Pathway engineering strategies for improved product yield in yeast-based industrial ethanol production

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1. Introduction

In 2020, 99 billion liters of ethanol were produced by yeast-based fermentation of agriculture-derived carbohydrates [1]. Of this volume, approximately 30% was produced from Brazilian cane sugar (mainly consisting of sucrose) and approximately 54% from corn starch-derived glucose, mainly in the United States of America [1]. Ethanol is predominantly used as a renewable ‘drop-in’ transport fuel and ethanol-based value chains towards other compounds, including jet fuel and polyethylene, are under development [2,3].

Despite a plethora of academic and industrial studies on alternative microbial platforms [4], Saccharomyces cerevisiae remains the organism of choice for industrial ethanol production from carbohydrates. Factors that contribute to its popularity include rapid fermentation of glucose and sucrose to ethanol, insensitivity to phages, a long history of safe use and sucrose to ethanol, insensitivity to phages, a long history of safe use and polyethylene, are under development [2,3].

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anaerobic growth.

In the absence of growth, survival of yeast cells requires cellular maintenance metabolism, which encompasses use of ATP for growth-independent processes that maintain structural integrity and viability [11]. In anaerobic yeast cultures, this ATP is exclusively generated via alcoholic fermentation (Fig. 1). In contrast, growth of yeast cells not only requires ATP but also organic precursors for biomass components, whose biosynthetic pathways compete for carbon with ethanol production (Fig. 1). Anaerobic growth occurs in all current industrial processes for ethanol production and the resulting surplus yeast biomass is valorized by its inclusion in a by-product stream sold as an animal feed supplement [12].

Growth is coupled to formation of glycerol, a second important byproduct of anaerobic yeast metabolism, by redox-cofactor

Fig. 1. Schematic representation of the distribution of substrate over biomass, glycerol, ethanol and CO₂ in anaerobically growing S. cerevisiae. NADH/NAD⁺ redox-cofactor coupling and use of ATP for sugar phosphorylation, biomass formation and maintenance are indicated by dotted and dashed arrows, respectively. Glucose, fructose and (after hydrolysis) sucrose are converted into pyruvate via the Emden-Meyerhoff glycolysis, yielding 2 NADH and 2 ATP per glucose equivalent. ATP is used for cellular maintenance and synthesis of biomass (growth). NADH is primarily re-oxidized via alcoholic fermentation, but a surplus of NADH formed during biomass synthesis is re-oxidized via the production of glycerol.
metabolism. Formation of \textit{S. cerevisiae} biomass from sugar, ammonium or urea and other nutrients is coupled to a net reduction of NAD$^+$ to NADH \cite{13,14} (Fig. 1). Anaerobic \textit{S. cerevisiae} cultures cannot re-oxidize this NADH by mitochondrial respiration and instead rely on NADH-dependent reduction of the glycolytic intermediate dihydroxyacetone-phosphate to glycerol-3-phosphate, in a reaction catalysed by NAD$^+$-dependent glycerol-3-phosphate dehydrogenase (Gpd1, Gpd2, EC 1.1.1.8 \cite{15,16}). Glycerol-3-phosphate is then hydrolyzed by glycerol-3-phosphate-phosphatase (Gpp1, Gpp2, EC 3.1.3.21 \cite{17}) to yield phosphate and glycerol (Fig. 1). In processes based on wild-type \textit{S. cerevisiae} strains, approximately 4% of the potential ethanol yield on carbohydrate feedstocks was estimated to be lost to glycerol \cite{18}. Based on current ethanol production volumes, this loss would correspond to approximately 4 billion liters of ethanol per year.

The aim of this paper is to review the current body of knowledge on pathway engineering strategies that focus on maximizing ethanol yields on glucose or sucrose by altering the ratio of ethanol, biomass and glycerol formation in \textit{S. cerevisiae}. This scope excludes a large body of metabolic engineering research aimed at expanding the sugar- and polysaccharide substrate range of \textit{S. cerevisiae} to enable its nascent application for industrial-scale fermentation of lignocellulosic hydrolysates generated from agricultural residues or energy crops (reviewed in Refs. \cite{4,19–21}). However, the discussed strategies can, in principle, be applied in such ‘second-generation’ bioethanol processes as well as in ‘first-generation’ processes based on corn starch or cane sugar, once other metabolic engineering strategies have been successfully addressed.

2. Process conditions

Growth of anaerobic laboratory cultures of wild-type \textit{S. cerevisiae} strains under different conditions provided insight in how distribution of sugar over biomass, glycerol and ethanol can be influenced and have therefore been a key source of inspiration for the design of metabolic engineering strategies.

In anaerobic, sugar-limited cultures of \textit{S. cerevisiae}, maintenance-energy requirements are essentially growth-rate independent \cite{22–24}. The fraction of the consumed sugar that is fermented to ethanol therefore increases with decreasing specific growth rate \cite{11} (Fig. 2A). This correlation is clearly demonstrated in anaerobic retentostat cultures of \textit{S. cerevisiae}, in which all biomass is retained in the culture and only cell-free effluent leaves the reactor. In such systems, near-theoretical ethanol yields on glucose were demonstrated during prolonged growth at near-zero specific growth rates \cite{22}.

As an alternative to reducing the specific growth rate, the fraction of the sugar substrate that is fermented to ethanol by actively growing anaerobic cultures to meet maintenance-energy requirements can be increased by changing cultivation conditions. In particular, addition of weak organic acids such as lactate, acetate, propionate or benzoate to anaerobic batch and chemostat cultures grown at low pH, was shown to lead to lower biomass yields and higher ethanol yields \cite{13,25–29}. These results reflect an increased maintenance energy requirement for intracellular pH homeostasis, caused by an influx of protons into the yeast cytosol as a result of weak acid diffusion (Fig. 2B) \cite{30}. In anaerobic yeast cultures, countering this ‘weak acid uncoupling’ and maintenance of intracellular pH homeostasis critically depends on ATP-dependent proton export by the plasma membrane ATPase (Pma1,
increased ATP requirements for cellular maintenance. In addition, high furfural and hydroxymethyl-2-furaldehyde [21, 35] requirements by increasing permeability of the yeast plasma membrane.

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Experiments on disaccharide metabolism by anaerobic S. cerevisiae cultures provided a first demonstration that ethanol yields can be modified by changing the mechanism of sugar import. In contrast to transport of glucose, which occurs via facilitated diffusion by Hxt transporters [40,41], uptake of its dimer maltose by S. cerevisiae is mediated by Malx2 transporters and involves symport with a single proton [32,42]. After intracellular hydrolysis of maltose by a Malx2 maltase (EC 3.2.1.20, maltose + H_2O → 2 glucose), alcoholic fermentation of the resulting two glucose molecules yields 4 molecules of ATP.

However, since one of these ATP molecules has to be used to enable expulsion of the symported proton by Pma1, which has a stoichiometry of 1H^+/ATP [32,42], the net ATP yield from maltose fermentation is only 1.5 ATP per glucose equivalent (Fig. 2C). Indeed, based on hextose units, ethanol and biomass yields of S. cerevisiae in anaerobic maltose-limited chemostat cultures were shown to be 16% higher and 25% lower, respectively, than in corresponding glucose-limited cultures [31]. These observations highlighted the potential for engineering redox-cofactor metabolism to improve ethanol yields.

3. Engineering of energy coupling

3.1. Introduction of futile coupling

Several metabolic engineering strategies have been explored to increase the use of sugar for cellular maintenance energy requirements by introducing metabolic ‘futile cycles’, whose net effect is the hydrolysis of ATP to ADP and inorganic phosphate with a concomitant release of heat (Fig. 2E). Such ‘ATP wasting’ cycles can either be introduced by constitutive expression of ATPases or by creating more complicated futile cycles that cause a net hydrolysis of ATP. Overexpression of the soluble F1 unit of the Escherichia coli H^+ -ATPase in S. cerevisiae [48,49] led to a 10% increase of the anaerobic ethanol yield on glucose relative to a reference strain, but also caused a 26% decrease of the specific growth rate [48]. Overexpression of PHOS or PHO8, which encode aspecific phosphatases (EC 3.1.3.1/2) [50,51] was similarly reported to cause increased ATP turn-over. PHO8 overexpression was reported to cause a 17% higher ethanol yield on glucose, without affecting growth rate [50]. Simultaneous activity of ATP-generating glycolytic and ATP-consuming gluconeogenic enzymes leads to textbook examples of futile metabolic cycles. Though not tested with the specific aim to improve ethanol yields, overexpression of the gluconeogenic enzyme fructose-1,6-bispophosphate (Fbp1, EC 3.1.3.11: fructose-1,6-bispophosphate + H_2O → fructose-6-phosphate + P_i) increased glucose consumption (19%) and CO_2 (10%) and ethanol (14%) production rates of aerobic suspensions of non-growing cells [52]. An even more pronounced effect on the ethanol production rate (22%) was found when the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.49: oxaloacetate + ATP → phosphoenolpyruvate + ADP + CO_2) was simultaneously overexpressed [52,53]. More recently, E. coli PEPCK (pckA) was overexpressed together with the yeast anaerobic enzyme pyruvate carboxylase (PyC2, EC 6.4.1.1: pyruvate + ATP + CO_2 → oxaloacetate + ADP + Pi) [54]. Simultaneous activity of these enzymes results in hydrolysis of two ATP molecules for the formation of phosphoenolpyruvate (PEP) from pyruvate. Since, in glucose-grown cultures, the glycolytic enzyme pyruvate kinase (Pyk2, Cdc19, EC 2.7.1.40) converts PEP back to pyruvate with the formation of only a single ATP, the net result of this futile cycle is the hydrolysis of one ATP. The potential of this strategy was demonstrated by more ethanol production, related to yeast biomass, by the overexpression strain than by the control strain [54].

An inherent risk of the constitutive expression of futile cycles is that, in industrial processes, situations may occur in which a too large drain of the cellular ATP content can no longer be compensated for by faster alcoholic fermentation. In extreme situations, net ATP synthesis might even decrease below maintenance energy-requirements and cause cell death. Careful ‘tuning’ of the in vivo activity of engineered futile cycles can, in principle, address this problem in cultures grown under constant conditions in the laboratory. However, such tuning would be much more difficult to achieve in large-scale industrial processes, which are highly dynamic, for example as a consequence of changing sugar and ethanol concentrations. Application-oriented pathway-engineering studies therefore mostly focus on strategies that, instead, aim at a fixed, stoichiometric reduction of the ATP yield from ethanol fermentation.

3.2. Decreasing the ATP stoichiometry of yeast glycolysis

The bacterium Zymomonas mobilis employs the Entner-Doudoroff (ED) pathway for alcoholic fermentation. Instead of the 2 mol ATP/mol glucose generated in yeast glycolysis, this pathway has a net ATP yield of only 1 mol ATP/mol glucose [55,56]. As a consequence, high ethanol yields can be achieved in growing Z. mobilis cultures [55,56]. A now expired patent proposed functional expression of the ED pathway in S. cerevisiae (Fig. 2F) [57]. However, experimental studies failed to achieve the high in vivo activities of 6-phosphogluconate dehydrogenase (PGDH, EC 4.2.1.12: 6-phosphogluconate → 2-dehydro-3-deoxy-glucono-6-phosphate) in S. cerevisiae that would be required to demonstrate an impact on ethanol yield [58,59]. A limiting activity of PGDH, which contains an [4Fe-4S] iron-sulfur cluster [60], was attributed to the well-documented difficulties in expressing heterologous iron-sulfur-cluster enzymes in the yeast cytosol [61].

An alternative approach to reduce the ATP yield of glycolysis in S. cerevisiae was based on functional expression of a heterologous, non-phosphorylating, NADP^+ -dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN, EC 1.2.1.9: glyceraldehyde-3-phosphate + NADP^+ → 3-phosphoglycerate + NADPH), which bypasses the ATP-generating phosphoglycerate kinase reaction (Pgk1, EC 2.7.2.3: 1,3-bisphosphoglycerate + ADP → 3-phosphoglycerate + ATP) [62-64]. Strains engineered with this strategy increased the ethanol yield in anaerobic cultures by 3% [64] and 7.6% [62]. This increase was partly attributed to a lower ATP yield of glycolysis and partly to changes in redox-cofactor metabolism (see 4.2).

3.3. Altering topology and energy coupling of disaccharide metabolism and transport

In contrast to maltose which, as described above, is taken up by proton symport prior to hydrolysis [31,42], sucrose metabolism in wild-type S. cerevisiae strains is predominantly initiated by its extracellular hydrolysis to glucose and fructose, catalysed by invertase (Suc2, EC
cultures, generation of glycerol-3-phosphate by the Gpd1 and Gpd2 genes of the wild-type S. cerevisiae strain backgrounds and under different (semi-) anaerobic cultivation conditions, been shown to affect specific growth rates, activities of glycerol-3-phosphate dehydrogenase, by deletion of either GPD1 or GPD2 prevents anaerobic growth [74,75]. Anaerobic growth of gpd1Δ gpd2Δ strains can be rescued by supplementation of compounds such as acetaldehyde or acetoin, which can be reduced by intracellular NADH-dependent dehydrogenases [74,75]. Glyceraldehyde-3-phosphate dehydrogenase (gpd1) and glycerol-3-phosphate dehydrogenase (gpd2) genes of the wild-type S. cerevisiae strain, while its glycerol yield on glucose was 38% lower and its ethanol yield was 10% higher [43]. The increased ethanol yield was attributed to a combination of reduced NADH formation and increased ATP consumption in ammonium assimilation. In the second strategy, deletion of GDH1 was combined with overexpression of the NADH-dependent glutamate dehydrogenase GDH2 (EC 1.4.1.2: 2-oxoglutarate + NH4+ + NADH → glutamate + NAD+). This approach led to a 30% lower glycerol yield. However, the ethanol yield was hardly affected and the biomass yield was 12% higher than that of the reference strain. This observation was attributed to a reduced loss of carbon via CO2 formation in the oxidative pentose-phosphate pathway [18], which is the main source of NADPH in S. cerevisiae [85,86]. Since NADH re-oxidation in the first step of ammonium assimilation cannot completely replace glycerol formation, the GS-GOGAT strategy, as successfully implemented by Nissen et al. (2000) [43], left room for further reduction of glycerol yields.

4.2. Expression of NADP+–dependent, non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (gapN)

In S. cerevisiae, the oxidative step in glycolysis is catalysed by the strictly NADP+–dependent oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate by isoenzymes of glyceraldehyde-3-phosphate dehydrogenase (Tdh1, 2 or 3, EC 1.2.1.12). Based on stoichiometric modelling of yeast metabolism, Bro et al. (2006) identified expression of a heterologous non-phosphorylating, NADP+–dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN), which generates 3-phosphoglycerate instead of 1,3-diphosphoglycerate, as a promising option to increase ethanol yields (Fig. 3 C). Initial experimental verification of this model prediction by expression of Streptococcus mutans gapN showed a 44% lower glycerol yield in anaerobic, glucose-grown batch cultures than in a reference strain. No negative impact on specific growth rate or biomass yield was observed, but also the ethanol yield on glucose was not significantly altered [64]. Subsequent studies in which expression of Bacillus cereus gapN was tested, reported a 3.5% higher final ethanol concentration and a 23% reduction of the glycerol yield on sugar relative to a reference strain [63]. Expression of Bacillus cereus gapN in combination with deletion of GPD1, yielded a strain that exhibited a 49% lower glycerol yield and 8% higher ethanol yield than the wild-type reference strain. However, the engineered strain was found to be highly sensitive to osmotic stress, thereby precluding its use in high-gravity industrial ethanol fermentation. When osmotolerance was restored by overexpression of TPS1 and TPS2, which encode trehalose-6-phosphate synthase (EC 2.4.1.15: glucose-6-phosphate + UDP-glucose → UDP + trehalose-6-phosphate) and trehalose-6-phosphate phosphatase (EC 3.1.3.12: trehalose-6-phosphate + H2O → trehalose + P0), near-wild-type anaerobic growth rates were reported along with an up to 8% higher ethanol yield and 73%
Fig. 3. Schematic representation of pathway engineering strategies for minimizing formation of glycerol as ‘redox’ sink for re-oxidation of NADH generated in biosynthetic reactions during anaerobic growth of S. cerevisiae. A: Biosynthetic reactions require a net input of ATP and NADPH, while yielding NADH. Ammonium assimilation is the key contributor to NADH production, and replacing the NADP$^{+}$-dependent step by an NADH-dependent step, can reduce the NADH production in biosynthetic reactions. B: Native glycerol pathway. C: Bypass of NAD$^{+}$-dependent glyceraldehyde-3-phosphate dehydrogenase by heterologous non-phosphorylating, NADP$^{+}$-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN). D: Non-oxidative bypass of NAD$^{+}$-dependent glyceraldehyde-3-phosphate dehydrogenase by heterologously expressed phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). E: Re-oxidation of NADH by A-ALD-expressing strain, using exogenous acetate as electron acceptor. F: Re-oxidation of NADH enabled by combined expression of heterologously expressed NADH-dependent acetylating acetaldehyde dehydrogenase (A-ALD), phosphoketolase and phosphotransacetylase. G: Re-oxidation of NADH enabled by combined expression of heterologously expressed A-ALD and pyruvate-formate lyase. H: Combined expression of a heterologous NADH-dependent glycerol dehydrogenase and the native dihydroxyacetone kinase enables ethanol formation from glycerol when combined with strategies D, E, F and/or G.
lower glycerol yield, respectively [62]. In a further study [87], expression of gapN from Streptococcus mutans was combined with deletion of FPS1, which encodes a membrane channel protein involved in glycerol export, in some strains combined with overexpression of UTR1, which encodes S. cerevisiae NADH kinase (EC 2.7.1.86: ATP + NADH → ADP + P↓ + NADPH) [88]. While lower glycerol yields and higher ethanol yields were observed in micro-aerobic cultures, the engineered strains were unable to grow under fully anaerobic conditions.

4.3. NADH-dependent reduction of acetate to ethanol

In many fermentative bacteria, acetylating acetaldehyde dehydrogenase (A-ALD, EC1.2.1.10: acetyl-CoA + NADPH → acetaldehyde + CoA + NAD⁺) catalyzes a key reaction in alcoholic fermentation, that is followed by NADH-dependent reduction of acetaldehyde to ethanol [59]. The potential of using the combination of A-ALD and yeast alcohol dehydrogenase from Candida tropicalis to re-oxidize NADH in anaerobic S. cerevisiae cultures, and thereby replace glycerol as NADH redox sink for ethanol, was explored by expressing the A-ALD-encoding E. coli gene mphF in a gpdΔ strain [90]. Like other gpdΔ S. cerevisiae strains, the resulting strain did not grow anaerobically on glucose as sole carbon source. However, anaerobic growth was restored by addition of acetate to growth media (Fig. 3E). In anaerobic S. cerevisiae cultures, acetate is activated to acetyl-CoA the acetyl-CoA synthetase isoenzyme Acs2 (EC 6.2.1.1: acetate + ATP + CoA → acetyl-CoA + AMP + P↓) [91]. In anaerobic bioreactor batch cultures supplemented with 2 g/L acetate, the engineered strain did not produce glycerol and showed a 13% higher apparent ethanol yield on glucose (note that part of the produced ethanol was derived from acetate rather than from glucose). Under these conditions, the mphF-expressing strain grew at 44% of the specific growth rate of the GPD1 GPD2 reference strain [90]. Introduction, in the same gpdΔ genetic background, of a single copy of an expression cassette for eutE, an alternative E. coli A-ALD gene, increased specific growth rate to 84% of that of the reference strain [92]. When E. coli EutE was expressed in a GPD1 GPD2 S. cerevisiae strain, a mere 10% reduction of the amount of glycerol produced per gram biomass was observed in anaerobic, glucose-grown batch cultures supplemented with acetate. This observation indicated that the native glycerol pathway effectively competed with E. coli EutE for NADH in this genetic context. Deletion of GPD2, which encodes the redox-regulated isoenzyme of glycerol-3-phosphate dehydrogenase in S. cerevisiae, led to an 80% reduction of glycerol production, with a corresponding increase in acetate consumption [92].

Acetate is a common constituent and inhibitor of yeast performance in the hydrolysates of lignocellulosic biomass that are explored as feedstocks for ‘second-generation’ yeast-based ethanol production [93]. Since, in such processes, expression of A-ALD offers an option to convert an inhibitor into additional product, further pathway engineering strategies were explored to increase the amount of NADH available for acetate reduction and to improve robustness of engineered gpdΔ strains, A-ALD-expressing strains. To enable additional NADH generation, the native S. cerevisiae NADP⁻-dependent 6-phosphogluconate dehydrogenases Gnd1 and Gnd2 (EC 1.1.1.44: 6-phosphogluconate + NADP⁺ → ribulose-5-phosphate + CO₂ + NADH) were replaced by the NAD⁺-dependent enzyme GndA from Methylobacterium flagellatus (EC 1.1.3.343). To force flux through the resulting, now partially NADH-coupled oxidative pentose-phosphate pathway, ALD6, which encodes NAD⁺-dependent acetaldehyde dehydrogenase (EC 1.2.1.5: acetaldehyde + NAD⁺ → acetaldehyde + NADH), was deleted. This metabolic engineering strategy resulted in a 29% higher acetate consumption per gram biomass than in the parental gpdΔ strain, EutE-expressing strain [94]. Relative to a congenic GPD1 GPD2 reference strain, the engineered strain showed a 13% higher ethanol yield and a 29% lower specific growth rate.

An alternative strategy to boost the acetate-reducing capacity of EutE-expressing strains focused on changing the cofactor preference of alcohol dehydrogenase, which in S. cerevisiae is strictly NADH-dependent [95]. Relative to an industrial S. cerevisiae strain expressing Bifidobacterium adolescentis EutE in a gpdΔ strain background, a further engineered strain that expressed an NADPH-dependent alcohol dehydrogenase from Entamoeba histolytica, combined with overexpression of S. cerevisiae NADP-dependent glucose-6-P dehydrogenase (Zwf1, EC 1.1.1.49: glucose-6-phosphate + NADP⁺ → 6-phospho-glucono-1,5-lactone + NADPH) and acetyl-CoA synthetase (Acs2) showed an almost 3-fold higher acetate consumption [95].

A different strategy to increase the potential for acetate reduction by A-ALD expressing strains is to enable anaerobic co-conversion of glycerol, which is left in the final phases of fermentation or obtained from post-distillation stills [96], to ethanol. In the patent literature, an NADH-specific glycerol dehydrogenase from E. coli (gldA, EC 1.1.1.6: glycerol + NAD⁺ → dihydroxyacetone + NADH + H⁺) was expressed together with an additional copy of DAK1, encoding dihydroxyacetone kinase (EC 2.7.1.29: dihydroxyacetone + ATP → dihydroxyacetone phosphate + ADP) [97,98]. Combined with enzymes from the lower half of glycolysis, pyruvate decarboxylase and alcohol dehydrogenase, GldA and Dak1 enable conversion of glycerol to ethanol with the formation of 1 mol of NADH (Fig. 3H). When, besides sugars, glycerol and acetate are present as an additional substrates in A-ALD expressing cultures, glycerol conversion to ethanol acts as source of NADH enabling more acetate reduction. Indeed, high apparent ethanol yields of 0.48–0.50 g ethanol per gram of glucose were reported for S. cerevisiae strains in which gldA and DAK1 overexpression was combined with expression of E. coli mphF or EutE [97,98].

4.4. Integration of acetyl-CoA reduction by A-ALD in yeast sugar metabolism

Organic acid concentrations in ‘first generation’ feedstocks for yeast-based ethanol production are generally around 1.3 g/L, [37,99], which limits the potential impact of the replacement of glycerol production by reduction of exogenous acetate via an engineered A-ALD pathway. In such settings, NADH re-oxidation by A-ALD could still replace glycerol production if acetyl-CoA is formed from glucose by pathways that yield fewer than 2 mol of NADH per mole of acetyl-CoA. The patent literature describes two strategies to achieve this goal, of which the first is based on heterologous expression of a bacterial pyruvate formate-lyase (PFL; EC 2.3.1.54: pyruvate → acetyl-CoA + formate) in A-ALD-expressing S. cerevisiae [100,101] (Fig. 3E). PFL, which is an oxygen-sensitive enzyme, was shown to be able to functionally replace the native pathway for acetyl-CoA synthesis in anaerobic S. cerevisiae cultures [102,103]. Synthesis of acetyl-CoA via glycolysis and PFL yields only one NADH per acetyl-CoA and thus enables a net reduction of one NADH when combined with ethanol production via A-ALD and yeast alcohol dehydrogenase. To prevent NADH formation by the yeast formate dehydrogenases Fdh1 and Fdh2 (EC 1.17.1.9: formate + NAD⁺ → CO₂ + NADH; [104]), it was proposed to delete Fdh1 and Fdh2 from PFL/A-ALD-expressing strains [100,101].

A second strategy for coupling A-ALD to sugar metabolism proposed in the patent literature [105] is based generation of acetyl-CoA through phosphoketolase (EC 4.1.2.9) and phosphotransacetylase (EC 2.3.1.8) (Fig. 3F). In this strategy, xylulose-5-phosphate is first formed from glucose in a redox-cofactor neutral manner via the enzymes of the non-oxidative pentose-phosphate pathway. This sugar phosphate is then converted into glyceraldehyde-3-phosphate and acetyl phosphate by a heterologously expressed phosphoketolase (PK, EC 4.1.2.9: xylulose-5-phosphate + P↓ → acetyl-phosphate + glyceraldehyde-3-phosphate + H₂O). Subsequently, a heterologously expressed phosphotransacetylase (PTA, EC 2.3.1.8: acetyl-phosphate + CoA → acetyl-CoA + P↓) converts acetyl phosphate to acetyl-CoA. This pathway has been successfully used for the ATP-efficient generation of acetyl-CoA as a precursor for aerobic product formation by engineered S. cerevisiae strains [106,107]. While the exact impact on ethanol yields will depend
on strain and process characteristics, both pathways have the theoretical potential to completely replace the role of glycerol formation in NADH re-oxidation.

4.5. Expression of Calvin-cycle enzymes

Phosphoribulokinase (PRK, EC 2.7.1.19: ribulose-5-phosphate + ATP → ribulose-1,5-biphosphate + ADP) and ribulose-1,5-biphosphatase carboxylase/oxygenase (Rubisco, EC 4.1.1.39: ribulose-1,5-biphosphate + CO₂ + H₂O → 2 glyceraldehyde-3-phosphate + 2H⁺) are the two key enzymes of the Calvin cycle for autotrophic CO₂ fixation. By capturing CO₂, these enzymes together have the potential to generate a redox-cofactor-neutral bypass of the oxidative glyceraldehyde-3-phosphate dehydrogenase reaction in glycolysis when ribulose-5-phosphate, the substrate of phosphoribulokinase, is generated from glucose via the reactions of the non-oxidative pentose-phosphate pathway (Fig. 3D). In theory, this bypass should enable the use of ethanol formation as a redox sink for NADH generated in biosynthetic reactions. This hypothesis was tested by Guadalupe-Medina et al. (2013) [108], who demonstrated the presence of a functionally active Rubisco in cell extracts of an engineered S. cerevisiae strain that co-expressed the Thioecuclis denitrificans type-II Rubisco CbbM with the E. coli chaperonins GroEL and GroES. Co-expression of CbbM, GroEL, GroES with spinach phosphoribulokinase was shown to result in a 90% lower glycerol yield and a 10% higher ethanol yield in anaerobic sugar-limited chemostat cultures grown at a dilution rate of 0.05 h⁻¹ and sparged with CO₂-enriched nitrogen [108]. In line with the low affinity of CbbM for CO₂ [109], a less pronounced effect on glycerol and ethanol yields was observed when cultures were sparged with pure nitrogen gas.

Papapetridis et al. (2018) observed that an S. cerevisiae strain that combined constitutive expression of PRK, Rubisco, GroEL and GroES showed only a modest reduction of glycerol in fast-growing anaerobic batch cultures on glucose than the slow-growing chemostat cultures studied by Guadalupe-Medina et al. (2013). To improve competition of the Rubisco pathway for NADH with the native glycerol pathway, GPD2 was deleted and the four key enzymes of the non-oxidative pentose phosphate pathway were overexpressed. In addition, PRK was expressed from a weaker, anaerobically inducible promoter to avoid reported toxic effects of PRK overexpression in microorganisms [110,111] during aerobic pre-cultivation. The resulting strain retained a wild-type growth rate in anaerobic, glucose-grown batch cultures, while showing an 86% lower glycerol yield and 15% higher ethanol yield on glucose than a congenic reference strain [77].

The strategies discussed above were first designed to reduce or eliminate the need for glycerol formation in alcoholic fermentation of disaccharides or hexoses. However, they can similarly be employed in conversion of other sugars into ethanol. Xylose-utilizing S. cerevisiae have been engineered either based on the functional expression of the fungal xylose reductase (XR, EC 1.1.1.307: xylitol + NAD(P)⁺ → xylitol + NAD(P)⁺) and xylitol dehydrogenase (XDH, EC 1.1.1.9: xylitol + NAD⁺ → xylulose + NADH), or the expression of a bacterial xylose isomerase (EC 5.3.1.5: xylitol → xylulose). A key challenge in the strategy based on XR and XDH is that XR typically prefers NADPH as cofactor, while XDH exclusively uses NAD⁺ [112]. As a consequence of this cofactor imbalance, xylitol is formed as a byproduct. Changing the cofactor preference of ammonium assimilation as demonstrated by Nissen et al. (2000) [43] facilitated re-oxidation of NADH generated in the XDH reaction and improved ethanol yield in an XR/XDH-based S. cerevisiae strain [113]. Combined functional expression, of PRK and Rubisco [114,115]; phosphoketolase and phosphotransacetylase [116]; or GAPN [64] were similarly applied to improve redox co-factor balancing in XR/XDH-based strains and, thereby, ethanol yields on xylose.

5. Model-based comparison of maximum theoretical impact of individual engineering strategies

Experimentally determined ethanol yields achieved with the pathway engineering strategies discussed in paragraphs 3 and 4 (Table 1) can be influenced by experimental conditions as well as by the S. cerevisiae genetic background into which genetic modifications were introduced, for example due to different biomass compositions. To eliminate these factors, different pathway strategies were implemented in a stoichiometric model of the core metabolic network of S. cerevisiae [117] and used to calculate growth stoichiometries of anaerobic, sugar-grown cultures (Table 2). Although the resulting estimates cannot be used to predict performance of strategies in specific strain backgrounds or processes, they do enable comparison of the maximum impact of the different strategies and identification of trade-offs.

To evaluate pathway engineering strategies aimed at reducing the ATP yield from sugar fermentation, two scenarios were simulated. In the first, glucose import required a net input of 0.5 ATP, which corresponds to the ATP yield per hexose unit in strains that combine sucrose-proton symport with intracellular sucrose hydrolysis [70]. The second scenario, in which glucose import required 1 ATP, corresponds to a situation in which hexose transport occurs via symport with a proton or, alternatively, glucose is fermented via an alternative glycolytic pathway with a

| Strategy | Genotype | Glycerol yield | Growth rate | Ethanol yield | Reference |
|----------|----------|----------------|-------------|--------------|-----------|
| Altered cofactor specificity of ammonium assimilation | gdhΔ1 GLN1 GLT1↑ | -38% (g/g glucose) | -10% | +10% (g/g glucose) | [18] |
| | gdhΔ1 GDH2↑ | -30% (g/g glucose) | -5% | +3% (g/g glucose) | [18] |
| NADH-dependent reduction of acetate to ethanol | gpdΔ1 gpdΔ2 Ec-mpbF | -100% (g/g glucose) | -56% | +13% (g/g glucose) | [90] |
| (Ec → E. coli) | gpdΔ1 gpdΔ2 Ec-aceE | -100% (g/g glucose) | -7% | +9% (g/g glucose) | [94] |
| NADH-dependent reduction of acetate to ethanol with increased NADH generation via pentose-phosphate pathway | gpdΔ2 gpdΔ1 gndΔ4 aldΔ4 gpdΔ1 gpdΔ1 Ec-aceE | -100% (g/g glucose) | -29% | +11% (g/g glucose) | [94] |
| NADH re-oxidation via expression of Calvin-cycle enzymes, optimized for anaerobic growth rate (S. oleracea, Td – Thioecuclis denitrificans) | gpdΔ2 RPE1 TKL1 TKL1 Δ TKL1 Δ TKL1 ↑ So-prk Td-cbbM (9 copies) Ec-groES, Ec-groEL | -86% (g/g glucose) | 0% | +15% (g/g glucose) | [77] |
| Reduced NADH and ATP formation in glycolysis by expression of gapN | Sm. gapN | -40% (g/g glucose) | 0% | +2% (g/g glucose) | [64] |
| (Sm – Streptococcus mutans) | gpdΔ1 Sm.gapN TFS1↑ TFS2↑ | -73% (g/g glucose) | 0% | +8% (g/g glucose) | [62] |
net ATP yield of 1 mol/mol glucose (e.g. the Entner-Doudoroff pathway) in combination with a glucose facilitator. At a specific growth rate of 0.30 h⁻¹, simulation of these scenarios gave predicted increases of ethanol yield on hexose equivalents of 8.1% and 16.2%, respectively (Table 2). Due to a larger impact of a constant maintenance-energy requirement at low growth rate [22,118], predicted benefits of these engineering strategies declined as the specific growth rate approached zero (Table 2). An important consequence of these two strategies was that, at each specific growth rate, specific rates of sugar conversion were 35% and 100% higher, respectively, than in the reference situation (Supplementary Table 1). Especially at high specific growth rates, which are important for supporting high volumetric productivities in industrial batch processes, achieving such high conversion rates may be challenging due to the requirement for a large resource allocation to glycolytic proteins [119,120] or for membrane space to accommodate the required number of sugar transporters [121]. In addition, concomitant reductions of the biomass yield on sugar by 25% and 50%, respectively (Supplementary Table 2) may cause economic trade-offs when surplus yeast biomass is sold as a co-product for application in animal feed products [12].

To assess the maximum theoretical impact on ethanol yield of the strategies focused on redox cofactor balancing, glycerol production was set to zero, so that re-oxidation of NADH generated in biosynthesis occurred exclusively via the engineered pathways. At a specific growth rate of 0.3 h⁻¹, the PFL/A-ALD, PK/PTA/A-ALD and PRK/Rubisco strategies yielded predicted improvements of the ethanol yield on glucose of 8.7%, 9.7% and 11.9%, respectively. The predicted differences between the impacts of the three strategies can be predominantly attributed to the different net ATP and ethanol yields for NADH re-oxidation via these pathways. Due to different ATP and carbon efficiencies of these heterologous pathways, implementation of these redox engineering strategies in the stoichiometric model also led to higher predicted biomass yields on glucose and correspondingly lower specific rates of glucose consumption (Supplementary Tables 1 and 2). Thus, in contrast to strategies aimed at reducing the ATP stoichiometry of sugar fermentation, their industrial implementation should not be affected by a potentially limited capacity of sugar fermentation and/or transport or by a trade-off with revenues from surplus yeast biomass. As observed for the strategies aimed at engineering ATP coupling of sugar dissimilation, the impact of the redox-engineering strategies on ethanol yield declined with decreasing specific growth rate and, at the lowest simulated growth rate (0.001 h⁻¹), the predicted increase of ethanol yield on glucose was only approximately 1%.

For several of the strategies, experimental studies (Table 1) yielded larger improvements of the ethanol yield than the maximum theoretical improvements shown in Table 2. In addition to differences in biomass composition and ethanol yields of reference S. cerevisiae strains, these differences may reflect unintended impacts of genetic modifications on cellular energy requirements. For example, high-level expression of heterologous proteins has been associated with increased cellular energy requirements [122,123] which, in anaerobic cultures, can contribute to higher ethanol yields. In addition, alteration of the expression of membrane proteins may potentially lead to increased ATP dissipation, for example by futile cycling of glucose through overexpressed Mal11 and Hxt transporters.

6. Discussion and outlook

As outlined in this review, multiple pathway engineering strategies have been demonstrated to improve ethanol yields on sugars in anaerobic laboratory cultures of S. cerevisiae by altering the ratio of the formation of ethanol, biomass and glycerol. However, observations made under controlled conditions in laboratory-scale media are not necessarily representative for industrial processes. Even in anaerobic glucose-limited cultures of wild-type S. cerevisiae, ethanol yields on glucose approach the theoretical maximum of 2 mol ethanol/mol glucose at near-zero growth rates [22]. Consequently, predicted benefits of all investigated pathway engineering strategies strongly depend on specific growth rate (Table 2). In industrial batch processes, the impact of the described engineering strategies on ethanol yield is likely to be highest during the initial phase in which vigorous growth occurs. Conversely, during the final phases of a batch fermentation process, where growth has essentially ceased and high ethanol concentrations lead to an increased maintenance energy requirement, their impact may well be negligible.

In addition to the inherent dynamics of industrial processes, development of industrial strains should take into account trade-offs between ethanol yield and other performance indicators. In particular, an improved product yield should not go at the expense of productivity. With few exceptions, academic studies reported that S. cerevisiae strains which were successfully engineered for improved ethanol yield grew slower than their non-engineered parental strains (Table 1). The extensive synthetic biology toolbox for genetic modification of S. cerevisiae, including approaches such as multiplexed Cas9-mediated genome editing and in vivo assembly and chromosomal integration of synthetic DNA fragments [124,125], is therefore intensively used to explore options for maximizing ethanol yields by engineering its metabolic network. In addition, pathway engineering in this yeast benefits from the availability of genome-scale metabolic models (for reviews see Refs. [126,127]), which allow for fast predictions of the impact of
genetic interventions on distribution of fluxes in metabolic networks. A dedicated study on PRK/Rubisco based strains [77] illustrates that restoring the specific growth rate of engineered strains to wild-type levels may require substantial additional engineering. Alternatively, adaptive laboratory evolution and/or reverse engineering of evolved strains [128,129] can be used for this purpose. Another important trade-off concerns cellular robustness. Until engineering strategies are available that fully restore osmotolerance in glycerol-negative strains, strategies aimed at reducing glycerol production should not completely eliminate glycerol production [76]. In addition to targeted engineering strategies, robustness may be increased by using natural and industrial Saccharomyces strains with a high innate tolerance to industrially relevant stress factors in strain improvement programmes [130,131]. Temperature, pH, pCO2, ethanol concentration and their dynamics in large-scale industrial processes may affect the impact of engineering strategies, thus requiring process-specific strain optimization. The economic significance of small differences in ethanol yield, combined with the use of non-defined industrial media and dynamic industrial processes, raises non-trivial challenges in setting up high-throughput cultivation and analysis systems that faithfully predict strain performance in real-life applications. Although companies tend not to disclose the genetic make-up of industrial strains, the introduction of multiple ‘high-ethanol-yield’ Saccharomyces strains into USA-based ethanol plants [132–134] indicates that at least some of the strategies discussed in this review already contribute to profitability and sustainability of industrial ethanol production. Introduction into Brazil, the second-largest ethanol-producing economy, may involve additional challenges related to the use of non-aspectively operated, extended production campaigns. This mode of operation not only poses high demands on the genetic stability of engineered strains, for example to prevent recovery of glycerol production by strains with down-regulated GPD1 and/or GPD2 expression, but also on their ability to compete with ‘wild’ strains entering the process [135].

Improving ethanol yield on fermentable sugars is by no means the only target of metabolic engineering studies related to yeast-based ethanol production. Other targets of intensive research include the reduction of processing costs by expression of polysaccharide hydrolases [136], extending substrate range to convert more fermentable substrates to ethanol [137,138], improving performance at high temperature to improve heat economy and cope with process temperature profiles [139], increasing yeast tolerance to process inhibitors and ethanol [139,140], improving osmotolerance of engineered strains with reduced glycerol formation [16,141] and simplification of nutritional requirements of industrial strains [142–145]. In addition, integration of corn-fiber from 1.5G processes [21] and reducing the need for antibiotics [139,146] are actively explored. Combination of these and other relevant traits with strategies for improving product yield, in Saccharomyces and potentially also in other yeast species [130,147,148] will, in the coming years, continue to generate interesting challenges for academic and industrial research.

Author contributions
AA: Formal analysis, Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing SV: Investigation, Data Curation, Writing – Original Draft, Writing – Review & Editing, Visualization WG Formal analysis MJ: Writing – Review & Editing JP: Conceptualization, Writing – Original Draft, Writing – Review & Editing RM: Writing – Original Draft, Writing – Review & Editing, Project administration.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.12.010.

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