for the laboratory-evolution cultures of strain IMX2744 and the anaerobic bioreactor batch of strains IMX2744 and IMS1247 on 50 g L^{-1} of glucose and 1 g L^{-1} of acetic acid, which were sparged with pure N_2. The outlet gas stream was cooled to 4 °C in a condenser to minimize evaporation. Oxygen diffusion was minimized by use of Norprene tubing (Saint-Gobain, Amsterdam, The Netherlands) and Viton O-rings (ERIKS, Haarlem, The Netherlands) (Mooiman et al., 2021). Inocula for bioreactor cultures were prepared in 500-mL shake flasks containing 100 mL SMD. A first preculture, inoculated with a frozen stock culture and grown aerobically at 30 °C for 15–18 h, was used to inoculate a second preculture. Upon reaching mid-exponential phase (OD_{660} of 3–6), the second preculture was used to inoculate a bioreactor culture at an initial OD_{660} of 0.2–0.4. For inoculation of the co-culture, the OD_{660} was used to calculate how much volume from each strain needed to be added into the reactor. DNA isolated from the mix containing both strains, used for inoculation was sequenced to determine the estimated starting inoculum ratio.

Laboratory evolution of *S. cerevisiae* IMX2744 was performed in SBR set-ups on SM supplemented with 50 g L^{-1} glucose and 1 g L^{-1} acetic acid. Cultures were sparged at 0.5 L min^{-1} with pure N_2. When, after having peaked, the CO_2 concentration in the off-gas had decreased to 60% of the highest CO_2-value measured during the preceding batch cycle, an effluent pump was automatically switched on for 20 min. After this emptying phase only 0.05 L broth remained in the reactor. The effluent pump was then stopped and the inflow pump activated to supply fresh sterile medium from a 20-L reservoir vessel. This refill phase was stopped via an electrical level sensor calibrated at a 1-L working volume.

4.6. Analytical methods

The optical density of cultures was measured at 660 nm on a Jenway 7200 spectrophotometer (Bibby Scientific, Staffordshire, UK). Biomass dry weight was measured as described previously (Guadalupe-Medina et al., 2013). Metabolite concentrations were determined by high-performance liquid chromatography and a first-order evaporation rate constant of 0.008 h^{-1} was used to correct ethanol concentrations (Guadalupe-Medina et al., 2013). Acetaldehyde concentrations in culture broth were determined after derivatization with 2,4-dinitrophenylhydrazine as described previously (van Aalst et al., 2023a). As carbon recoveries could not be accurately calculated due to the high concentration of CO_2 in the inlet gas of bioreactor cultures, electron recoveries were used instead (Roels, 1980).

4.7. Whole-genome sequencing

Genomic DNA was extracted from a 100-mL aerobic, late-exponential-phase (OD_{660} of 10–15) shake-flask culture on SMD of *S. cerevisiae* strain IMS1247, using a Qiagen Blood & Cell Culture DNA kit and 100/G Genomics-tips (Qiagen, Hilden, Germany). Custom paired-end sequencing of genomic DNA was performed by Macrogen (Amsterdam, The Netherlands) on a 350-bp PCR-free insert library using Illumina SBS technology. Sequence reads were mapped against the genome of *S. cerevisiae* CEN. PK113-7D (Salazar et al., 2017) to which a virtual contig containing p*TDH3-eutE* had been added, and processed as described previously (van Aalst et al., 2022b).

To determine relative abundancy of strains IMX2736 and IMX2503 in bioreactor batch co-cultivation experiments, 50-mL culture samples were centrifuged for 10 min at 5000×*g* and biomass pellets temporarily stored at −20 °C. These pellets were used for genomic DNA extraction and Illumina sequencing. Sequence reads were mapped against the genome of *S. cerevisiae* CEN. PK113-7D to which a virtual contig containing p*TDH3-eutE*, p*DAN1-PRK*, p*TDH3-cbbM*, p*TPI1-groES* and p*TEF1-groEL* had been added. Unique SNPs in strains IMX2736 and IMS1247 were identified and used to quantify the percentage of each strain present in the sample.

4.8. Statistical analysis

Significance was assessed by performing a two-sided unpaired Student's t-test. Differences were considered to be significant if a p-value <0.05 was obtained.

Funding

DSM Bio-based Products & Services B.V. (Delft, The Netherlands).

Author contributions

AA: Validation, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. **IM:** Investigation, Writing – Review & Editing. **MJ:** Writing – Review & Editing. **RM:** Conceptualization, Supervision, Writing – Review & Editing. **JP:** Conceptualization, Supervision, Writing – Original Draft, Writing – Review & Editing.

Declaration of competing interest

The PhD project of AA is funded by DSM Bio-based Products & Services B.V. (Delft, The Netherlands). Royal DSM owns intellectual property rights of technology discussed in this paper.

Data availability

Short read DNA sequencing data were deposited at NCBI under BioProject accession number PRJNA972873. All measurement data are available in Additional files.

Acknowledgements

We want to thank Rinke van Tatenhove-Pel for stimulating discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2023.09.010.

References

Albertyn, J., Hohmann, S., Thevelein, J.M., Prior, B.A., 1994. *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell Biol. 14, 4135–4144.

Andrei, M., Munos, J.W., 2017. Inventors; Danisco US Inc, Assignee. Altered Host Cell Pathway for Improved Ethanol Production. US 2017088861A1.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J.M., Adler, L., 1997. The two isoenzymes for yeast NAD+-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. EMBO J. 16, 2179–2187.

Argyros, A., Sillers, W., Barrett, T., Caiazza, N., Shaw, A., 2015. Inventors; Lallemand Hungary Liquidity Management, Assignee. Methods for the Improvement of Product Yield and Production in a Microorganism through the Addition of Alternate Electron Acceptors. US 008956851B2.

Bachmann, H., Pronk, J.T., Kleerebezem, M., Teusink, B., 2015. Evolutionary engineering to enhance starter culture performance in food fermentations. Curr. Opin. Biotechnol. 32, 1–7.

Bakker, B.M., Overkamp, K.M., van Maris, A.J., Kötter, P., Luttik, M.A., van Dijken, J.P., et al., 2001. Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 25, 15–37.

Basso, L.C., Basso, T.O., Rocha, S.N., 2011. Ethanol production in Brazil: the industrial process and its impact on yeast fermentation. Biofuel Production-Recent Developments and Prospects 1530, 85–100.

Batista, F.R., Meirelles, A.J., 2009. A strategy for controlling acetaldehyde content in an industrial plant of bioethanol. IFAC Proc. Vol. 42, 928–933.

Beber, M.E., Gollub, M.G., Mozaffari, D., Shebek, K.M., Flamholz, A.I., Milo, R., et al., 2022. eQuilibrator 3.0: a database solution for thermodynamic constant estimation. Nucleic Acids Res. 50, 603–609.

Bergman, A., Siewers, V., Nielsen, J., Chen, Y., 2016. Functional expression and evaluation of heterologous phosphoketolases in *Saccharomyces cerevisiae*. Amb. Express 6, 1–13.

Bertani, G., 2004. Lysogeny at mid-twentieth century: P1, P2, and other experimental systems. J. Bacteriol. 186, 595–600.

Blomberg, A., Adler, L., 1992. Physiology of osmotolerance in fungi. Adv. Microb. Physiol. 33, 145–212.

Brown, R.C., Wright, M.M., 2009. Biomass Conversion to Fuels and Electric Power. Cornell University Library's Initiatives in Publishing (CIP), pp. 53–64.

Buckenhüskes, H.J., 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev. 12, 253–271.

Cohen, G., Kreutzer, N., Mowat, K., Hassan, A.A., Dvorak, B., 2021. Compliance with hand sanitizer quality during the SARS-CoV-2 pandemic: assessing the impurities in an ethanol plant. J. Environ. Manag. 297, 113329.

Cori, C.F., 1983. Embden and the glycolytic pathway. Trends Biochem. Sci. 8, 257–259.

de Bont, J., Teunissen, A., 2012. Inventors; Yeast Company B.V., Assignee. Yeast Strains Engineered to Produce Ethanol from Glycerol. WO 2012/067510 A1. International patent WO 2012/067510 A1.

de Jong-Gubbels, P., van den Berg, M.A., Steensma, H.Y., van Dijken, J.P., Pronk, J.T., 1997. The *Saccharomyces cerevisiae* acetyl-coenzyme A synthetase encoded by the *ACS1* gene, but not the *ACS2*-encoded enzyme, is subject to glucose catabolite inactivation. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 153, 75–81.

de Smidt, O., du Preez, J.C., Albertyn, J., 2008. The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. FEMS Yeast Res. 8, 967–978.

Della-Bianca, B.E., Gombert, A.K., 2013. Stress tolerance and growth physiology of yeast strains from the Brazilian fuel ethanol industry. Antonie Leeuwenhoek 104, 1083–1095.

Della-Bianca, B.E., Basso, T.O., Stambuk, B.U., Basso, L.C., Gombert, A.K., 2013. What do we know about the yeast strains from the Brazilian fuel ethanol industry? Appl. Microbiol. Biotechnol. 97, 979–991.

Embden, G., Deuticke, H., Kraft, G., 1933. Über die intermediären Vorgänge bei der Glykolyse in der Muskulatur. Klin. Wochenschr. 12, 213–215.

Entian, K.-D., Kötter, P., 2007. 25 yeast genetic strain and plasmid collections. Methods Microbiol. 36, 629–666.

Eriksson, P., André, L., Ansell, R., Blomberg, A., Adler, L., 1995. Cloning and characterization of *GPD2*, a second gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD^+) in *Saccharomyces cerevisiae*, and its comparison with *GPD1*. Mol. Microbiol. 17, 95–107.

Flikweert, M.T., de Swaaf, M., van Dijken, J.P., Pronk, J.T., 1999. Growth requirements of pyruvate-decarboxylase-negative *Saccharomyces cerevisiae*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 174, 73–79.

Froger, A., Hall, J.E., 2007. Transformation of plasmid DNA into *E. coli* using the heat shock method. JoVE e253.

Gietz, R.D., Woods, R.A., 2001. Genetic transformation of yeast. Biotechniques 30, 816–831.

Guadalupe-Medina, V., Almering, M.J., van Maris, A.J., Pronk, J.T., 2010. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. Appl. Environ. Microbiol. 76, 190–195.

Guadalupe-Medina, V., Wisselink, H.W., Luttik, M.A., de Hulster, E., Daran, J.-M., Pronk, J.T., et al., 2013. Carbon dioxide fixation by Calvin-Cycle enzymes improves ethanol yield in yeast. Biotechnol. Biofuels 6, 1–12.

Guadalupe-Medina, V., Metz, B., Oud, B., van der Graaf, C.M., Mans, R., Pronk, J.T., et al., 2014. Evolutionary engineering of a glycerol-3-phosphate dehydrogenase-

negative, acetate-reducing *Saccharomyces cerevisiae* strain enables anaerobic growth at high glucose concentrations. Microb. Biotechnol. 7, 44–53.
Guan, X., Rubin, E., Anni, H., 2012. An optimized method for the measurement of acetaldehyde by high-performance liquid chromatography. Alcohol Clin. Exp. Res. 36, 398–405.
Guo, Z-p, Zhang, L., Ding, Z-y, Shi, G-y, 2011. Minimization of glycerol synthesis in industrial ethanol yeast without influencing its fermentation performance. Metab. Eng. 13, 49–59.
Holst, B., Lunde, C., Lages, F., Oliveira, R., Lucas, C., Kielland-Brandt, M.C., 2000. *GUP1* and its close homologue *GUP2*, encoding multimembrane-spanning proteins involved in active glycerol uptake in *Saccharomyces cerevisiae*. Mol. Microbiol. 37, 108–124.
IFF, 2022. Your Needs Change, Your Yeast Can't. Ethanol Producer Magazine.
Ingledew, W., Lin, Y., 2011. Ethanol from starch-based feedstocks. In: Comprehensive Biotechnology, 2 ed. Elsevier, Amsterdam, pp. 37–49.
Ingledew, W.M., 2003. Water reuse in fuel alcohol plants: effect on fermentation. Is a 'zero discharge' concept attainable? In: Jacques, K., Lyons, T., Kelsall, D. (Eds.), The Alcohol Textbook, 4 ed., pp. 343–354 Nottingham.
Ingledew, W., 2017. Yeast stress and fermentation. In: Walker, G., Abbas, C.A., Ingledew, W.M., Pilgrim, C. (Eds.), The Alcohol Textbook, 6 ed. Duluth, pp. 273–285.
Ingram, L.O.N., Buttke, T.M., 1985. Effects of alcohols on micro-organisms. Adv. Microb. Physiol. 25, 253–300.
Jacobus, A.P., Gross, J., Evans, J.H., Ceccato-Antonini, S.R., Gombert, A.K., 2021. *Saccharomyces cerevisiae* strains used industrially for bioethanol production. Essays Biochem. 65, 147–161.
Kumar, D., Jansen, M., Basu, R., Singh, V., 2020. Enhancing ethanol yields in corn dry grind process by reducing glycerol production. Cereal Chem. 97, 1026–1036.
Lindemann, S.R., Bernstein, H.C., Song, H.-S., Fredrickson, J.K., Fields, M.W., Shou, W., et al., 2016. Engineering microbial consortia for controllable outputs. ISME J. 10, 2077–2084.
Mans, R., van Rossum, H.M., Wijsman, M., Backx, A., Kuijpers, N.G., van den Broek, M., et al., 2015. CRISPR/Cas9: a molecular Swiss army knife for simultaneous introduction of multiple genetic modifications in *Saccharomyces cerevisiae*. FEMS Yeast Res. 15.
Meadows, A.L., Hawkins, K.M., Tsegaye, Y., Antipov, E., Kim, Y., Raetz, L., et al., 2016. Rewriting yeast central carbon metabolism for industrial isoprenoid production. Nature 537, 694–697.
Mehta, K., Leung, D., Lefebvre, L., Smith, M., 1990. The *ANB1* locus of *Saccharomyces cerevisiae* encodes the protein synthesis initiation factor eIF-4D. J. Biol. Chem. 265, 8802–8807.
Mooiman, C., Bouwknegt, J., Dekker, W.J., Wiersma, S.J., Ortiz-Merino, R.A., De Hulster, E., et al., 2021. Critical parameters and procedures for anaerobic cultivation of yeasts in bioreactors and anaerobic chambers. FEMS Yeast Res. 21 foab035.
Narendranath, N.V., Thomas, K.C., Ingledew, W.M., 2001. Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J. Am. Soc. Brew. Chem. 59, 187–194.
Nevoigt, E., Stahl, U., 1997. Osmoregulation and glycerol metabolism in the yeast *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 21, 231–241.
Nijkamp, J.F., van den Broek, M., Datema, E., de Kok, S., Bosman, L., Luttik, M.A., et al., 2012. De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN. PK113-7D, a model for modern industrial biotechnology. Microb. Cell Factories 11, 1–17.
Nissen, T.L., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J., 2000a. Optimization of ethanol production in *Saccharomyces cerevisiae* by metabolic engineering of the ammonium assimilation. Metab. Eng. 2, 69–77.
Nissen, T.L., Hamann, C.W., Kielland-Brandt, M.C., Nielsen, J., Villadsen, J., 2000b. Anaerobic and aerobic batch cultivations of *Saccharomyces cerevisiae* mutants impaired in glycerol synthesis. Yeast 16, 463–474.
Norbeck, J., Påhlman, A.-K., Akhtar, N., Blomberg, A., Adler, L., 1996. Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*: identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. J. Biol. Chem. 271, 13875–13881.
Papapetridis, I., van Dijk, M., Dobbe, A., Metz, B., Pronk, J.T., van Maris, A.J., 2016. Improving ethanol yield in acetate-reducing *Saccharomyces cerevisiae* by cofactor engineering of 6-phosphogluconate dehydrogenase and deletion of *ALD6*. Microb. Cell Factories 15, 1–16.
Papapetridis, I., Van Dijk, M., Van Maris, A.J., Pronk, J.T., 2017. Metabolic engineering strategies for optimizing acetate reduction, ethanol yield and osmotolerance in *Saccharomyces cerevisiae*. Biotechnol. Biofuels 10, 1–14.
Papapetridis, I., Goudriaan, M., Vázquez Vitali, M., De Keijzer, N.A., Van Den Broek, M., Van Maris, A.J., et al., 2018. Optimizing anaerobic growth rate and fermentation kinetics in *Saccharomyces cerevisiae* strains expressing Calvin-cycle enzymes for improved ethanol yield. Biotechnol. Biofuels 11, 1–17.
Phibro Animal Health Corporation. https://fermfacts.com/ferm-facts/yeast-health/. (Accessed 1 December 2022).
Rasmussen, M.L., Koziel, J.A., Jane, J-l, Pometto III, A.L., 2015. Reducing bacterial contamination in fuel ethanol fermentations by ozone treatment of uncooked corn mash. J. Agric. Food Chem. 63, 5239–5248.
Renewable Fuels association: annual ethanol production. https://ethanolrfa.org/markets-and-statistics/annual-ethanol-production. (Accessed 7 January 2022).
Roell, G.W., Zha, J., Carr, R.R., Koffas, M.A., Fong, S.S., Tang, Y.J., 2019. Engineering microbial consortia by division of labor. Microb. Cell Factories 18, 1–11.
Roels, J., 1980. Simple model for the energetics of growth on substrates with different degrees of reduction. Biotechnol. Bioeng. 22, 33–53.
Russell, I., 2003. Understanding yeast fundamentals. In: Jacques, K., Lyons, T., Kelsall, D. (Eds.), The Alcohol Textbook, 4 ed., pp. 85–120 Nottingham.
Salazar, A.N., Gorter de Vries, A.R., van den Broek, M., Wijsman, M., de la Torre Cortés, P., Brickwedde, A., et al., 2017. Nanopore sequencing enables near-complete de novo assembly of *Saccharomyces cerevisiae* reference strain CEN. PK113-7D, vol. 17. FEMS Yeast Research.
Secches, T.O., Santos Viera, C.F., Pereira, T.K., Santos, V.T., Ribeirodos Santos, J., Pereira, G.A., et al., 2022. Brazilian industrial yeasts show high fermentative performance in high solids content for corn ethanol process. Bioresources and Bioprocessing 9, 1–9.
Shong, J., Diaz, M.R.J., Collins, C.H., 2012. Towards synthetic microbial consortia for bioprocessing. Curr. Opin. Biotechnol. 23, 798–802.
Solis-Escalante, D., Kuijpers, N.G., Nadine, B., Bolat, I., Bosman, L., Pronk, J.T., et al., 2013. amdSYM, a new dominant recyclable marker cassette for *Saccharomyces cerevisiae*. FEMS Yeast Res. 13, 126–139.
Stanley, G., Pamment, N., 1993. Transport and intracellular accumulation of acetaldehyde in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 42, 24–29.
Stanley, G., Douglas, N., Every, E., Tzanatos, T., Pamment, N., 1993. Inhibition and stimulation of yeast growth by acetaldehyde. Biotechnol. Lett. 15, 1199–1204.
Tabañag, I.D.F., Chu, I.-M., Wei, Y.-H., Tsai, S.-L., 2018. Ethanol production from hemicellulose by a consortium of different genetically-modified *Sacharomyces cerevisiae*. J. Taiwan Inst. Chem. Eng. 89, 15–25.
van Aalst, A.C., de Valk, S.C., van Gulik, W.M., Jansen, M.L., Pronk, J.T., Mans, R., 2022a. Pathway engineering strategies for improved product yield in yeast-based industrial ethanol production. Synthetic and Systems Biotechnology 7, 554–566.
van Aalst, A.C., Mans, R., Pronk, J.T., 2022b. An engineered non-oxidative glycolytic bypass based on Calvin-cycle enzymes enables anaerobic co-fermentation of glucose and sorbitol by *Saccharomyces cerevisiae*. Biotechnology for Biofuels and Bioproducts 15, 1–15.
van Aalst, A.C., Jansen, M.L., Mans, R., Pronk, J.T., 2023a. Quantification and mitigation of byproduct formation by low-glycerol-producing *Saccharomyces cerevisiae* strains containing Calvin-cycle enzymes. Biotechnology for Biofuels and Bioproducts 16, 1–17.
van Aalst, A.C., Geraats, E.H., Jansen, M.L., Mans, R., Pronk, J.T., 2023b. Optimizing the balance between heterologous acetate- and CO_2-reduction pathways in anaerobic cultures of *Saccharomyces cerevisiae* strains engineered for low glycerol production. bioRxiv 5.
van Zuyl, F., Kauers, K., 2017. Contaminant microbes and their control. In: Walker, G., Abbas, C.A., Ingledew, W.M., Pilgrim, C. (Eds.), The Alcohol Textbook, 6 ed. Duluth, pp. 377–400.
Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P., 1990. Physiology of *Saccharomyces cerevisiae* in anaerobic glucose-limited chemostat cultures. Microbiology 136, 395–403.
Verduyn, C., Postma, E., Scheffers, W.A., van Dijken, J.P., 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast 8, 501–517.
Verhoeven, M.D., de Valk, S.C., Daran, J.-M.G., van Maris, A.J., Pronk, J.T., 2018. Fermentation of glucose-xylose-arabinose mixtures by a synthetic consortium of single-sugar-fermenting *Saccharomyces cerevisiae* strains. FEMS Yeast Res. 18, foy075.
Zhou, K., Qiao, K., Edgar, S., Stephanopoulos, G., 2015. Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nat. Biotechnol. 33, 377–383.