Supplemental File 1

Maximal theoretical acetate uptake stoichiometry

Acetate can be converted into ethanol via AMP-forming ACS (EC 6.2.1.1), ADA (EC 1.2.1.10), and ADH (EC 1.1.1.1). For each molecule of acetate, AMP-ACS converts 1 ATP to 1 AMP. However, to simplify our stoichiometric calculations, we will assume that this equals the conversion of 2 ATP to 2 ADP. Note that while this substitution is generally valid when considering reaction stoichiometries (1), it should not be used for calculations involving reaction thermodynamics and kinetics. For all equations in this document, we also leave out $NAD(P)^+$, H^+, H_2O, ADP, and inorganic phosphate, and all reactions involving ATP will be interconversions of ATP and ADP. We now can express the conversion of acetate into ethanol as:

$$1\ acetate + 2\ NAD(P)H + 2\ ATP \rightarrow 1\ ethanol\ (eq.1)$$

Since this pathway requires ATP and an input of electrons, it cannot operate without supporting reactions. Here we will calculate the maximal theoretical acetate uptake stoichiometry under anaerobic conditions, when ethanolic fermentation from glucose provides both the necessary ATP and NAD(P)H.

Ethanolic fermentation of glucose via glycolysis, pyruvate decarboxylase and alcohol dehydrogenase is redox neutral in *S. cerevisiae*, and produces ATP with equimolar amounts of ethanol and CO_2:

$$1\ glucose \rightarrow 2\ ethanol + 2\ CO_2 + 2\ ATP\ (eq.2)$$

To generate the NAD(P)H required for acetate consumption, *S. cerevisiae* can divert glucose from glycolysis into the oxidative pentose phosphate pathway (oxPPP). The end product of glycolysis is pyruvate. In contrast, when a molecule of glucose is run through the oxPPP, the resulting products are fructose-6-P (F6P) and glyceraldehyde-3-phosphate (G3P):

$$1\ glucose + 1\ ATP \rightarrow 1\ G6P \rightarrow \frac{2}{3} F6P + \frac{1}{3} G3P + 1\ CO_2 + 2\ NAD(P)H\ (eq. 3)$$

When all F6P is recycled into the oxPPP, the equation becomes:

$$1\ glucose + 1\ ATP \rightarrow 1\ G3P + 3\ CO_2 + 6\ NAD(P)H\ (eq. 4)$$

The G3P can be converted to ethanol, resulting in the overall reaction:

$$1\ glucose \rightarrow 1\ ethanol + 4\ CO_2 + 6\ NAD(P)H + 1\ ATP\ (eq. 5)$$

When we compare equations 2 (glycolysis) and 5 (oxPPP), we see that use of the oxPPP effectively exchanges ethanol and ATP for NAD(P)H and CO_2, according to the following stoichiometry:

$$1\ ethanol + 1\ ATP \rightarrow 2\ CO_2 + 6\ NAD(P)H\ (eq. 6)$$

Equation 5 shows production of ethanol and CO_2. In theory, the G3P produced in equation 4 could also be recycled into the oxPPP and converted to CO_2, which would increase the NAD(P)H yield on glucose and eliminate ethanol production. However, G3P can only be converted to F6P through the gluconeogenic enzyme fructose-1,6-bisphosphatase (FBP), which hydrolyses a phosphate bond without generating ATP. In contrast, the glycolytic enzyme phosphofructokinase (PFK) expends ATP in the conversion of F6P to G3P. Recycling G3P in the oxPPP therefore requires an additional ATP investment that is undesirable as long as there is a net flux from F6P to G3P, as antagonistic action of FBP and PFK would just result in the conversion of ATP to ADP. As we will see below, maximum acetate uptake won't be limited by the NAD(P)H yield of the oxPPP on glucose. There will be a net flux from F6P to G3P, and no extra ATP will need to be invested to achieve a higher NAD(P)H yield in the oxPPP.

Equations 1 (x, acetate to ethanol), 2 (y, glycolysis), and 5 (z, oxPPP) can be solved as a system of linear equations for ATP and NAD(P)H:

$$ATP:\ -2x + 2y + z = 0$$

$$NAD(P)H: -2x + 6z = 0$$

A redox-neutral solution with zero net ATP production is available at an equation stoichiometry for x:y:z of 6:5:2. Adjusting the ratio of equations 1, 2, and 5 accordingly gives:

$$6\ acetate + 12\ NAD(P)H + 12\ ATP \rightarrow 6\ ethanol\ (eq.7)$$

$$5\ glucose \rightarrow 10\ ethanol + 10\ CO_2 + 10\ ATP\ (eq.8)$$

$$2\ glucose \rightarrow 2\ ethanol + 8\ CO_2 + 12\ NAD(P)H + 2\ ATP\ (eq.9)$$

This results in an overall conversion of:

$$7\ glucose + 6\ acetate \rightarrow 18\ ethanol + 18\ CO_2\ (eq.10)$$

At this stoichiometry, no ATP is available for cellular growth or maintenance. Per gram of glucose, 0.29 g acetate is converted, and the ethanol yield on glucose is 0.66 g g^{-1} (up from 0.51 g g^{-1} for ethanolic fermentation via glycolysis, per equation 1). Of the consumed glucose, 29% is directed into the oxPPP, and 71% into glycolysis. Since we assumed no G3P recycling for the oxPPP, this means that there is a net flux of 0.71 mol F6P to G3P per mol of glucose consumed.

Balancing cofactors with NADPH-ADH

In the calculations above, we worked with a single NAD(P)H pool of reduced redox cofactors. If we look at the actual cofactor specificity of the involved reactions in *S. cerevisiae*, the equations 1, 2, and 5 can be rewritten as:

$$1\ acetate + 2\ NADH + 2\ ATP \rightarrow 1\ ethanol\ (eq.11)$$

$$1\ glucose \rightarrow 2\ ethanol + 2\ CO_2 + 2\ ATP\ (eq.12)$$

$$1\ glucose \rightarrow 1\ ethanol + 4\ CO_2 + 6\ NADPH + 1\ ATP\ (eq.13)$$

Here we assume that ADA, ADH, and glyceraldehyde-3-P dehydrogenase are NADH-specific, and that glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase are NADPH-specific. It is apparent from these equations that there is a cofactor imbalance: conversion of acetate to ethanol consumes NADH, while the oxPPP produces NADPH.

The introduction of an NADPH-specific ADH would give the cell flexibility to use either NADH or NADPH as the cofactor for alcohol dehydrogenase. Since use of NADPH-ADH effectively replaces NADH consumption by NADPH consumption, we can represent its action by a transhydrogenase-like conversion:

$$1\ NADPH \rightarrow 1\ NADH\ (eq. 14)$$

This allows us to solve equations 11 to 14 as a set of linear equations for ATP, NADH, and NADPH. With equation 11 (x, acetate to ethanol), 12 (y, glycolysis), and 13 (z, oxPPP), and 14 (w, transhydrogenase):

$$ATP:\ -2x + 2y + z = 0$$

$$NADH: -2x + w = 0$$

$$NADPH: 6z - w = 0$$

A redox-neutral solution with zero net ATP production is available at an equation stoichiometry for x:y:z:w of 6:5:2:12. Adjusting the ratio of equations 11 to 14 accordingly gives:

$$6\ acetate + 12\ NADH + 12\ ATP \rightarrow 6\ ethanol\ (eq. 15)$$

$$5\ glucose \rightarrow 10\ ethanol + 10\ CO_2 + 10\ ATP\ (eq. 16)$$

$$2\ glucose \rightarrow 2\ ethanol + 8\ CO_2 + 12\ NADPH + 2\ ATP\ (eq. 17)$$

$$12\ NADPH \rightarrow 12\ NADH\ (eq. 18)$$

The net reaction of equations 15 to 18 equals equation 10:

$$7\ glucose + 6\ acetate \rightarrow 18\ ethanol + 18\ CO_2\ (eq.10)$$

Thus, at maximum redox-balanced acetate uptake, 12 of the 18 ethanol molecules have to be produced via NADPH-ADH, or 2/3rds of the total ADH flux. Assuming that the use of NADPH-ADH is evenly distributed over the various pathways, equation 18 can be incorporated into equations 15-17 as:

$$6\ acetate + 8\ NADH + 4\ NADPH + 12\ ATP \rightarrow 6\ ethanol\ (eq.19)$$

$$5\ glucose + 6\tfrac{2}{3}\ NADPH \rightarrow 10\ ethanol + 10\ CO_2 + 6\tfrac{2}{3}\ NADH + 10\ ATP\ (eq.20)$$

$$2\ glucose \rightarrow 2\ ethanol + 8\ CO_2 + 1\tfrac{1}{3}\ NADH + 10\tfrac{2}{3}\ NADPH + 2\ ATP\ (eq.21)$$

Benefit of acetate uptake for glycerol displacement

Wild type *S. cerevisiae* strains can produce glycerol from glucose via glycolysis, with the following stoichiometry:

$$\tfrac{1}{2}\ glucose + 1\ NADH + 1\ ATP \rightarrow 1\ glycerol\ (eq.22)$$

One of the reasons for producing glycerol anaerobically is to reoxidize the surplus NADH produced during biomass formation. If yeast cells are engineered to produce less glycerol, and instead reoxidize this NADH by converting acetate to ethanol, higher ethanol yields on glucose can be achieved. Not only because of the extra ethanol produced from acetate, but also because producing less glycerol saves glucose and ATP. This recovered glucose can also be fermented to ethanol. By combining equations 1, 2 and 22, the exchange can be presented as:

$$1\ glycerol + \tfrac{1}{2}\ acetate \rightarrow 1\tfrac{1}{2}\ ethanol + CO_2 + 1\ ATP\ (eq.23)$$

This gives an effective yield of ethanol on acetate of 2.30 g g^{-1}, or a yield of ethanol on avoided glycerol of 0.75 g g^{-1}. This ethanol on acetate yield compares favorably to the yield of equation 10, where

consumption of 6 mol acetate results in the additional formation of 4 mol ethanol, or 0.51 g ethanol (g acetate)$^{-1}$. That yield is lower than the equimolar conversion of acetate into ethanol (0.77 g g^{-1}; see equation 1) since without a 'free' source of NADH, additional glucose needs to be converted to CO_2 via the oxPPP to provide the necessary redox cofactors for acetate conversion, with a lower ethanol yield on glucose as a result.

References

1. **Hardie DG, Hawley SA**. 2001. AMP-activated protein kinase: the energy charge hypothesis revisited. BioEssays **23**:1112–1119.