Development of a Platform Strain for Production of Adipic Acid Yields Insights into the Localized Redox Metabolism of *S. cerevisiae*

by

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A thesis submitted in conformity for the degree of Master of Applied Science

Graduate Department of Chemical Engineering and Applied Chemistry

University of Toronto

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2013

Abstract

Adipic acid is a commodity chemical that is currently derived from petroleum and has a global production value of 2.2 billion pounds per annum. Although it is not naturally synthesized, several pathways have been proposed for its biosynthesis from glucose. This work describes efforts to construct a platform strain of *S. cerevisiae* for production of alpha-ketoglutarate, a TCA cycle intermediate and a metabolite common to several proposed pathways, through cofactor engineering. Specifically, we investigated the disruption of glucose-6-phosphate dehydrogenase (*ZWF1*) and an aldehyde dehydrogenase (*ALD6*) gene, key reactions in the generation of cytosolic NADPH, in backgrounds with an interrupted TCA cycle through disruption of succinyl-CoA ligase (*LSC2*). The resulting strains showed improvements in growth rate and the production of alpha-ketoglutarate, suggesting the mechanism of recovery to be the use of cytosolic NADP(+) dependent isocitrate dehydrogenase for generation of NADPH and thus a coupling of alpha-ketoglutarate production to NADPH synthesis.
Acknowledgements

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I may not have gone where I intended to go,
but I think I have ended up where I needed to be.

- Douglas Adams
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2PG</td>
<td>2-phosphoglycerate</td>
</tr>
<tr>
<td>3PG</td>
<td>3-phosphoglycerate</td>
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<tr>
<td>6PGN</td>
<td>6-phosphogluconate</td>
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<tr>
<td>6PGNL</td>
<td>6-phosphogluconolactone</td>
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<tr>
<td>ACA</td>
<td>acetaldehyde</td>
</tr>
<tr>
<td>AcCoA</td>
<td>acetyl-coenzyme A</td>
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<tr>
<td>AKG</td>
<td>alpha-ketoglutarate</td>
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<tr>
<td>CIT</td>
<td>citrate</td>
</tr>
<tr>
<td>DHAP</td>
<td>dihydroxyacetonephosphate</td>
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<tr>
<td>DPG</td>
<td>diphosphoglyceroldehyde</td>
</tr>
<tr>
<td>E4P</td>
<td>erythrose-4-phosphate</td>
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<td>ethanol</td>
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<td>F1,6P</td>
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</tr>
<tr>
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<td>fumarate</td>
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<td>G3P</td>
<td>glyceraldehyde-3-phosphate</td>
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<td>GLC</td>
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<td>ICT</td>
<td>isocitrate</td>
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<tr>
<td>OAA</td>
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<td>phosphoenolpyruvate</td>
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<tr>
<td>Met</td>
<td>methionine</td>
</tr>
<tr>
<td>ZWF1</td>
<td>glucose-6-phosphate dehydrogenase</td>
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<tr>
<td>LSC2</td>
<td>succinyl-CoA ligase 3-subunit</td>
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<td>IDP1</td>
<td>mitochondrial NADP$^+$ dependent isocitrate dehydrogenase</td>
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<td>cytosolic NAD$^+$ dependent isocitrate dehydrogenase</td>
</tr>
<tr>
<td>ALD6</td>
<td>cytosolic NAD$^+$ dependent aldehyde dehydrogenase</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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1 Literature Review

1.1 NADPH as a Major Biosynthetic Cofactor in *S. cerevisiae*

Cofactors such as NAD\(^+\) and NADP\(^+\) play critical roles in the metabolism of *S. cerevisiae* and have a dramatic impact on the product profile of a strain. Whereas NAD\(^+\) is typically used as a cofactor in catabolic pathways, NADP\(^+\) is generally involved in anabolic reactions. An interesting nuance of *S. cerevisiae* is that as a compartmentalized eukaryote, subcellular compartments are surrounded by a membranes impermeable to NAD(P)\(^+\) and NADP(H) (van Dijken, 1986). Consequently, cofactor pools are separate for each subcellular compartment, and thus redox based reactions exist in each to generate and consume NAD(P)\(^+\). In addition to cofactor based reactions in subcellular compartments, *S. cerevisiae* contains NAD\(^+\) and NADH kinases to catalyze the phosphorylation of NAD\(^+\) to NADP\(^+\) and NADH to NADPH, respectively, in both the mitochondria and cytosol (Miyagi et al., 2009). *S. cerevisiae* contains three NAD\(^+\) kinases; Utr1p, Yef1p and Pos5p, of which the two former are present in the cytosol and the latter is present in the mitochondria (Miyagi et al., 2009). Although the cytosolic NAD kinases Utr1p and Yef1p also have activity as NADH kinases, it has been previously demonstrated that their contribution to overall cytosolic NADPH levels is negligible (Shi et al., 2005).

Indeed, the major sources of cytosolic NADPH are catabolic reactions involved in the central carbon metabolism, namely: glucose-6-phosphate dehydrogenase (ZWF1p), aldehyde dehydrogenase (ALD6p) and NADP-dependent isocitrate dehydrogenase (IDP2p) (Miyagi et al., 2008). As well, glutamate dehydrogenases have been previously demonstrated to play an important role in the balance of cytosolic redox (Scalcinati et al., 2012).

ZWF1p catalyzes the first step in the oxidative pentose phosphate pathway; the
conversion of glucose-6-phosphate (G6P) to 6-phosphogluconolactone (6PGNL), and in the process reduces one molecule of NADP$^+$ to NADPH. It is important to note that downstream of ZWF1 in the oxidative pentose phosphate pathway, phosphogluconate dehydrogenase (GND) catalyzes the conversion of 6-phosphogluconate (6PGN) to ribulose-5-phosphate (Ru5P), reducing an additional one molecule of NADP$^+$ to NADPH. Thus, the oxidative pentose phosphate pathway plays a critical role in the synthesis of cytosolic NADPH, as for every one molecule of G6P entering the pathway, two molecules of NADPH are produced. Highlighting the importance of the oxidative pentose phosphate pathway, a study by Frick et al., (2005) observed through C13 measurements that the amount of glucose flux that is directed to the pathway varies from 10% under oxygen limited conditions to over 50% in glucose limiting conditions (Frick, 2005). Accordingly, the disruption of ZWF1 is deleterious to the strain, causing extreme NADPH starvation. A particular limitation under these conditions is the synthesis of sulfur containing amino acids, such as methionine and homocysteine, which are NADPH intensive, requiring four molecules of NADPH to synthesize one molecule of each amino acid (Figure 2) (Thomas & Surdin-Kerjan, 1997). As a result, a ZWF1 deletion strain may be rescued by the addition of methionine to the growth medium (Grabowska & Chelstowska, 2003).

In order to compensate for reduced availability of NADPH, flux through ALD6p is
dramatically higher in ZWF1 deletion strains, increasing the conversion of acetaldehyde to acetate and thus increasing NADPH pools (Grabowska & Chelstowska, 2003). ALD6p is one of five aldehyde dehydrogenases present in S. cerevisiae, and encodes for an NADP\(^+\) dependent aldehyde dehydrogenase, which is thought to play a role in synthesis of cytosolic acetyl-CoA (Meaden et al., 1994). The deletion of ALD6 in S. cerevisiae YPH499 (MAT A) has been reported to significantly reduce growth rate and carbon source utilization (Meaden et al., 1994).

IDP2 is one of three isozymes of isocitrate dehydrogenase that have been characterized in S. cerevisiae, of which all three differ in their localization and cofactor specificity. The alternate isozymes, IDH1/2 and IDP1, are both mitochondrial isocitrate dehydrogenases and are NAD\(^+\) and NADP\(^+\) dependent, respectively, and the former is the primary isocitrate dehydrogenase used during growth on glucose (Loftus et al., 1994). In a 1994 study by Loftus et al., the authors disrupted IDP1 and IDP2 in S. cerevisiae S173-6B (MAT A, leu2-3,112, his3-1, ura3-57, trp1-289) and found there to be no significant differences in terms of growth rate and auxotrophies related to the wild-type strain, suggesting that the remaining IDH1/2 flux is sufficient for production of \(\alpha\)-ketoglutarate (Loftus et al., 1994).

In 2008, Grabowska and Chelstowska reported a study that investigated the roles of alternative NADPH synthesizing reactions in a strain of S. cerevisiae lacking the ZWF1 gene. While ALD6 has increased activity in the absence of glucose-6-phosphate dehydrogenase to alleviate cytosolic NADPH starvation, the authors demonstrated that the growth rate of the \(\Delta ZWF1\) strain could be improved by overexpression of ALD6 and the methionine auxotrophy in this case could be alleviated (Grabowska & Chelstowska, 2003). However, the authors demonstrate that the overexpression of ALD6 was unable to completely restore the growth of the \(\Delta ZWF1\) phenotype. Further, the authors overexpressed IDP2 and found that it did not increase the growth
rate of the mutant strain. Additionally, the authors attempted to construct a $\Delta ZWF1 \Delta ALD6$ deletion strain, but were unable to do so and as a result, concluded that the $ALD6$ gene is a critical source of NADPH in strains lacking the $ZWF1$ gene.

1.2 Engineering the Redox Metabolism of *S. cerevisiae*

The metabolism of many microorganisms contains metabolic intermediates that are of high industrial importance both as products and as precursors for downstream processes. During growth of microorganisms however, such compounds are produced in titres too low to be of industrial value. Metabolic engineering seeks to alter biochemical reactions within the metabolic network such that the production of a desired chemical is maximized, generally by minimizing by-product synthesis and directing metabolic flux to product pathways (Nevoigt, 2008). The use of *S. cerevisiae* as a host organism for production of chemicals offers significant advantages over bacterial hosts such as *E. coli*, mainly in its tolerance of inhibitory compounds and superior growth rate in low pH conditions, aiding in reduced contamination probability and ease of downstream
product separation (Nevoigt, 2008; Matsushika et al., 2009). Strains of *S. cerevisiae* have been engineered for the production of many chemical products which have been reviewed extensively elsewhere (Ostergaard et al., 2000; Pscheidt & Glieder, 2008; Nevoigt, 2008).

Engineering of the redox metabolism in *S. cerevisiae* is an interesting approach that provides an additional layer of complexity and methods to improve pathway flux to metabolic engineering strategies, and has been the focus of several studies in the past. In a 2003 study, Verho and colleagues improved xylose fermentation in yeast by the overexpression of a fungal NADP\(^+\) dependent glyceraldehyde-3-phosphate dehydrogenase (*GDP1*) and observed that the resulting strain had decreased levels of xylitol, an unwanted byproduct resulting from cofactor imbalance in the base strain, and increased ethanol yield (Verho et al., 2003). The impact of NADH availability in *S. cerevisiae* has also been investigated by Heux et al., 2006 through the expression of a water-forming NADH oxidase from *Lactococcus lactis*. In the engineered strain, the authors observed a NADH pools to be one-fifth and the NADH:NAD ratio to be six times that of the wild-type strain. As would be expected in a strain with limited NADH availability, characterization revealed a marked decrease in the formation of reduced products (e.g. ethanol, glycerol, succinate and hydroxyglutarate) with a concomitant increase in excretion of oxidized products (e.g. acetate, acetaldehyde and acetoin) (Heux et al., 2006). Recently, a combination of upstream metabolite availability and cofactor balance was used to improve production of \(\alpha\)-santalene in *S. cerevisiae* (Scalcinati et al., 2012). In this study, the authors reasoned that as synthesis of \(\alpha\)-santalene nets production of NADH and consumption of NADPH, a change in the NADH:NADPH ratio would be beneficial to drive pathway flux. By deleting and overexpressing a NADP\(^+\) dependent and a NAD\(^+\) dependent glutamate dehydrogenase, (*GDH1* and *GDH2*, respectively) the authors demonstrated the yield of \(\alpha\)-santalene could be increased.
1.3 Production of Adipic Acid

Current methods of adipic acid synthesis rely on petrochemical feedstock for the air-oxidation of cyclohexane and cyclohexanol in the presence of nitric acid (Park & Goroff, 1993). However, a byproduct of this process is N\textsubscript{2}O, a gas commonly thought to be a contributor to global warming and ozone depletion (Sato et al., 1998) (Werpy & Petersen, 2004). In 1998, the production of adipic acid accounted for 5-8% of anthropogenic emissions of N\textsubscript{2}O (Sato et al., 1998). Additionally, increasing volatility in the petrochemical market has generated interest in producing biomass-derived value-added chemicals. As a result of such limitations, bioprocesses are being heavily investigated as alternative approaches to adipic acid production. In this section, previously proposed and demonstrated pathways for the biosynthesis of adipic acid from petrochemical and biomass feedstock are reviewed.

1.3.1 Bioprocesses for adipate synthesis from petrochemical feedstock

To circumvent the production of environmentally sensitive by-products, bioprocesses for the synthesis of adipic acid from petrochemical feedstock have been investigated. Particularly interesting in this application is the metabolism of \textit{Acinetobacter} NCIB 9871, which has been reported to oxidize cyclohexanol to adipate (Donoghue & Trudgill, 1975). The organism contains genes that encode for enzymes capable of converting cyclohexanol to \(\varepsilon\)-caprolactone and subsequently oxidizing the compound to adipic acid. Following the production of adipic acid, \textit{Acinetobacter} sp. Degrades the compounds to succinyl-CoA and acetyl-CoA for use in primary metabolism (Donoghue & Trudgill, 1975). A patent by Cheng et al (2004) claims the genes cluster of \textit{Acinetobacter} that encodes for the enzymes required for this pathway (Cheng et al., 2004). A recombinant strain of \textit{E. coli} harbouring the \textit{Acinetobacter} gene cluster for this pathway has been shown to catalyze these reactions with a yield of 1.1-2.3 mol\textsubscript{Adipate}/mol\textsubscript{Cyclohexanol} with glucose as the carbon source (Cheng et al., 2004). A patent by Brzostowicz et al. (2002) similarly claims nucleotide sequences for an identical pathway in a wild-type strain of \textit{Brevibacterium} sp. (Brzostowicz & Rouviere, 2002).
Additionally, prospective petrochemical feedstocks have been expanded to include aromatic compounds. In a patent by Maxwell in 1982, the production of cis,cis-muconic acid, a precursor to adipic acid, from toluene by organisms of the Pseudomonas genus is claimed (Maxwell, 1982). A strain of Pseudomonas putida was shown to convert toluene to cis,cis-muconic acid with a productivity of $30 \text{ mg gDW}^{-1} \text{ hr}^{-1}$ (Maxwell, 1982). Although such pathways circumvent the production of environmentally sensitive by-products, such as N$_2$O, these processes still remain subject to the fluctuating availability and price of petrochemical feedstock.

1.3.2 Hybrid biochemical pathways for adipate synthesis from renewable biomass

In efforts to reduce the reliance of adipic acid production on the volatile market of petrochemical feedstock, processes for its production from renewable biomass has previously been investigated. Microorganisms have demonstrated potential in the production of biomass-derived compounds that may serve as precursors for chemical synthesis of adipate, in addition to bio-catalysts for the conversion of chemically derived compounds to adipate, illustrating their effectiveness in hybrid biochemical processes. A patent by Faber in 1982 claims a process for the chemical synthesis of hexanediol from biomass derived 5-hydroxymethylfurfural and its subsequent oxidation to adipate by Gluconobacter oxydans subsp. Oxydans (Faber, 1983). Notably, a biosynthetic pathway for the conversion of glucose to cis,cis-muconic acid was demonstrated in recombinant E. coli by Niu and colleagues in 2002. The pathway incorporates genes from Acinetobacter sp. and Klebsiella pneumonia for the conversion of glucose to catechol (1,2-dihydroxybenzene) through the shikimate pathway, followed by the oxygenation of catechol to cis,cis-muconic acid (Niu et al., 2002). The pathway demonstrated by Niu et al. (2002) is claimed by multiple patents (Frost & Draths, 1997) (Frost & Draths, 1996). The authors demonstrate that following the purification of cis,cis-muconic acid, the product may be chemically converted to adipic acid by a hydrogenation reaction (10% Pt/C, H$_2$, 3400 kPa, 25°C). The study found that
a yield of 24% mol/mol of cis,cis-muconic acid on glucose is achieved through this pathway, and attained a further 97% mol/mol yield of adipate on cis,cis-muconic acid in the hydrogenation reaction (Niu et al., 2002). In a recent study, Curran et al. demonstrated the expression of this pathway in a strain of S. cerevisiae engineered to reduce feedback inhibition of the shikimate pathway, in addition to driving metabolic flux through upper glycolysis via deletion of the ZWF1 and over-expression of TKL1 (Curran et al., 2012). The authors attained a final titer of 140 mg/L muconic acid in shake-flask cultures.

Although the approaches demonstrated by Niu et al. (2002) and Curran et al. (2012) are significant advancements towards the synthesis of adipic acid from renewable biomass, disadvantages of the pathway include a low ATP yield attributing to low cell growth rate and the requirement of oxygen for the pathway, precluding fermentation processes (Burgard et al., 2010).

1.3.3 Bioprocesses for adipate synthesis from renewable biomass

More recently, the total biosynthesis (the conversion of substrate to final product in a single process) of adipic acid directly from biomass feedstock has been the focus of much attention in the biotechnology sector. A total of eight unique pathways are proposed in four patents, all of which have been filed in the previous three years (Baynes & Geremia, 2010; Burgard et al., 2010; Raemakers-Franken et al., 2010; Picataggio & Beardslee, 2011).

From 2-oxoglutarate

In their 2010 patent, Burgard and colleagues detail six candidate pathways for the total biosynthesis of adipate from glucose (Burgard et al., 2010). Of the pathways proposed in the patent, two share 2-oxoglutarate as a starting substrate, referred to herein as Pathway 1 and Pathway 2 (Figure 2(A), Figure 2(B) respectively). In pathway 1, the authors describe the three-step elongation of 2-oxoglutarate to 2-oxoadipate,
via the isopropylmalate synthase (IPMS) chain extension, catalyzed by the enzymes homocitrate synthase, homoaconitase and homoisocitrate dehydrogenase. The IPMS chain extension reaction scheme has been suggested for the production of alkanes ranging in length from 4-9 carbons (Li et al., 2010). The second step in this pathway consists of an oxidoreductase step, reducing 2-oxoadipate to 2-hydroxyadipate. In a recent study, Parthasarathy and colleagues demonstrated that (R)-2-hydroxyglutarate dehydrogenase was able catalyze the NAD\(^+\) dependent reduction of 2-oxoadipate to 2-hydroxyadipate (Parthasarathy et al., 2011). The subsequent step dehydrates 2-hydroxyadipate to yield the unsaturated dicarboxylic acid hex-2-enedioate. Following this reaction, it is proposed that hex-2-enedioate may be converted to adipate through a reaction catalyzed by either an enoate reductase enzyme or through chemical hydrogenation (Niu et al., 2002; Burgard et al., 2010). The theoretical maximum yield of this pathway was calculated to be 0.83 and 0.36 mol\(_{Adipate}/mol_{Glucose}\) in \textit{E. coli} under aerobic and anaerobic conditions respectively.

In Pathway 2 as outlined by Burgard and colleagues, similar reactions are used to convert 2-oxoadipyl-CoA to adipic acid. In this pathway a CoA-transferase enzyme affords the CoA-activated form of 2-oxoadipate. The pathway proceeds through steps similar to the previously described pathway, in that 2-oxoadipyl-CoA is converted to 2-hydroxyadipyl-CoA and subsequently to 5-carboxy-2-pentenoyl-CoA by an oxidoreductase and dehydratase enzyme, respectively. The dehydration step in this pathway however, differs compared to the 2-ketoadipate pathway previously discussed, as the \(\alpha\)-dehydration of a CoA-activated substrate is known to be of a radical mechanism, requiring an electron-drawing group for the removal of the beta-proton (Schweiger & Buckel, 1984). The theoretical maximum yield of Pathway 2 was calculated to be 0.81 and 0.32 mol\(_{Adipate}/mol_{Glucose}\) in \textit{E. coli} under aerobic and anaerobic conditions respectively.
Figure 3: Pathways for the biosynthesis of adipic acid proposed in patent literature.
In a patent by Baynes and colleagues, an alternative pathway to produce adipic acid from 2-oxoadipate is proposed (Pathway 3, Figure 2(C)) (Baynes & Geremia, 2010). A similar approach has been claimed in a patent prepared by Raemakers-Franken and colleagues (Raemakers-Franken et al., 2010). In this pathway, 2-oxoadipate is formed from 2-oxoglutarate by the IPMS chain extension, and is further extended by the same reaction scheme to 2-oxopimelate. In the subsequent reaction step, 2-oxopimelate undergoes a decarboxylation reaction to yield adipate-6-semialdehyde. In the final step, adipate-6-semialdehyde undergoes an oxidation reaction catalyzed by a 6-oxohexanoate dehydrogenase. The ChnE enzyme has been previously reported to catalyze this reaction in the metabolism of *Acinetobacter* sp. (Iwaki et al., 1999). The theoretical maximum yield of this pathway is calculated to be 0.68 and 0.20 mol$_{\text{Adipate}}$/mol$_{\text{Glucose}}$ in *E. coli* under aerobic and anaerobic conditions respectively.

In the patent prepared by Raemakers-Franken et al., the authors provide experimental evidence for this pathway. They demonstrate that a recombinant strain of *E. coli* harboring a plasmid with genes from methanogenic bacteria encoding for homoaconitase and homoisocitrate dehydrogenase synthesized up to 650 mg/L of 2-oxopimelate from glycerol. Moreover, a recombinant *E. coli* harboring an aminotransferase from *Vibrio flurialis* and α-keto acid decarboxylase from *Lactococcus lactis* produced up to 33 mg/L adipic acid after cultivation in shake flasks and resuspension in TY medium with 1% glycerol and 500 mg/L 2-oxopimelate. The final reaction step, converting adipate-6-semialdehyde to adipic acid is thought to be catalyzed by an endogenous aldehyde dehydrogenase (Raemakers-Franken et al., 2010).

**From 3-oxoadipate**

Two additional pathways described by Burgard and colleagues stem from 3-oxoadipyl-CoA and are referred to herein as Pathway 4 and Pathway 5, respectively (Figure 2(D)). The primary metabolite from which these pathways begin, 3-oxoadipate, is a natural intermediate of the adipate degradation pathway of *Penicillium chrysogenum*, which
culminates in the cleavage of 3-oxoadipyl-CoA to yield succinyl-CoA and acetyl-CoA (Thykaer et al., 2002). Additionally, 3-oxoadipyl-CoA is an intermediate of catechol and protocatechuate degradation pathways of bacterium and eukaryotes (Harwood & Parales, 1996). The patent by Burgard et al. proposes that if this degradation reaction is reversed, 3-oxoadipyl-CoA may be obtained from readily available TCA intermediates. In the first step of the 3-oxoadipyl-CoA pathway following the formation of 3-oxoadipyl-CoA, the compound is reduced to 3-hydroxyadipyl-CoA by an oxidoreductase enzyme. The subsequent steps consist of a dehydration step to yield 5-carboxy-2-pentenoyl-CoA, reduction of 5-carboxy-2-pentenoyl-CoA to adipyl-CoA, and the conversion of adipyl-CoA to adipate by a CoA-transferase. As in the previously discussed pathway, the reduction of the unsaturated compound may be carried out either enzymatically by a 2-enoate reductase enzyme or by chemical hydrogenation (Burgard et al., 2010; Niu et al., 2002; Stuermer et al., 2007).

A similar pathway that has been proposed by Burgard et al, 2010 is one in which 3-oxoadipyl-CoA is first converted to 3-oxoadipate by an acyl-transferase before being converted to hex-2-enedioate by the same pathway as described above (Figure 2(E)). Subsequently, hex-2-enedioate is converted chemically or enzymatically to adipate (Burgard et al., 2010).

Pathways 4 and 5 have the highest theoretical maximum yield of the pathways examined in this study, achieving 0.92 mol$\text{Adipate}$/mol$\text{Glucose}$ in $E. \text{coli}$ under aerobic conditions (Table 1). Moreover, a recent study by Dellomonaco and colleagues demonstrated that the $\beta$-oxidation pathway in $E. \text{coli}$, incorporating a (1) thiolase, (2) hydroxyacyl-CoA dehydrogenase, (3) enoyl-CoA hydratase and (4) enoyl-CoA reductase, may be reversed to afford the efficient formation of $C_{n+2}$ alcohols from a thiolase, dehydrogenase and enoyl-CoA dehydratase (Dellomonaco et al., 2011). This suggests that the formation of 3-oxoadipyl-CoA from succinyl-CoA and acetyl-CoA is likely a feasible reaction step. Interestingly, the patent by Burgard and colleagues
further discusses that the theoretical maximum yield can be increased to $1 \text{ mol}_{\text{Adipate}}/\text{mol}_{\text{Glucose}}$, in the case where chemical hydrogenation is used in lieu of enzymatic reduction (thereby eliminating the consumption of NADH), and if the host is assumed to be 100% efficient. While the scenario of a 100% efficient host is infeasible, it underscores the potential high-yields attainable in processes using such a pathway.

**From Lysine**

An additional pathway for adipate biosynthesis by means of a lysine degradation pathway is described by Burgard et al, (2010) and is referred to as Pathway 6 (Figure 2(F)). The authors describe a pathway wherein lysine is degraded to 6-amino-hex-2-enoate by a carbon-nitrogen lyase, and further to 6-aminocaproic acid by an oxidoreductase. Following this step, 6-aminocaproate may undergo a transaminase-catalyzed reaction to produce adipate semialdehyde, which may be oxidized by an alcohol dehydrogenase to produce adipate. The theoretical maximum yield of Pathway 6 was calculated to be 0.87 and 0.57 $\text{mol}_{\text{Adipate}}/\text{mol}_{\text{Glucose}}$ in *E. coli* for aerobic and anaerobic cases, respectively.

**From Malonyl-CoA**

In a recent patent, a recombinant pathway for the production of adipic acid through hexanoic acid was proposed (Figure 2(G)) (Picataggio & Beardslee, 2011). This pathway (Pathway 7) synthesizes adipic acid via the fatty acid metabolism and stems from the metabolites malonyl-CoA and acetyl-CoA. The key enzymatic step in this pathway is the conversion of two malonyl-CoA and one acetyl-CoA to hexanoate by the enzyme hexanoate synthase (HexS; EC 2.3.1.85). Following the synthesis of hexanoate, the compound undergoes three consecutive enzymatic oxidation reactions on the $\omega$-carbon to yield adipic acid. The theoretical maximum yield of Pathway 7 under aerobic conditions was calculated to be 0.37 $\text{mol}_{\text{Adipate}}/\text{mol}_{\text{Glucose}}$ in *E. coli*. 
Table 1: Comparison of maximum theoretical molar yields of adipate on glucose for various biosynthetic pathways.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>E. coli (iJR904)</th>
<th>E. coli (iAF1260)</th>
<th>S. cerevisiae (iMM904)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathway 1</td>
<td>0.82 / 0.48</td>
<td>0.83 / 0.36</td>
<td>0.42 / 0.28</td>
<td>Burgard, Pharkya, &amp; Osterhout, 2010</td>
</tr>
<tr>
<td>Pathway 2</td>
<td>0.81 / 0.45</td>
<td>0.81 / 0.32</td>
<td>0.36 / 0.22</td>
<td>Burgard, Pharkya, &amp; Osterhout, 2010</td>
</tr>
<tr>
<td>Pathway 3</td>
<td>0.66 / 0.28</td>
<td>0.68 / 0.20</td>
<td>0.23 / 0.15</td>
<td>Baynes &amp; Geremia, 2010; Raemakers-Franken et al., 2010</td>
</tr>
<tr>
<td>Pathway 4</td>
<td>0.92 / 0.92</td>
<td>0.85 / 0.42</td>
<td>0.92 / 0.92</td>
<td>Burgard, Pharkya, &amp; Osterhout, 2010</td>
</tr>
<tr>
<td>Pathway 5</td>
<td>0.92 / 0.92</td>
<td>0.83 / 0.37</td>
<td>0.92 / 0.92</td>
<td>Burgard, Pharkya, &amp; Osterhout, 2010</td>
</tr>
<tr>
<td>Pathway 6</td>
<td>0.86 / 0.59</td>
<td>0.87 / 0.57</td>
<td>0.32 / 0.22</td>
<td>Baynes &amp; Geremia, 2010; Burgard et al., 2010</td>
</tr>
<tr>
<td>Pathway 7</td>
<td>0.73 / 0</td>
<td>0.37 / 0</td>
<td>0.19 / 0</td>
<td>Picataggio &amp; Beardslee, 2011</td>
</tr>
<tr>
<td>Muconate</td>
<td>0.83 / 0</td>
<td>0.83 / 0</td>
<td>0.19 / 0</td>
<td>Frost &amp; Draths, 1997; Frost, &amp; Draths, 1996</td>
</tr>
</tbody>
</table>

Reported as mAdipate/molGlucose (aerobic / anaerobic) Theoretical yields of pathways involving the reduction of hex-2-enedioate to adipate were calculated under the assumption that the reaction is catalyzed in vivo rather than by chemical hydrogenation. Maximum yields were modeling using the E. coli metabolic models iJR904 (Reed, Vo, Schilling, & Palsson, 2003) and iAF1260 (Feist, et al., 2007) and the S. cerevisiae metabolic model iMM904 (Mo et al., 2009).

Maximum theoretical yield was calculated using flux balance analysis (FBA) (Varma & Palsson, 1994). The production of adipic acid in each pathway was used as the objective function was maximized while the minimum growth rate ($\mu_{min}$) and ATP maintenance requirement were set to zero. In aerobic cases, the oxygen uptake was set to 18.2 mmol/gDW/hr and 2 mmol/gDW/hr for E. coli and S. cerevisiae, respectively. In anaerobic cases, the iMM904 model was supplemented with additional sterols to permit growth.

2 Motivation and Statement of Objectives

At a fundamental level, metabolic engineering offers an alternative to consumption of petroleum for production of value-added chemicals. It has been previously noted that as a result of increasing global rate of oil consumption and decreasing rate at which new fossil fuel resources are discovered, alternative sources of transportation fuels and chemicals are highly sought after (van Maris et al., 2006). Through the application of metabolic engineering approaches such as gene deletions and heterologous expression of pathways or regulation in industrial organisms such as S. cerevisiae, the production of a target compound may be achieved at an industrial scale. However, there exist many fundamental problems in engineering strains for biosynthesis of specific compounds, ranging from the limitation of metabolism to achieve certain compounds of interest, or the generation of significant driving forces such as cofactor availability, to achieve high production rates. For example, there has been considerable industrial interest
in engineering microbes for the production of adipic acid, a precursor for Nylon(6,6) with a large market value. Although several patents have been filed for the production of adipic acid, pathways for its synthesis are not known to exist naturally in any organism, and efforts are underway to discover novel enzymes for completion of such a pathway.

Computational algorithms have been used with metabolic models in the past to couple the growth of a strain to the production of a target compound through genetic disruptions and modifications, however there has been significantly less attention given to using cofactors availability to couple both growth rate and chemicals synthesis. Cofactor engineering offers significant advantages in a platform strain as the redox imbalance may be channeled to synthesize a variety of compounds.

The focus of this study is on one aspect of strain construction for synthesis of adipic acid from glucose; the engineering of a pathway upstream of adipic acid biosynthesis pathways by harnessing cofactor availability. A common precursor of many of the identified potential adipic acid biosynthesis pathways is α-ketoglutarate, a metabolic intermediate in the TCA cycle that is synthesized by both NAD⁺ and NADP⁺ dependent isocitrate dehydrogenases in the mitochondria and NADP⁺ dependent isocitrate dehydrogenase in the cytosol. As a result of engineered oxidative stress in the form of NADPH starvation in the cytosol, and due to separate pools of NAD(P)⁺ in subcellular compartments, cytosolic reactions that net NADPH will be forced to be upregulated. Thus, the growth of the constructed strain will essentially be tied to the production of α-ketoglutarate in order to maintain cytosolic NADPH levels.

NADPH is of particular interest to couple production as a far greater number of moles are required for S. cerevisiae anabolism as compared to other biomass precursors. For example, a study by Frick and Wittmann in 2005 calculated the metabolic demand of NADPH and α-ketoglutarate to be 10 088 and 1008 µmol gDW⁻¹, re-
spectively, illustrating that a substantial increase in α-ketoglutarate synthesis may be achieved by this approach (Frick, 2005). Moreover, strains of *S. cerevisiae* lacking the *ZWF1* and other key genes in the NADPH metabolism have not previously been characterized for their product profiles. The characterization of the growth rates and product profiles of such strains would give insight into the localized redox metabolism of *S. cerevisiae*, and thereby enhance future efforts to engineer strains for the overproduction of target metabolites.
Statement of Objectives

1. To improve synthesis of α-ketoglutarate in *S. cerevisiae* CEN.PK 113-1A by coupling its synthesis to cytosolic NADPH pools;

The production of α-ketoglutarate is desirable not only for the purposes of adipic acid biosynthesis, but it is also a platform chemical from which other industrially relevant compounds may be produced in microorganisms. These compounds include, but are not limited to, L-glutamate, succinate, butanediol and itaconic acid.

2. To characterize the impact of cytosolic NADPH starvation on the metabolism of *S. cerevisiae* through deletion of key reactions in the central carbon metabolism;

While there have been studies that examine the impact of the inactivation of genes that are vital to the production of NADPH in *S. cerevisiae*, they have done so purely from a biochemistry viewpoint and have focused their examination on the levels of cofactors in the strains. While this is fundamentally interesting, the full characterization of such strains would be of value to metabolic engineering endeavours in improving our understanding of the strain.

3. To improve understanding of cofactor engineering and balance and its application to developing strains for the overproduction of value added compounds.

Traditional metabolic engineering has focused on diverting flux towards a product by means of by-product elimination and improvement of upstream precursor availability through gene regulation. Conversely, there have been relatively few studies that couple the inactivation and regulation of target genes to modification of cofactor pools to drive pathway flux. Improving understanding of cofactor engineering would aid in future engineering of production strains.
3 Materials and Methods

3.1 Strains, media and culture conditions

In metabolic engineering experiments, *S. cerevisiae* CEN.PK 113-1A, a haploid strain (MAT α) procured from the laboratory of Dr. Vince Martin (Concordia University) was used. In all cases, strains were maintained on YPD growth medium. Fresh cultures from frozen stock were used in all experiments. Mutant strains of *S. cerevisiae* CEN.PK 113-1A were maintained on YPD plates supplemented with the appropriate antibiotic marker (hygromycin 300 µg/mL, geneticin 200 µg/mL). Flask cultivations of mutant strains were carried out in mineral medium, as described by Hahn-Hagerdal et al. (2005), supplemented with 20 g/L glucose (Hahn-Hagerdal et al., 2005). The growth medium of some mutants, where indicated, was supplemented with 20 mg/L methionine. Strains were further characterized in mineral medium in MiniReactors (Applikon Biotechnology) with a working volume of 300 mL and pH controlled to 5.5 using 3M KOH.

Mutant strains of *S. cerevisiae* CEN.PK 113-1A were maintained on YPD plates supplemented with the appropriate antibiotic marker (hygromycin 300 µg/mL, geneticin 200 µg/mL). Flask cultivations of mutant strains were carried out in mineral medium supplemented with 20 g/L glucose. The growth medium of some mutants, where indicated, was supplemented with 20 mg/L methionine. Strains were further characterized in mineral medium in MiniReactors (Applikon Biotechnology) with a working volume of 300 mL and pH controlled to 5.5 using 3M KOH.

3.2 PCR generation of gene-disruption cassettes

Plasmids used for gene disruption cassettes, pUG6 (geneticin) and pAG34 (hygromycin), were procured from the Euroscarf (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) in strains of *E. coli* DH5α. Plasmids were isolated using a Sigma Miniprep extraction kit (Sigma Aldrich) and were used as described by Gueldener et al., (2002) and
Goldstein et al., (1999) to generate gene disruption cassettes. Forward and reverse primers were designed to have 50 bp homology to the target gene. The sequences cagctgaagcttcgtacgc and gcataggccactagtggatctg were added to the 3’ ends of the forward and reverse primers, respectively, as homology sequences to the plasmid templates.

Following an initial denaturation step (95°C for 2 minutes) the PCR cycle used was: (1) 95°C for 30 seconds, (2) 55°C for 10 seconds, (3) 72°C for 1 min 45 seconds and 3 minutes for using pUG6 and pAG34 for templates, respectively. Correct amplification of cassettes was confirmed in all cases by gel electrophoresis.

### 3.3 Transformation of gene-disruption cassettes

Disruption cassettes were transformed using a modified version of the PEG/LiAc method (Geitz et al., 2007). For a detailed transformation protocol, see Appendix A. Transformants grown from initial plating were replated on selective media to eliminate background expression. Successful deletions and correct locus integrations were confirmed by PCR using primers flanking the cassette on the genome and using a reverse primer designed to be partially inside the cassette (see Table 2 for a list of primers used in this study).

### 3.4 Analysis

Metabolites were measured using HPLC (Aminex HPX-87H column, 25 mM H2SO4, 25°C). Growth was monitored through optical density measurements using a spectrophotometer (Genesys 20, Thermo Scientific).
4 Results and Discussion

4.1 Strain Designs for Coupling NADPH Synthesis to $\alpha$-ketoglutarate Production

There exist several reactions in the cytosol of *S. cerevisiae* through which NADPH may be produced:

i) Glucose-6-phosphate dehydrogenase (*ZWF1*)
ii) Aldehyde dehydrogenase (*ALD6*)
iii) NADP$^+$ dependent isocitrate dehydrogenase (*IDP2*)
iv) Succinate semialdehyde dehydrogenase (*UGA2*)

In order to couple the production of NADPH to synthesis of alpha-ketoglutarate, we sought to engineer strains with limited pools of cytosolic NADPH. As a result, the strain would be forced to increase flux through *IDP2* to satisfy NADPH requirements. Specifically, we targeted the *ZWF1* and *ALD6* genes in order to reduce NADPH availability. Another consideration in strain design was the use of various isoforms of isocitrate dehydrogenase to produce $\alpha$-ketoglutarate. It has been previously shown that the NAD$^+$-dependent *IDH1/2* (mitochondrial) is the dominant isoform during aerobic growth on glucose, and further reported results suggest that isocitrate pools in the cytosol are severely limited (Loftus et al., 1994).

To address the limitations of diminished isocitrate pools and insufficient flux of *IDP2*, our approach involved activation of the glyoxylate cycle. The glyoxylate cycle in *S. cerevisiae* is an interesting pathway in that it functions exclusively in the cytosol, despite the TCA cycle being localized in the mitochondria (Figure 4). In addition, the isocitrate required for the first step of the pathway must be transported out of the mitochondria, as the conversion of citrate to isocitrate occurs exclusively in the mitochondria. A previous computationally-based strain design for tyrosine biosynthesis suggested the activation of the glyoxylate cycle through gene deletions that forced
oxaloacetate (OAA) to be produced by the pathway. In this design, this was accomplished by deleting each of (1) the mitochondrial malate dehydrogenase to prevent OAA from being made in the mitochondria; (2) the malate transporter to prevent malate from being shuttled to the cytosol and subsequently oxidized to oxaloacetate; and (3) the pyruvate carboxylase gene to prevent synthesis of oxaloacetate in the cytosol (Figure 4A). It was reasoned that the increase in flux through the glyoxylate cycle improved tyrosine production by improving NADPH availability (Cautha, 2012).
Figure 4: Engineering approaches to activate the glyoxylate cycle. Activation of the glyoxylate cycle by (A) isolation of oxaloacetate synthesis (Cautha, 2012) and (B) inactivation of succinyl-CoA ligase.
Table 2: Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type α</td>
<td>CEN.PK 113-1A</td>
<td>Dr. Vince Martin, Concordia University</td>
</tr>
<tr>
<td>ΔLSC2</td>
<td>CEN.PK 113-1A  LSC2::hphMX</td>
<td>This study</td>
</tr>
<tr>
<td>ΔZWFI</td>
<td>CEN.PK 113-1A  ZWF1::kanMX</td>
<td>This study</td>
</tr>
<tr>
<td>ΔALD6</td>
<td>CEN.PK 113-1A  ALD6::hphMX</td>
<td>This study</td>
</tr>
<tr>
<td>ΔLSC2 ΔALD6</td>
<td>CEN.PK 113-1A  LSC2::kanMX  ALD6::hphMX</td>
<td>This study</td>
</tr>
<tr>
<td>ΔZWFI ΔLSC2</td>
<td>CEN.PK 113-1A  ZWF1::kanMX  LSC2::hphMX</td>
<td>This study</td>
</tr>
</tbody>
</table>

However, in our analysis, we reasoned that the identical activation of the glyoxylate cycle could be achieved by inactivation of succinyl-CoA ligase (LSC1/2) alone. Deletion of the β-subunit of succinyl-CoA ligase (LSC2) (Figure 4B) prevents the conversion of succinyl-CoA to succinate in the mitochondria. Thus, in order to synthesize oxaloacetate to complete the TCA cycle, the cell must either use pyruvate carboxylase or export isocitrate from the mitochondria for conversion to isocitrate via the glyoxylate cycle. We reasoned that the former case is far less beneficial towards the growth of the cell, in that pyruvate is converted to oxaloacetate at the cost of one molecule of ATP, rather than being converted to acetyl-CoA and used towards biomass production. Through natural selection, the deletion of LSC2 should induce the glyoxylate cycle in *S. cerevisiae*, and as a result, isocitrate pools will be increased in the cytosol. To explore the implications of NADPH limitation coupled with activation of the glyoxylate cycle in *S. cerevisiae*, we made deletion combinations of ZWF1, ALD6 and LSC2. Strains constructed in this work are listed in Table 2.
4.2 Synthetic Rescue of a $\Delta ZWF1$ Strain by Activation of the Glyoxylate Cycle

As an initial characterization, strains were cultivated in mineral medium supplemented with 2% glucose in shake flasks. Growth rate and acetate secretion were measured to ascertain the impact of gene deletions on the metabolism. The deletion of $LSC2$ appeared to have no effect on the growth rate and acetate yield of strains as compared to the wild-type strain (Figure 5). However, the growth rates of strains harbouring the $\Delta ZWF1$ genotype were reduced significantly, and were rescued by the addition of methionine to the growth media (Figure 5A). Moreover, we determined the acetate yield of the $\Delta ZWF1$ deletion strain to be eight fold-higher than the wild-type strain, demonstrating the up-regulation of $ALD6$ to compensate for NADPH starvation. In cultivations with additional methionine in the growth media, acetate yield was observed to decrease substantially, suggesting that the NADPH limitation was somewhat alleviated in these cases.

We then disrupted $LSC2$ in the $\Delta ZWF1$ background. Interestingly, while acetate yield was increased eight-fold in the $\Delta ZWF1$ strain, an increase of only five-fold in the $\Delta ZWF1 \Delta LSC2$ strain was observed, with a concomitant increase in the growth rate of the double deletion over the single (Figure 5B). This is counterintuitive in that the deletion of a gene usually is accompanied by no change or a reduction in growth rate rather than an increase in strain fitness. In order to further investigate the mechanism of the synthetic rescue, we cultured both strains in the presence of methionine, effectively alleviating the the NADPH limitation. We found that the deletion of $LSC2$ in a $\Delta ZWF1$ background only improved strain fitness in media where exogenous methionine has not been added (Table 3). This result suggests that the mechanism of the rescue observed in the double deletion strain is a result of an increase in NADPH pools.
Figure 5: Growth rate and acetate yields of mutant strains lacking *ZWF1* in shake flasks. Error bars indicate the standard deviation of two replicates.

While the upregulation of *ALD6* is known to be utilized in Δ*ZWF1* strains to compensate for NADPH starvation, this was likely not the mechanism of recovery in the Δ*ZWF1* Δ*LSC2* strain, as the acetate yield is observed to decrease in the double deletion strain relative to the single deletion. An alternative reaction that may be used is the cytosolic NADP⁺ dependent isocitrate dehydrogenase (*IDP2*), which is a homolog of the mitochondrial isocitrate dehydrogenase (*IDH1/2*), the dominant isoform used during growth on glucose. Moreover, there is no gene encoding an enzyme to convert citrate to isocitrate in the cytosol and as a result, activity of IDP2p appears to be negligible during regular growth. Accordingly, isocitrate used in this reaction must be synthesized in and transported out of the mitochondria.
Table 3: Comparison of growth characteristics of $\Delta ZWF1$ and $\Delta ZWF1 \Delta LSC2$ strains.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>$\mu$ met$^-$</th>
<th>$\mu$ met$^+$</th>
<th>Fold Change$^a$</th>
<th>$Y_{Ac/G}$ met$^-$</th>
<th>$Y_{Ac/G}$ met$^+$</th>
<th>Fold Change$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.32 ± 0.0063</td>
<td>0.34</td>
<td>- -</td>
<td>1</td>
<td>1</td>
<td>- -</td>
</tr>
<tr>
<td>$\Delta ZWF1$</td>
<td>0.12 ± 0.0024</td>
<td>0.21 ± 0.0098</td>
<td>1.82 ± 0.12</td>
<td>8.2 ± 0.42</td>
<td>3.6 ± 0.44</td>
<td>0.56 ± 0.078</td>
</tr>
<tr>
<td>$\Delta ZWF1\Delta LSC2$</td>
<td>0.16 ± 0.0072</td>
<td>0.22 ± 0.0043</td>
<td>1.37 ± 0.03</td>
<td>5.2 ± 0.29</td>
<td>3.9 ± 0.47</td>
<td>0.25 ± 0.035</td>
</tr>
</tbody>
</table>

* Acetate yields normalized to wild-type.

$^a$ Fold change between met$^-$ and met$^+$ cultivations.

We hypothesize that through the perturbation of $LSC2$, the mitochondrial TCA cycle is interrupted and the organism must utilize the glyoxylate cycle for the production of oxaloacetate. In order to complete the first step of the glyoxylate cycle, isocitrate lyase, isocitrate must be transported from the mitochondria to the cytosol. As a result, cytosolic isocitrate is available for the activity of $IDP2$ and thus an additional source of NADPH is available, resulting in increased strain fitness. This theory is also able to account for the reduction in acetate production, as NADPH is now produced by isocitrate dehydrogenase, resulting in the formation of $\alpha$-ketoglutarate, which would be of more benefit to biomass production as compared to acetate.

Interestingly, in aerobic batch cultivations of the $\Delta ZWF1$ with exogenous methionine added, two distinct growth rates were observed (Figure 6B). Although the precise mechanism of this growth profile is unknown, it is likely a result of NADPH stress. Cultivations of $\Delta ZWF \Delta LSC$ met$^-$ showed a similar trend as the $\Delta ZWF$ met$^+$ strain, supporting the hypothesis that the deletion of $LSC2$ acts as a synthetic rescue in the strain.

The hypothesis of glyoxylate cycle activation and cytosolic isocitrate availability also explains results published in a study by Grabowska and Chelstowska in 2003, which investigated the $\Delta ZWF1$ phenotype and attempted to rescue the phenotype by overexpressing reactions that produced NADPH (Grabowska & Chelstowska, 2003).
Moreover, the authors demonstrated that the double deletion of \( \Delta ZWF1 \) and \( ALD6 \) is not viable, and thus concluded that \( ALD6 \) is an indispensable source of NADPH when the oxidative pentose phosphate pathway is disrupted. As well, the authors constructed a strain of \( S. \ cerevisiae \) with the \( \Delta ZWF1 \) genotype, and found that while growth could be rescued by overexpression of \( ALD6 \), they determined that there was no benefit to fitness when \( IDP2 \) was overexpressed. However, this is a result that is anticipated given the fact that \( IDH1/2 \) is almost exclusively used for growth on glucose (Loftus et al., 1994), compounded with the lack of enzymes to catalyze the conversion of citrate to isocitrate in the cytosol. As a result, under aerobic growth on glucose, there would be no cytosolic pools of isocitrate and thus, no substrate for which \( IDP2 \) to utilize, regardless of its expression level. Therefore, no improvement in strain fitness should be observed with the overexpression of \( IDP2 \) in a \( \Delta ZWF1 \) background.

Indeed, the rescue of NADPH starved strains by IDP2p is further supported by a study published in 2005 by Minard and McAlister-Henn, which investigated sources
of NADPH as a function of carbon source (Minard & McAlister-Henn, 2005). In this study, the authors were able to construct a ΔZWF1 ΔALD6 double deletion strain, which was previously thought to be non-viable due to cytosolic NADPH limitations (Grabowska & Chelstowska, 2003), by culturing the strain on lactate, a non-fermentable carbon source. When grown on non-fermentable carbon sources, S. cerevisiae is known to use the glyoxylate cycle as key source of oxaloacetate (Pronk et al., 1996). During cultivation studies in which the double deletion strain was first cultured on YP-lactate (2%) and then transferred to YP-glucose (2%), the authors noted that when growth resumed following depletion of glucose and diauxic lag, Idp2p (cytosolic NADPH-dependent isocitrate dehydrogenase; encoded by IDP2) levels were elevated. In their manuscript, the authors note that other genes such as Ald4p and Ald5p that might compensate for the disruption of ALD6. However, this is likely only true to the extent of acetyl-CoA synthesis, as Ald4p and Ald5p are mitochondrial NADP⁺-dependent aldehyde dehydrogenases and thus cannot contribute directly to the regeneration of NADPH in the cytosol. With these considerations in mind, it is very likely that the strain was able to recover and grow by utilizing IDP2 to generate cytosolic NADPH. Isocitrate pools in this case would again be provided via the glyoxylate cycle, which would be active following growth with lactate as a carbon source.

These results strongly suggest that the reason behind the viability of the ΔZWF ΔALD6 genotype recovered on lactate is due to the activation of the glyoxylate shunt, which in turn necessitates transport of isocitrate from the mitochondria to the cytosol. The presence of cytosolic isocitrate enables use of the NADP⁺ dependent isocitrate dehydrogenase, thereby increasing cytosolic NADPH pools, which is likely the cause of the rescue of the ΔZWF1 ΔLSC2 strain.

A corollary of this hypothesis is that the activation of the glyoxylate cycle during growth on non-fermentable carbon sources is due to NADPH limitations. It is a distinct possibility that the glyoxylate cycle is preferential during growth under
these conditions, as opposed to the oxidative pentose phosphate pathway, which would require activation of gluconeogenic pathways. This is supported by a recent study by Zampar et al. (2013) that found increased concentration of the metabolic intermediates of the glyoxylate cycle and decreased metabolites of the pentose-phosphate pathway following the depletion of glucose and aerobic consumption of ethanol (Zampar et al., 2013). The authors as well proposed that this could be a result of gluconeogenic pathways being metabolically expensive for the cell.
4.3 Batch Cultivation and Production of α-ketoglutarate

Mutant and wild-type strains of *S. cerevisiae* were cultured under aerobic conditions in batch reactors in a working volume of 300 mL with pH controlled to 5.5 using 3M KOH. Similar to the case in baffled flask cultures, we found the growth rate of strains lacking the *ZWF1* gene to have severely reduced growth rate (Figure 7).

![Graphs showing growth rate, glucose concentration, α-ketoglutarate concentration, acetate concentration, pyruvate concentration, and ethanol concentration over time for wild-type, Δ*ZWF1*, and Δ*ZWF1* supplemented with 20 mg/L methionine strains.]

Figure 7: Characterization of the Δ*ZWF1* deletion in batch cultivation of *S. cerevisiae* CEN.PK 113-1A. Wild-type (open diamonds); Δ*ZWF1* (open triangles); Δ*ZWF1* supplemented with 20 mg/L methionine (open squares).

In our cultivations, the Δ*ZWF1* strain grew non-exponentially and ceased to grow after approximately 32 hours, and after consuming approximately 10% of glucose available. This result suggests that NADPH is severely limiting in these strains, and is in agreement with previous studies that have found the strain to be auxotrophic.
for methionine. HPLC analysis indicated that \( \alpha \)-ketoglutarate was indeed produced by this strain with yields of approximately 2.5-fold that of wild-type (Figure 7C). However, due to the poor growth rate of the cell, the titres quantified in the growth media were below that of the wild-type strain.

In order to improve the growth rate of the \( \Delta ZWF1 \) strain, 20 mg/L methionine was added to the growth medium. We observed a marked improvement in the growth and substrate consumption profiles. Interestingly, the culture appeared to grow in an exponential manner for 10 hours, followed by a reduced growth phase, suggesting that exogenous methionine was able to rescue the strain until it had diminished and the strain again entered an NADPH starved growth regime. With the addition of methionine, we had previously thought that the production of \( \alpha \)-ketoglutarate would be reduced, as the driving force for its synthesis (NADPH starvation) would be reduced with the addition of methionine to the growth media. However, contrary to our hypothesis, we observed a substantial increase in \( \alpha \)-ketoglutarate secretion in the strain (Figure 7C). These results suggest that despite the addition of methionine being able to rescue the growth rate of the mutant strain, it does not wholly diminish the cofactor stress, and thus flux through \( IDP2 \) remains high.

Despite the coupling of \( \alpha \)-ketoglutarate synthesis to production of NADPH, we observed very low titres in cultivations (approximately 350 \( \mu \)M). This result is not unexpected, as \( \alpha \)-ketoglutarate is a central metabolite that may be directed towards production of biomass; however a high flux through \( IDP2 \), as is suggested by the large increase in secreted \( \alpha \)-ketoglutarate, would be beneficial for a platform strain for adipic acid production. Interestingly, in both cases of the mutant strain, acetate and pyruvate are formed as byproducts in significant amounts. While increased acetate secretion would be expected in the deletion strain, we did not anticipate an increase in pyruvate synthesis. It is possible that increased pyruvate synthesis is due to a bottleneck in the downstream metabolism, possibly a result of a maximum flux limit
through \textit{ALD6}. The increase in the formation of these products highlights the degree to which organisms can be robust to metabolic perturbations, through activation and up-regulation of multiple pathways. The formation of acetate and pyruvate should be addressed in future strain engineering, as they represent significant amounts of carbon that is directed away from $\alpha$-ketoglutarate. Interestingly, we also observed a decrease in the ethanol yield of the strain, possibly due to the channeling of flux from acetaldehyde to acetate rather than ethanol due to NADPH stress. Nonetheless, the product profiles and yields of the $\Delta ZWF1$ strain clearly demonstrate the coupling of NADPH synthesis to the production of $\alpha$-ketoglutarate.

We further characterized the $\Delta ZWF1$ $\Delta LSC2$ strain (Figure 8), which should have increased fitness and $\alpha$-ketoglutarate synthesis as a result of glyoxylate cycle activation. We observed that the double deletion of $\Delta ZWF1$ $\Delta LSC2$ had a superior growth rate as compared to the $\Delta ZWF1$ strain, demonstrating that $LSC2$ acts as a synthetic rescue for the strain. Comparison of yields revealed a decrease in the acetic acid formation as compared to the single deletion case, suggesting that the mechanism of rescue is based on improving NADPH synthesis (Figure 9).

Additionally, the $\Delta ZWF1$ $\Delta LSC2$ strain produced a higher titer of $\alpha$-ketoglutarate as compared to the single deletion strain. The addition of methionine to the growth medium had a similar impact on cultivation as in the single deletion case; strain fitness was improved while no reduction in $\alpha$-ketoglutarate synthesis was observed. In fact, the $\Delta ZWF1$ $\Delta LSC2$ strain supplemented with methionine showed the highest improvement in yield of $\alpha$-ketoglutarate (Figure 9). Curiously, in the presence of methionine, $\Delta ZWF1$ $\Delta LSC2$ produces acetate in similar yields as compared to the $\Delta ZWF1$ strain grown without methionine. Nonetheless, these results conclusively demonstrate that the inactivation of succinyl-CoA ligase acts, as predicted in this work, as a synthetic rescue for the $\Delta ZWF1$ strain.
Figure 8: Characterization of the \( \Delta ZWF1 \Delta LSC2 \) double deletion strain in batch cultivation of \( S. \) \textit{cerevisiae} CEN.PK 113-1A. Wild-type (open diamonds); \( \Delta ZWF1 \Delta LSC2 \) (open triangles); \( \Delta ZWF1 \Delta LSC2 \) supplemented with 20 mg/L methionine (open squares).
4.4 Impacts of the Deletion of *ALD6* on the Metabolism of *S. cerevisiae*

In addition to the oxidative pentose phosphate pathway, the ALD6p is a major enzyme involved in the generation of cytosolic NADPH, producing approximately one-half the amount that is done so by the oxidative pentose phosphate pathway (Frick, 2005). Based on the previous results shown for the deletion of *ZWF1*, we expected that interruption of *ALD6* would have a similar, albeit lesser, effect by reducing the ability of the strain to produce NADPH. However, we found that the deletion of *ALD6* did not diminish the growth rate or biomass yield of the strain.

![Figure 9: Comparison of growth rates and yields of mutant strains cultured in glucose mineral media. Values normalized to wild-type. Glycerol yields for Δ*ALD6* deletion strains not shown.](image)

This finding is in contrast to a previous study by Meaden and colleagues in 1994 which found that the deletion of *ALD6* decreased growth rate of a *MAT A* haploid of *S. cerevisiae* by over two-thirds (Meaden et al., 1994). Interestingly, the deletion of *ALD6* did not appear to incur significant oxidative stress in the mutant strain, as suggested by the production of α-ketoglutarate and other oxidized metabolites at a lesser rate than that of wild-type (Figure 10). Additionally, excretion of acetate
was nearly eliminated in the strain, which was an anticipated result as \( ALD6 \) is a major isoform of aldehyde dehydrogenase. The elevated growth rates and reduced titers of oxidized metabolites suggests that although \( ALD6 \) is thought to be a major contributor to cytosolic NADPH, the metabolic network is robust in the sense that other reactions may easily compensate for its absence with minimal perturbation. Table 4 summarizes the absolute yields determined from the characterization of all mutant strains constructed in this work.

Table 4: Comparison of cultivation characteristics of mutant strains constructed in this work. Aerobic growth on glucose in 300 mL working volume.

<table>
<thead>
<tr>
<th>Strain</th>
<th>( Y_{X/Glc} ) (mg\textsubscript{DW}/mM\textsubscript{Glc})</th>
<th>( Y_{AKG/Glc} )</th>
<th>( Y_{Ac/Glc} )</th>
<th>( Y_{EtOH/Glc} )</th>
<th>( Y_{Pyr/Glc} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type( ^\dagger )</td>
<td>9.23 ± 0.35</td>
<td>1.02 ± 0.23</td>
<td>37.5 ± 6.9</td>
<td>1394 ± 72</td>
<td>37.9 ± 2.0</td>
</tr>
<tr>
<td>( \Delta ZWF1 )( ^\ddagger )</td>
<td>10.3 ± 1.0</td>
<td>2.76 ± 0.45</td>
<td>114 ± 5.7</td>
<td>1119 ± 128</td>
<td>60.1 ± 6.8</td>
</tr>
<tr>
<td>( \Delta ZWF1 ) \text{met} ( ^\ddagger )</td>
<td>11.4 ± 2.5</td>
<td>3.00 ± 0.32</td>
<td>108 ± 1.8</td>
<td>855 ± 82</td>
<td>59.2 ± 5.0</td>
</tr>
<tr>
<td>( \Delta ZWF1 ) \text{ΔLSC2} ( ^\dagger )</td>
<td>10.5 ± 1.4</td>
<td>3.19 ± 0.31</td>
<td>89.4 ± 6.3</td>
<td>627 ± 48</td>
<td>57.1 ± 12.5</td>
</tr>
<tr>
<td>( \Delta ZWF1 ) \text{ΔLSC2} \text{met} ( ^\ddagger )</td>
<td>10.8 ± 1.4</td>
<td>3.68 ± 0.58</td>
<td>136 ± 12</td>
<td>1064 ± 64</td>
<td>58.4 ± 10</td>
</tr>
<tr>
<td>( \Delta ALD6 )( ^\dagger )</td>
<td>10.4 ± 0.19</td>
<td>0.49 ± 0.12</td>
<td>27.0 ± 0.40</td>
<td>1404 ± 276</td>
<td>44.7 ± 0.60</td>
</tr>
<tr>
<td>( \Delta ALD6 ) \text{ΔLSC2} ( ^\dagger )</td>
<td>10.2 ± 1.1</td>
<td>0.42 ± 0.032</td>
<td>27.2 ± 1.3</td>
<td>1738 ± 174</td>
<td>37.7 ± 12</td>
</tr>
<tr>
<td>( \Delta ALD6 ) \text{ΔLSC2} \text{met} ( ^\ddagger )</td>
<td>11.1 ± 2.6</td>
<td>0.59 ± 0.13</td>
<td>25.0 ± 2.5</td>
<td>1040 ± 104</td>
<td>37.0 ± 3.7</td>
</tr>
</tbody>
</table>

\( Y_{X/Glc} \) represents biomass yield.

Yields of wild-type calculated at 16 hours into batch, prior to depletion of glucose. Yields of mutant strains calculated at 24 hours into all batches, prior to depletion of glucose. Values reported as mM\textsubscript{Product}/mM\textsubscript{Glucose} unless otherwise noted. Error represents the standard deviation between two (\( ^\dagger \)) or three (\( ^\ddagger \)) replicates.
Figure 10: Characterization of the $\Delta ALD6$ deletion in batch cultivation of S. cerevisiae CEN.PK 113-1A. $\Delta ALD6$ (open diamonds); $\Delta ALD6 \Delta LSC2$ (open triangles); $\Delta ALD6 \Delta LSC2$ supplemented with 20 mg/L methionine (open squares).
5 Conclusions and Outlook

In this work, we sought to construct a platform strain of *S. cerevisiae* for adipic acid biosynthesis by overproducing α-ketoglutarate, a common precursor of several proposed adipic acid synthesis pathways. We constructed strains of *S. cerevisiae* CEN.PK 113-1A deficient in cytosolic NADPH synthesizing pathways were experimentally constructed in efforts to couple the production of α-ketoglutarate to the NADPH synthesis and thus, the growth of the organism, through the activity of cytosolic isocitrate dehydrogenase. Specifically, we examined the impact of deleting the glucose-6-phosphate dehydrogenase (*ZWF1*) and aldehyde dehydrogenase (*ALD6*), two key reactions in cytosolic NADPH synthesis, and succinyl-CoA ligase β-subunit (*LSC2*) to disrupt the TCA cycle.

Strains lacking the *ZWF1* gene had a dramatic decrease in growth rate and increase in acetate yield as expected due to NADPH starvation and increased flux through *ALD6* to generate NADPH. The strain was shown to be able to be rescued with the addition of methionine to the growth medium; a result consistent those previously reported in literature. We found that interrupting the TCA cycle in a Δ*ZWF1* by disrupting the *LSC2* gene increased growth rate and decreased acetate yield, suggesting the partial alleviation of NADPH starvation. We believe that the improvement in NADPH synthesis is due to activation of the glyoxylate cycle, and hence increased cytosolic isocitrate pools and activity of the NADP+-dependent isocitrate dehydrogenase.

As the product profiles of these strains have not been previously reported, we characterized their growth in aerobic batch cultivations in glucose mineral medium. We found that the production of acetate and α-ketoglutarate were greatly increased in the Δ*ZWF1* and Δ*ZWF1 ΔLSC2* strains, and that the strains themselves could not consume all substrate present due to their methionine auxotrophy. The addition of methionine in these cultures rescued the growth rate of the strains, but did not eliminate production of α-ketoglutarate, demonstrating that methionine does not wholly alleviate
NADPH starvation and the strain must continue to upregulate \textit{ALD6} and \textit{IDP2} to maintain viability. These results demonstrate coupling of $\alpha$-ketoglutarate synthesis to NADPH production. Notably, increases in concentrations of $\alpha$-ketoglutarate produced were achieved without the use of other metabolic engineering approaches, such as upregulation of upstream pathways and elimination of byproducts. It is probable that the inclusion of such approaches in cofactor engineered strains would greatly improve $\alpha$-ketoglutarate production.

We also determined that the \textit{ALD6} gene is not as crucial to strain viability as has been previously reported. In our constructed strains lacking the \textit{ALD6} gene, we observed no negative impact on growth rate or yield, minimal changes in ethanol profiles during aerobic cultivation and very little evidence of oxidative stress. However, we did observe significantly decreased concentrations of acetic acid in the growth media as compared to the wild-type strain. These findings indicate that alternative reactions are easily used by the strain to compensate for the perturbation, and also suggest that the deletion of \textit{ALD6} only becomes significant when the strain already has a reduced pool of cytosolic NADPH.

The results of this work illustrate the importance of cofactor availability and balance in metabolic engineering strategies. By targeting critical reactions in NADPH synthesis, we show that the viability and product profile of \textit{S. cerevisiae} may be dramatically shifted towards production of oxidized compounds such as acetate, pyruvate and $\alpha$-ketoglutarate. Broadly, the approaches highlighted in this work may be applied to strain designs to manipulate cofactor balance and availability towards improved production of target metabolites.
6 Recommendations

The work presented herein is an investigation into the role of cofactors in targeted overproduction of compounds via metabolic engineering. While an important step, additional research is required in order to develop strains that produce significantly more α-ketoglutarate in order to be commercially viable. Prospective follow-up studies are discussed in this section. Firstly, regardless of the increased titres of products observed in cultivations, one significant limitation of this work is the dramatic decrease in growth rate observed in the engineered strains. Specifically, our results show that the ΔZWF1 genotype is deleterious to the point where strains are only able to consume a fraction of glucose present in batch cultivation. In the past, adaptive evolution has been conducted on engineered strains in order to improve growth rate by natural selection. Improvement in the growth rates of the strains reported in this work by adaptive evolution would be very interesting, as in order to improve growth rate, improvement of NADPH synthesis would be required, likely to some extent through the use of IDP2 and thus, be linked with increasing α-ketoglutarate production. Alternatively, in order to decrease acetate formation by the ΔZWF1 strain, ALD6 would be a desirable target for further genetic perturbations. In this work we attempted to construct the ΔZWF1 ΔALD6 phenotype as was described by Minard et al (2005), but were unable to isolate the double deletion strain (Minard & McAlister-Henn, 2005). Characterization of such a strain would shed more light on the viability of using IDP2 as the sole reaction for production of cytosolic NADPH. Further, up regulation of IDP2 in a ΔZWF1 ΔALD6 ΔLSC2 strain background would likely improve α-ketoglutarate production significantly. Additionally, metabolic flux could be directed towards the TCA cycle through the inactivation of pyruvate decarboxylase (PDC).

Moreover, in this work the deletion of LSC2 is hypothesized to activate the glyoxylate cycle on the basis of carbon efficiency in the cell. While results presented in this study strongly suggest that this is the case, it may be verified by gene expression
data or 13C flux analysis would be a useful follow up study. Such experiments would also prove useful in identifying other pathways which may play a role in the localized redox metabolism, such as NADP$^+$ dependent succinate semialdehyde dehydrogenase, an enzyme of the glutamate degradation pathway. In addition, it would be interesting to overexpress IDP2 in the ΔZWF ΔLSC background and ascertain if there is growth improvement, which would also verify that the glyoxylate cycle is active. Following this, the overexpression of IDP2 in this background could be used to generate the ΔZWF1 ΔALD6 genotype as IDP2 serves as a mechanism by which the strain may be rescued.

Lastly, it would be of great interest to couple NADPH with production of alternative compounds to investigate if it might have a similar effect. Particularly intriguing is the production of succinate through the glutamate degradation pathway. A previous study has demonstrated that the disruption of both mitochondrial isocitrate dehydrogenases (IDH and IDP1) results in a viable mutant, which would as a result use IDP2 exclusively for this activity in the TCA cycle; this combination of deletions would be ideal in a ΔZWF1 mutant. From α-ketoglutarate, glutamate may be formed and then degraded by NADP$^+$ dependent succinate semialdehyde dehydrogenase, thus producing an additional molecule of NADPH and succinate. Production of succinate and acetate are equivalent in their benefit to overall production of NADPH. Considering that succinate may be further directed towards production of biomass, it is likely that the engineered strain will selectively produce succinate to compensate for NADPH limitations.
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Appendix A

High-efficiency Transformation of Yeast (courtesy of Nick Milne, TUDelft)
Adapted from Geitz and Schiestl (2007) Nat. Protoc. 2(1):31-34.
Updated by Patrick Hyland (2012).

1. Inoculate 50 mL YPD with 5-6 mL overnight culture (depending on growth rate). Incubate at 30°C, 250 rpm in a baffled flask.

2. When the culture reaches approximately an OD$_{600}$ of 2 relative to a blank of YPD (approximately 4-5 hours) harvest in sterile centrifuge tubes. Spin at 3000g for 5 min.

3. Carefully discard supernatant.

4. Resuspend in 25 mL autoclaved MILLIQ H$_2$O and centrifuge again at 3000g for 5 min. If not enough water is removed in this step, but volume is less than 1.5 mL, transfer to sterile eppendorf and spin at max speed for 15 seconds and aspirate off the water.

5. Carefully discard water and resuspend in 1.0 mL of 100 mM LiAc solution in 1 M sorbitol. Transfer solution to a sterile 1.5 mL eppendorf (solution should be approximately 1.5 mL). Pellet the cells and remove supernatant with pipette. Sorbitol is used to reduce osmotic stress. Weak LiAc is used in this step to make the cells competent; cells should not be in LiAc for more than 20-30 minutes, and can stay at room temperature.

6. Resuspend pellet in 400 µL 100 mM LiAc (in 1M sorbitol) solution. Total volume will be approximately 500 µL, enough for 10 transformations.

7. When ready for transformations (see step 9 for DNA preparation), pipette 50 µL of the cell suspension into a separate sterile eppendorf tube. For transformation of plasmid DNA, use 50 µL of mini prep.

8. Boil a 35-40 µl aliquot of ssDNA (10 mg/mL) for 5 minutes to denature it. See Geitz and Schiestl, (2007) for instructions to make the solution. * Put boiled ssDNA on ice immediately following boiling step SSDNA improves efficiency of transformation. Typical protocols use 2 mg/mL, however efficiency appeared to be higher with 10 mg/mL.

9. While ssDNA is boiling, thaw PCR reactions containing the cassettes. Add five reactions to one Amicon Ultra 0.5 mL 10K Centrifugal Filter (Millipore). Centrifuge at max speed for 8-10 minutes. This is a lot of DNA. With five reactions you should get 400+ transformants - likely 2-3 reactions would suffice

10. Add 300 µL of sterile water to the filters and spin again 8-10 minutes.
11. Place filters inverted into a new centrifuge tube and spin at max speed for 2 minutes. * Place DNA on ice.

12. The transformation mix is: - 240 µL of PEG 2200 (50% w/v) *
    - 36 µL of 1.0 M LiAc (in 1M sorbitol)
    - 25 µL of ssDNA (10 mg/mL)
    - 50 µL of DNA to be transformed (or amount recovered from filter)

    *concentration is critical for efficiency

    PEG2200 stabilizes the cell and prevents lysis. 1.0 M LiAc degrades the cell membrane for transformation.

13. Mix the PEG, LiAc together in a sterile eppendorf tube. When ready to transform cells, add ssDNA to the PEG/LiAc mix and then add directly to 50 µL cells. Mix well by aspirating.

14. Add DNA to PEG/LiAc/ssDNA/cell mix and mix well by aspirating or vortexing.

15. Incubate for 30 minutes at 30°C.

16. Heat shock for 30 minutes at 42°C

    30 minutes is used for CEN.PK. Optimum heat shock times will vary between strains used.

17. Let rest for approximately 5 minutes at room temperature.

18. Spin down cells at max speed for 15-20 seconds.

19. Gently remove supernatant using a pipette and add 1mL YPD. Transfer to a 15 mL sterile centrifuge tube and incubate for 2 hours at 30°C.

20. Spin down and resuspend in 400 µL YPD. Plate onto selective plates and incubate for 2 days at 30°C.

21. This protocol should have a very high number of transformants (200+ per plate).
## Appendix B

Table 5: Primers used in this work.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’ → 3’</th>
<th>Description</th>
</tr>
</thead>
</table>
| LSC2fwd     | CCGCGTAGTCAACATCTTTTGAG  
              | TTTTATATAAGATCGACGTTCCT-TTACGCGcatgaagttctgcgc | Forward cassette primer for LSC2 |
| LSC2rev     | TAATCCGCCAATAACGCACTGAT  
              | TACGTAAGCATAATATGTCCCT-GAAGCTCGCgcattgaggcccagtcgc | Reverse cassette primer for LSC2 |
| LSC2chkFwd  | CGCGTAGTCAACATCTTTCAG  | Forward check primer for LSC2 |
| LSC2chkRev  | GTACTAATCCGCAATAACGC | Reverse check primer for LSC2 |
| ZWF1fwd     | GTGGCTTTCTCTCTGGCCCTCTTC  
              | TCCCTTTTTTCACCCTCCCTCCCTCCCTCCTCCCTGCGcatgaagttctgcgc | Forward cassette primer for ZWF1 |
| ZWF1rev     | CAAAACAAATACACACGCAAGTA  
              | GAGAGGAGTTGTTGGGGGGGAGA-GATGCGcatagccactagtggatgc | Reverse cassette primer for ZWF1 |
| ZWF1chkFwd  | CCTAAAGTGGCTTCCTTCCTGC | Forward check primer for ZWF1 |
| ZWF1chkRev  | CCGATAATAGTGGCTTCCTGC | Reverse check primer for ZWF1 |
| ALD6fwd     | ATGACTAAGCTACATTCTTTTGACA  
              | CTCGCTGAACCTCAGTCAACACGATCAGTCACCTCCCTGCGcatgaagttctgcgc | Forward cassette primer for ALD6 |
| ALD6rev     | TTACAACCTTAATTTCTGACACCT  
              | TTACCGTGATCTGATGGTGATGGATGACTGCGcatagccactagtggatgc | Reverse cassette primer for ALD6 |
| ALD6chkFwd  | GGGATTCAAGACAAGCAACC | Forward check primer for ALD6 |
| ALD6chkRev  | CGGAAAGAATGCGAGGTTGG | Reverse check primer for ALD6 |
| hphRchk     | CCCCAATGTCAAGCAGCTTTCC  | Reverse check primer for hphMX3 cassette |
| kanMXb      | GGATGTATGGCTAAATGC | Reverse check primer for kanMX cassette (Gueldener et al, 2006) |