Rewiring yeast metabolism to synthesize products beyond ethanol

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Abstract:

Saccharomyces cerevisiae, Baker's yeast, is the industrial workhorse for producing ethanol and the subject of substantial metabolic engineering research in both industry and academia. *S. cerevisiae* has been used to demonstrate production of a wide range of chemical products from glucose. However, in many cases, the demonstrations report titers and yields that fall below thresholds for industrial feasibility. Ethanol synthesis is a central part of *S. cerevisiae* metabolism and redirecting flux to other products remains a barrier to industrialize strains for producing other molecules. Removing ethanol producing pathways leads to poor fitness, such as impaired growth on glucose. Here, we review metabolic engineering efforts aimed at restoring growth in non-ethanol producing strains with emphasis on relieving glucose repression associated with the Crabtree effect and rewiring metabolism to provide access to critical cellular building blocks. Substantial progress has been made in the past decade, but many opportunities for improvement remain.

The yeast *Saccharomyces cerevisiae* has long been an important species to humans, from its use in brewing and baking to its role as a model organism for studying eukaryotic biology. Over the past few decades, *S. cerevisiae* has also gained prominence as a platform for synthesizing chemical products due to several attractive attributes: a well-developed genetic toolkit [1,2]; fast growth (doubling time ~90 min [3]); and tolerance to a variety of industrial stressors [4]. To date, *S. cerevisiae* has been engineered to produce molecules used in a wide variety of applications including: biofuels, pharmaceuticals, food additives, beauty agents, bulk-chemicals and specialty-chemicals [5,6]. In order for these products to be produced economically, high titers, rates, and yields must be achieved in cultures grown on low-cost feedstocks. This means that metabolic engineering strategies must overpower the dominant native flux to ethanol. In other organisms, such as *Escherichia coli*, this can be simply accomplished by deleting the genes involved in ethanol biosynthesis [7]. Unfortunately, ethanol production is integrated with many central aspects of *S. cerevisiae* biology including regulation, substrate uptake, and energy generation. As such, substantial efforts have been undertaken in the metabolic engineering community to break *S. cerevisiae*'s reliance on and preference for ethanol production. These efforts are the subject of this article.

The significance of ethanol production is clearly seen in the Crabtree-Warburg effect, a central feature of *S. cerevisiae* metabolism. Under glucose rich conditions, Crabtree-positive yeast ferment sugars into ethanol even under aerobic conditions. The Crabtree effect, thoroughly reviewed elsewhere [8–10], is triggered by overflow metabolism at the pyruvate node (short-term effect) and by glucose-driven repression of respiratory enzymes (long-term effect). The evolutionary driving force behind the Crabtree effect is still debated [10,11], but the leading theory opines that it is an economical approach to maximize growth (biomass generation), while minimizing resource allocation to protein synthesis [12]. The theory is supported by the fact that the catalytic capacity of fermentation is higher than respiration (more ATP is produced per protein mass) [12]. Simulations have also captured the Crabtree effect and overflow metabolism by using an enzyme-constraint based approach to optimize biomass generation in a genome-scale metabolic model [12,13]. While advantageous in the natural world, these effects are not helpful to industrial chemical producing yeasts. Ethanol produced either via fermentation or the Crabtree effect reduces flux to desired chemical products and therefore needs to be by-passed to engineer industrially viable biocatalysts.

Efforts to eliminate ethanol production have proven challenging since ethanol synthesis plays an essential role in redox balance (i.e. reoxidizing NADH produced in glycolysis) and other aspects of *S. cerevisiae* biology. Non-ethanol producing strains have been created by deleting either the complete set of three pyruvate decarboxylases (*PDC1, PDC5, and PDC6*) [14] or the set of six alcohol dehydrogenases (*ADH1, ADH2, ADH3, ADH4, ADH5, and SFA1*) [15], which catalyze the final two catalytic steps in ethanol production respectively (**Figure 1**). Strains lacking these enzymes produce no ethanol but also experience severe physiological defects [16]. Complete deletion of Pdc activity prevents the production of acetaldehyde which in addition to being the substrate for ethanol production is also the primary precursor of cytosolic acetyl-CoA. Without supplementation of ethanol, acetate, or heterologous pathways discussed below, cells cannot produce sufficient acetyl-CoA flux to support fatty acid biosynthesis and other biosynthetic requirements. When supplemented, strains still grow slowly in part because cells are unable to rapidly replenish the NAD^+ supply required for glucose catabolism. In the presence of high glucose concentrations, this redox imbalance is exacerbated by native regulation of key catabolic

components. In summary, rerouting carbon flux away from ethanol requires an alternate, balanced, high-flux NADH-oxidizing pathway, an alternative cytosolic acetyl-CoA generation pathway, and fine-tuning of internal metabolic regulation.

This review highlights the recent advances towards abolishing the Crabtree effect in *S. cerevisiae* and redirecting flux to non-ethanol products. We summarize strategies to restore redox balance through respiration and alternative pyruvate-derived products as well as strategies to regenerate cytosolic acetyl-CoA in Pdc$^-$ strains. In addition, we tabulated recent examples of how these strategies have been successfully implemented in chemical producing strains. This review serves as a guide for understanding the facets of *S. cerevisiae*'s ethanol-dependencies, the strategies that have been publicly disclosed for circumventing these obstacles, and the remaining challenges to using *S. cerevisiae* for producing chemical products beyond ethanol.

1. Cytosolic acetyl-CoA generation in Pdc$^-$ *S. cerevisiae*

Pdc$^-$ strains are auxotrophic for C_2-compounds due to their inability to produce cytosolic acetyl-CoA with the native pyruvate dehydrogenase (Pdh) bypass consisting of pyruvate decarboxylase (Pdc), acetaldehyde dehydrogenase (Ald), and acetyl-CoA synthetase (Acs) (**Figure 1)**. Acetyl-CoA supply is further complicated by the fact that in eukaryotes it cannot transverse the membranes of subcellular compartments such that mitochondria-made acetyl-CoA cannot be directly exported to the cytosol. Some reports propose that evolved Pdc$^-$ strains circumvent this barrier through use of a CoA-transferase, Ach1p, that converts acetyl-CoA into acetate which can cross into the cytosol and subsequently be reactivated as acetyl-CoA at the cost of ATP [17]. Another report suggests that the native shuttle system is not sufficient [18]. Alternatively, the C_2-auxotrophy can be circumvented by providing a heterologous synthesis pathway or by supplementation with a C_2-compound (ethanol or acetate). Alternative acetyl-CoA producing pathways include: pyruvate-formate lyase (Pfl), acetylating acetaldehyde dehydrogenase (A-Ald), cytosolic-pyruvate dehydrogenase (Pdh_{cyto}), pyruvate oxidase (Po)/phosphotransacetylase (Pta), phosphoketolase (Pk)/phosphotransacetylase (Pta), threonine aldolase (Gly), carnitine shuttle (Cat), and citrate-oxaloacetate shuttle (Cit/Acl) (**Figure 1**) [19,20]. The genetics and biochemistry of these pathways is thoroughly reviewed in reference [20]. While many alternative pathways exist, heterologous expression by itself is not sufficient to restore growth in a Pdc$^-$ strain to wild-type levels. As discussed below, additional modifications are necessary, but overcoming acetyl-CoA auxotrophy is a critical first step.

2. Balancing NAD^+ regeneration with glycolysis in the absence of ethanol synthesis

It is widely known that ethanol is produced during yeast fermentation in order to regenerate the NAD^+ needed to enable glycolysis. Analogously, when oxygen is available, non-ethanol producing yeast grown in glucose-limited conditions regenerate NAD^+ through oxidative phosphorylation, which can support a low glycolytic flux. However, at high glucose concentrations, genes involved in oxidative phosphorylation are downregulated while genes involved in glucose transport and glycolysis are upregulated [21]. This phenomenon is controlled by the three glucose sensing systems (Rgt2p/Snf3p, Snf1p/Mig1p, and cAMP/PKA; see Figure 3 of reference [22] for a detailed depiction) and is associated with the Crabtree effect [9,22].

Elevated rates of glucose import ultimately lead to an increased glycolytic flux above the catalytic capacity of the respiratory chain, resulting in insufficient NAD^+ regeneration and a bottleneck in the catabolism of glucose [8]. The bottleneck is caused in part by the indirect repression of the mitochondrial pyruvate dehydrogenase (Pdh) by Mig1p [23]. Thus, under excess glucose conditions, non-ethanol producing strains do not grow due to the NADH/NAD^+ redox imbalance caused by a metabolic bottleneck at the pyruvate node. Strategies to overcome the imbalance include relieving glucose repression caused by the Crabtree-effect, restricting glucose uptake, downregulating glycolytic flux, providing alternative redox sinks, and throttling the delivery of glucose to cells with fed-batch bioreactors [24].

2.1 Adaptive Laboratory Evolution

Adaptive laboratory evolution (ALE) has been widely used to enhance growth of Pdc^- strains on glucose since the early 2000s. Recent efforts have tried to understand the role and optimize the function of ALE-created mutations in regulating glucose catabolism, ethanol production, and the Crabtree effect. Through multiple studies, researchers identified mutations or deletions in *MTH1*, a transcriptional repressor in the Rgt2p/Snf3p glucose-sensing cascade involved in hexose transporter (Hxt) expression [16,18,25,26]. These mutations reduce expression of the Hxts, thereby limiting glucose uptake and preventing the respiratory chain from being overloaded. Cells carrying a *mth1* mutation can grow on glucose because glucose-repression is alleviated, however, growth comes at a reduced rate (less than 30% of Pdc^+ strains), which is insufficient for industrial purposes. In an attempt to overcome the growth rate limitation, researchers performed extra rounds of ALE starting with strains carrying the *mth1* mutation and isolated a strain with a higher growth rate when grown on 2% glucose. The resulting strain had a mutation in *YAK1* and a growth rate that increased 2.5 fold over the base strain, albeit at rates still below the Pdc^+ parent [27]. *YAK1* encodes a serine/threonine protein kinase involved in regulating cell growth in the presence of glucose. In a separate study, downregulating *PYK1*, a.k.a. *CDC19*, which encodes the major pyruvate kinase, was also shown to relieve glucose repression in the context of another Pdc^- strain [28]. Pyk1p is considered a control node in glycolysis and its activity is tightly regulated by fructose-1,6-biphosphate (FBP) concentrations. Engineering the allosteric sites on Pyk1p could be an alternative strategy to the regulatory approaches discovered in the evolutionary studies. While ALE has provided the initial insights into the importance of the regulatory network in allowing Pdc^- strains to grow on glucose, the problem is not yet solved. Evolved mutants often have undesirable characteristics, such as lower than wild-type growth, that must be overcome before these strains can become useful platforms for bioproduction.

2.2 Rational re-wiring of native sugar metabolism regulation

Increasing the catalytic capacity of the respiratory chain by rational engineering is also a valid yet underdeveloped strategy for enabling Pdc^- strains to grow on glucose. The signaling and regulation involved in the Crabtree effect have been heavily studied, and recent work has shown that the Snf1p/Mig1p glucose repression pathway [29] as well as the ratio of glucose-6-phosphate (G6P) to FBP [30] is directly linked to the Crabtree phenotype. The Snf1p signaling pathway negatively regulates genes associated with the uptake and catabolism of non-glucose

sugars and expression of gluconeogenesis, respiratory, and Hxt genes by direct interaction with many transcription factors such as Mig1p, Rgt1, Msn2, and Cat8 [9,23]. Deletion of *SNF1*, *HXK2*, or *MIG1* increases respiratory capacity and reduces overflow metabolism, suggesting that these regulators are promising targets for shifting production from ethanol to other compounds [29,31]. This strategy is supported by increased transcript levels of Mig1p-mediated repressed genes in Pdc$^-$ strains that have undergone ALE [26]. Tps1p is also an important glycolytic regulator that has been recently linked to Snf1p signaling and may provide another engineering target [32]. The intentional rewiring of regulation is a promising strategy for increasing the growth rates of Pdc$^-$ strains by increasing the respiratory capacity rather than limiting sugar metabolism. Furthermore, it could allow for intentional tuning of metabolism while minimizing undesirable effects. Alternatively, non-Crabtree yeasts such as *Kluyveromyces. lactis*, which natively contain the desired regulatory structure, could be engineered to produce desired products.

2.3 NADH-oxidation coupled to pyruvate-derived metabolites

While strategies that reduce glucose import lead to improved growth, the glucose utilization rate is an important parameter in industrial bioprocessing that should be as large as possible. Alternatively, the NADH/NAD$^+$ redox balance in Pdc$^-$ strains can be maintained by producing products that require the same amount of reducing equivalents as ethanol. Pyruvate is a common metabolic node from which these products are derived (**Figure 2**). Products such as 2,3-butanediol [25,27,33–36] and lactate [37–41] can directly replace ethanol fermentation and each product has been produced in substantial quantities in Pdc$^-$ strains. Malate production is also redox-balanced with glycolysis, but requires ATP [42]. Other desirable products such as isobutanol and free fatty acids require NADPH reducing equivalents, necessitating a means for converting glycolytic NADH into NADPH [28,43]. This can be overcome by introducing a transhydrogenase reaction (NADPH + NAD$^+$ ⇌ NADP$^+$ + NADH) into *S. cerevisiae* [28,44,45]. Heterologous expression of an *E. coli* transhydrogenase in Pdc$^-$ *S. cerevisiae* has been shown to increase growth and pyruvate production [46]. Similarly, relocalization of *S. cerevisiae* malic enzyme to the cytosol creates a transhydrogenase cycle that has been shown to improve isobutanol production [47]. Alternatively, diverting carbon from glycolysis into the pentose phosphate pathway makes more NADPH available and can increase both free fatty acid [28] and isobutanol [48] production. Engineering NADPH-dependent enzymes to instead utilize NADH is another approach and has been demonstrated in isobutanol production, though with limited effect on production in *S. cerevisiae* [43,49].

2.4 Regenerating NAD$^+$ for anabolism

Though replacing ethanol with other products can balance glycolytic NADH generation, *S. cerevisiae* anabolism also produces NADH, which is normally balanced by glycerol production [50]. Glycerol production in *S. cerevisiae* can divert up to 8.5% of carbon, making its removal via *GPD1* and *GPD2* deletion a common metabolic engineering strategy, even when ethanol is the desired product [51]. However, glycerol is important in *S. cerevisiae* osmotic tolerance so completely blocking its synthesis is often undesirable [52]. Glycerol formation can be reduced by providing other routes to oxidize NADH, such as through succinate production, which requires two reducing equivalents per pyruvate. Succinate has been produced in Pdc$^-$ *S.*

cerevisiae in significant amounts, but only under aerobic conditions [53,54]. Other NADH oxidizing routes include heterologous expression of a water-forming NADH oxidase [33,36] or an alternative oxidase [8]. These enzymes require aerobic conditions to function, and thus, it is common to see them used under semi-aerobic conditions with a fermentation product. Carbon fixation is also a common sink of excess reducing power in photoautotrophs. When Calvin-cycle enzymes RuBisCo and phosphoribulokinase were heterologously expressed in *S. cerevisiae*, it coupled NADH oxidation with CO_2 reduction and significantly decreased glycerol production [55]. Acetate, which is present in high amounts in lignocellulosic hydrolysates, can also be fermented into ethanol to oxidize residual NADH via Ald and Adh and reduce glycerol formation (**Figure 2**) [56]. In many cases, it may be possible to couple NADH recycling with production of a desired product. In others, feedstocks may need to be optimized such that redox balance, ATP generation, and product synthesis are optimized.

3. Metabolic rewiring to reduce ethanol production without its complete elimination

As discussed above, eliminating ethanol production in yeast is difficult, especially if the adverse impacts on physiology limit desired applications. For this reason, researchers have also explored reducing, but not eliminating, ethanol production. Successful strategies include controlling glycolytic flux with a metabolic circuit, utilizing a reduced activity Pdc from a Crabtree-negative yeast [35,36], or co-catabolizing non-preferred carbon sources, such as xylose or glycerol.

3.1 Redirecting flux using metabolic circuits

Controlling when and where carbon flux is directed in a fermentation process can be achieved with dynamic control strategies, including inducible-metabolic valves [57–59]. In practice, the fermentation occurs in alternating decoupled phases (growth and production). During the growth phase, biomass generation is promoted via NAD^+ regeneration through ethanol fermentation as flux is directed through glycolysis. During the production phase, ethanol flux is restricted and resources are directed towards a product of interest. This switch is achieved by turning on transcription of pathway genes using either a chemical inducer or light via an optogenetics switch. The balance between growth and product synthesis has been successfully demonstrated with isobutanol production [60,61]. Expansion of this idea will depend on finding ways to minimize the amount of time cells spend in the biomass generation phase such that maximum titers, rates, and yields of products can be achieved.

3.2 Non-preferred carbon sources

Many of the challenges to removing ethanol production depend on glucose being present. Using alternative carbon sources, such as xylose or glycerol that do not induce the Crabtree effect, is another way to decrease ethanol production [62–64]. *S. cerevisiae* cannot naturally assimilate xylose, but xylose catabolism has been extensively studied and introduced into yeast by many groups [65,66]. In one case, isobutanol producing strains generated 2-fold less ethanol and 6-fold more isobutanol when grown on xylose instead of glucose [63]. Improved performance is not restricted to xylose. In another case, a strain produced ~1.5-fold more 1,2-propanediol than

ethanol when grown on glycerol [67]. Recently, there has also been interest in the generation of xylose-utilizing Pdc^- strains for generating pyruvate-derived products, but the strains suffer from low productivities due to slow-growth [68–70]. While catabolism of xylose and glycerol has proved beneficial in decreasing ethanol production in defined media on a lab scale, the ultimate application will be growth of Pdc- strains on lignocellulosic hydrolysates containing mixtures of sugars, organic acids, and inhibitors.

Table 1. Summary of strategies for non-ethanol chemical production in *S. cerevisiae*

Reference	Pdc- phenotype	Relieve Crabtree effect through ALE	Alternate acetyl-CoA routes	NAD^+ regeneration	Metabolic control of ethanol production	Alternate carbon source	Product(s)	Yield (g/g carbon source)	Titer (g/L)	Conditions (carbon source, medium, batch/fed-batch, vessel, conditions)
[71]	✓	✓	✓				*Acetate* *Pyruvate*	0.14[a] 0.007[a]	< 0.5 < 0.5	Glucose, minimal medium, batch, flask, aerobic
[25]	✓	✓		✓			**2,3-Butanediol** *Glycerol* *Acetoin*	0.28 0.09[a] 0.01[a]	96.2 >30[c] ~5 [c]	Glucose, YP medium, fed-batch, bioreactor, aerobic
[33]	✓			✓			**2,3-Butanediol** *Acetoin*	0.407 0.01[a]	72.91 1.38	Glucose, YP medium, fed-batch, flask, aerobic
[34]	✓			✓			**2,3-Butanediol** *Ethanol* *Glycerol* *Acetoin*	0.359 0.11[a] 0.069 0.052	32.31[c] ~10[c] 6.21[c] 4.68[c]	Glucose + ethanol, minimal media, batch, bioreactor, aerobic
[35]	✓		✓	✓			**2,3-Butanediol** *Glycerol* *Acetate*	0.404 0.088 0.006[a]	154.3 33.5 [c] 2.3	Glucose, YP medium, fed-batch, bioreactor, full aeration followed by oxygen limitation
[36]	✓	✓	✓	✓			**2,3-Butanediol** *Acetoin* *Acetate*	0.462 0.02[a] 0.0004[a]	108.6 4.6 0.1	Glucose, YP medium, fed-batch, bioreactor, full aeration followed by oxygen limitation
[27]	✓	✓		✓			**2,3-Butanediol** *Glycerol* *Succinate*	0.27 0.239[a] 0.013[a]	81.0 71.8 4.0	Glucose, synthetic defined medium, fed-batch, flask, mild aerobic
[72]	✓			✓			**n-Butanol** *Ethanol* *Glycerol*	0.012 0.10 0.17	0.130 ~1[c] ~2[c]	Glucose + pantothenate, SMD medium, flask, semi-anaerobic
[43]	✓	✓		✓			**Isobutanol** *Ethanol* *Glycerol* *Pyruvate* *2,3-butanediol* *Dihydroxyisovalerate* *Isobutyrate*	0.0074[a] 0.033[a] 0.26[a] 0.02[a] 0.325[a] 0.05[a] 0.03[a]	[b] [b] [b] [b] [b] [b] [b]	Glucose, SMG medium + Tween-80 + ergosterol, batch, serum bottle, microaerobic
[61]				✓	✓		**Isobutanol** **2-Methyl-1-butanol** *Ethanol* *Glycerol*	0.0535 0.01417 0.187 [b]	8.49 2.38 39.8 [b]	Glucose, SC-Ura, fed-batch, bioreactor, microaerobic
[28]	✓	✓	✓	✓			**Free fatty acids** *Glycerol* *Succinate*	0.1 0.5[a] 0.007[a]	25 [b] [b]	Glucose, minimal N_2-limited medium, fed-batch, bioreactor, aerobic

[42]		✓	✓		✓			**Malic acid** *Succinate* *Glycerol* *Pyruvate* *Fumarate*	0.31[a] 0.04[a] 0.13[a] 0.02[a] 0.01[a]	59 8 25 3 2	Glucose, synthetic medium, batch, flask, aerobic
[54]		✓	✓		✓			**Succinic acid** *Pyruvate* *Malate* *Glycerol*	0.044[a] 0.364[a] 0.022[a] 0.076[a]	2.2 18.2 1.1 3.8	Glucose + formate, mineral salt medium, fed-batch, deep-well plate, microaerobic
[60]					✓	✓		**Gluconate** *Ethanol* *Glycerol*	0.12[a] 0.05[a] [b]	2.31 1.01 [b]	Glucose, synthetic defined, batch, culture tube, semi-anaerobic
[41]		✓			✓			**Lactic acid** *Ethanol* *Glycerol*	0.6 0.02 0.01	80 1.6 2.6	Glucose, YP medium, fed-batch, bioreactor, aerobic
[40]					✓			**Lactic acid** *Ethanol*	0.8 0.02[a]	112.0 2.6	Glucose, YP medium, fed-batch, flask, aerobic
[69]		✓			✓		✓	**Lactic acid** *Acetate* *Pyruvate* *Xylitol*	0.50[a] 2.5[a] n.d. 0.02	7.8 2.5[c] n.d. 0.31[c]	2-stage sugar consumption (ethanol then xylose), YP medium, batch, bioreactor, aerobic followed by microaerophilic
[70]					✓		✓	**Lactic acid** *Ethanol* *Xylitol* *Glycerol* *Acetate*	0.67 0.01[a] 0.01[a] 0.01[a] 0.01[a]	60 <1 <1 <1 <1	Xylose, YP medium, batch, flask, aerobic
[68]		✓		✓	✓		✓	**2,3-Butanediol** *Glycerol* *Ethanol* *Xylitol*	0.46[a] 0.11[a] 0.08[a] 0.03[a]	96.8 23.3 16.3 5.9	Xylose + glucose, YP medium, fed-batch, bioreactor, microaerobic
[73]		✓			✓		✓	**2,3-Butanediol** *Glycerol* *Xylitol*	0.26[a] 0.26[a] 0.03[a]	43.6 45 4.7	Xylose + glucose, YP medium, fed-batch, bioreactor, microaerobic
[63]					✓		✓	**Isobutanol** *Ethanol* *Glycerol*	0.026[a] 0.275[a] 0.10[a]	2.6 ~27.5 [c] ~10 g/L[c]	Xylose, nutrient-rich Verduyn medium, fed-batch, bioreactor, aerobic
[62]					✓		✓	**Isobutanol** **2-Methyl-1-butanol** *Ethanol* *Glycerol*	0.0196 0.0053 0.32[a] [b]	3.10 0.79 <50[c] [b]	Xylose, synthetic medium, fed-batch, conical tube, semi-anaerobic
[67]					✓		✓	**1,2-Propanediol** *Ethanol*	0.129 0.09[a]	4.3 < 3[c]	Glycerol, YP medium, batch, bioreactor, aerobic

Bold = product of interest
Italics = by-product
n.d. = not detected
Absence of ethanol or glycerol in a row indicates it was not detected or that the yield or titer was <0.01

[a] = Yield not directly reported. Yield is either read from a graph, converted from a molar yield, calculated as g/g consumed carbon source, or calculated by dividing production rate by carbon source uptake rate.
[b] = Not reported
[c] = Titer not directly reported. Titer is either read from a graph, converted from molar concentration, or calculated as g/L fed carbon source, which is quantified by multiplying the reported yield (g/g carbon source) by the carbon source concentration (g/L).

4. Recent engineering strategies to increase production of non-ethanol products

The metabolic engineering community has leveraged many of the strategies described above to improve the production of non-ethanol products. In Table 1, we summarize recent studies by tabulating the approaches used and commonly reported performance metrics for several key products. Additional insight can be obtained in the highlighted references.

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5. Conclusion/perspectives

Redirecting flux away from ethanol production in yeast remains a challenging endeavor. Recent work shows that enhancing carbon flux through non-ethanol pathways can be achieved by several metabolic engineering strategies: 1.) relieving the Crabtree effect through regulatory modifications, 2.) regenerating NAD^+ by producing alternative reduced products, and 3.) providing routes for synthesizing cytosolic acetyl-CoA. In many cases, it has been far easier to throttle down ethanol production, rather than to completely eliminate it. Production of alternative chemical products in yeast has provided the scientific and economic motivation to deepen our understanding of the Crabtree effect and identify strategies for circumventing it. Deleting ethanol synthesis remains hard, but a promising strategy is emerging. This approach starts by providing alternative pathways for synthesis of acetyl-CoA, oxidation of NADH, and circumventing the Crabtree effect. Next, eliminate ethanol production by deleting all three *PDC* genes or control ethanol production with advanced genetic circuits. Lastly, the selective pressure to grow, particularly under fermentation conditions, provides a driving force for applying ALE to optimize strain performance. These steps have likely been applied in industry to optimize production of several commercially available products (e.g. lactate, isobutanol, …), but the secrets of the evolved strains remain to be elucidated by academic researchers. Herein, we have described several substantial advances in the academic literature. Each has room for further improvement. The pursuit of these improvements will hopefully deepen our understanding of regulation (particularly of central metabolism and substrate uptake), establish new tools for manipulating metabolism, and establish publicly available strains that can be rapidly engineered to produce any of the remaining chemical products desired by society.

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Figures

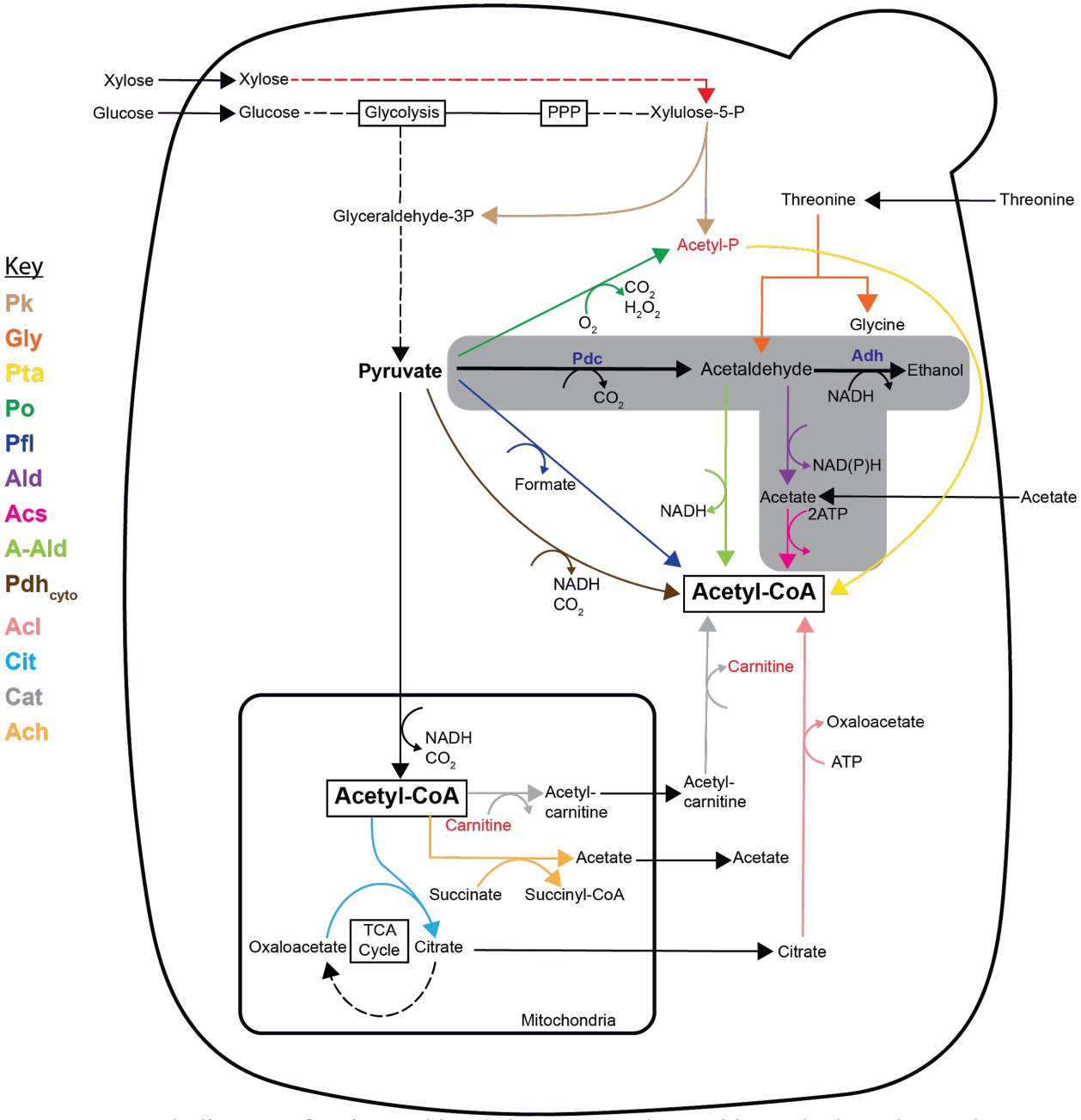

Figure 1. Metabolic map of native and heterologous acetyl-CoA biosynthetic pathways in *S. cerevisiae*. Naturally, cytosolic acetyl-CoA is derived from the pyruvate dehydrogenase (Pdh) bypass consisting of pyruvate decarboxylase (Pdc), acetaldehyde dehydrogenase (Ald, purple), and acetyl-CoA synthetase (Acs, pink); ethanol is also derived from an intermediate in this bypass (grayed box). Other native metabolic pathways include threonine aldolase (Gly, dark orange) and the mitochondrial shuttle system consisting of a CoA-transferase (Ach1, light orange). Heterologous tested pathways for the synthesis of cytosolic acetyl-CoA include the following: phosphoketolase (Pk, tan), phosphotransacetylase (Pta, yellow), pyruvate oxidase (Po, dark green), pyruvate-formate lyase (Pfl, dark blue), acetylating aldehyde dehydrogenase (A-

Ald, light green), pyruvate dehydrogenase (Pdh_{cyto}, brown), citrate lyase (Acl, light pink), citrate synthase (Cit, light blue), and carnitine acetyl-CoA transferase (Cat, gray). Non-native metabolites (acetyl-P and carnitine) and pathway (xylose catabolism) are indicated in red.

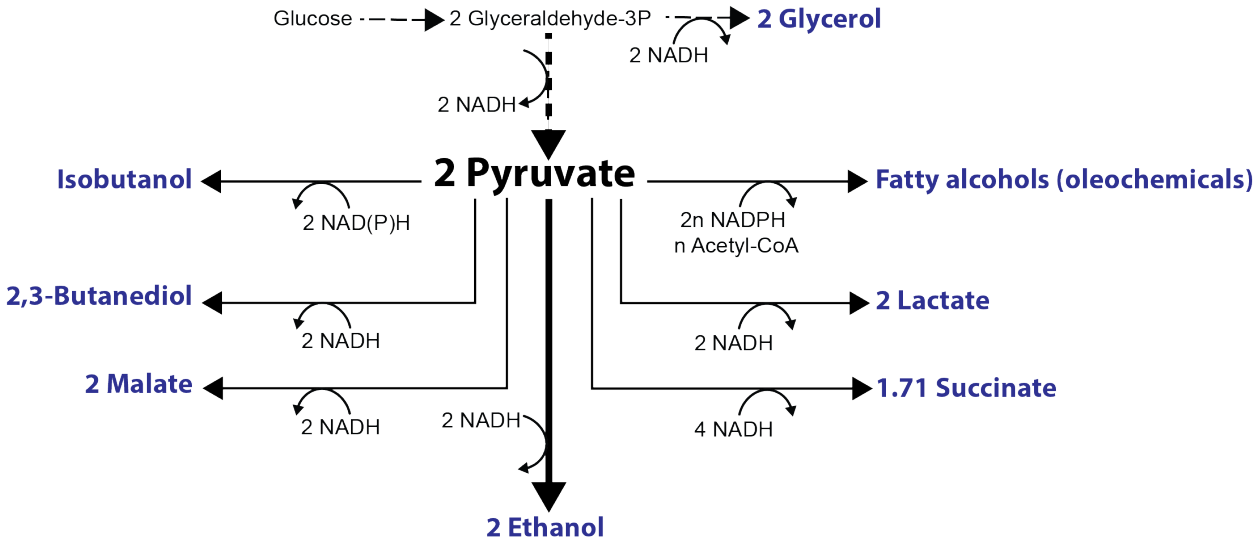

Figure 2. Schematic depicting pyruvate-derived products. Glycerol, malate, lactate, and succinate [74] production require NADH reducing equivalents and can theoretically replace ethanol fermentation while isobutanol and fatty alcohols/other oleochemicals require NAD(P)H reducing equivalents.