MINIREVIEW

Energy coupling in *Saccharomyces cerevisiae*: selected opportunities for metabolic engineering

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Abstract

Free-energy (ATP) conservation during product formation is crucial for the maximum product yield that can be obtained, but often overlooked in metabolic engineering strategies. Product pathways that do not yield ATP or even demand input of free energy (ATP) require an additional pathway to supply the ATP needed for product formation, cellular maintenance, and/or growth. On the other hand, product pathways with a high ATP yield may result in excess biomass formation at the expense of the product yield. This mini-review discusses the importance of the ATP yield for product formation and presents several opportunities for engineering free-energy (ATP) conservation, with a focus on sugar-based product formation by *Saccharomyces cerevisiae*. These engineering opportunities are not limited to the metabolic flexibility within *S. cerevisiae* itself, but also expression of heterologous reactions will be taken into account. As such, the diversity in microbial sugar uptake and phosphorylation mechanisms, carboxylation reactions, product export, and the flexibility of oxidative phosphorylation via the respiratory chain and H^+-ATP synthase can be used to increase or decrease free-energy (ATP) conservation. For product pathways with a negative, zero or too high ATP yield, analysis and metabolic engineering of the ATP yield of product formation will provide a promising strategy to increase the product yield and simplify process conditions.

Introduction

Accelerated by spectacular developments in recombinant-DNA technology, DNA sequencing, DNA synthesis, functional genomics, systems biology, and high-throughput experimentation, metabolic engineering increasingly provides viable alternatives for petrochemistry-based production of chemicals. Products of engineered microorganisms range from pharmaceuticals (e.g. the anti-malarial precursor artemisinic acid and hydrocortisone) to bulk chemicals (e.g. 1,3-propanediol) and fuels (e.g. isobutanol) (Nakamura & Whited, 2003; Szczebara *et al.*, 2003; Ro *et al.*, 2006; Atsumi *et al.*, 2008).

Especially for microbial processes aimed at the production of commodity chemicals, costs of the carbon substrate strongly influence process economy. For instance, sugar costs have been estimated to make up 70% of the cost price of bioethanol (Marini *et al.*, 1997; Pfromm *et al.*, 2010). Process economy therefore provides a strong incentive to push product yields of engineered microorganisms toward their thermodynamic and stoichiometric limits by optimization of product pathways.

Once pathways that lead to byproduct formation have been eliminated, several aspects of microbial metabolic networks determine the maximum feasible product yield on substrate. Firstly, the biochemical pathways operating in an industrial microorganism can constrain conversion coefficients for carbon and other elements. For example, if conversion of glucose to acetyl-CoA, a key intermediate of lipid synthesis, exclusively proceeds via a $C_3 \rightarrow C_2$ decarboxylation step (e.g. via pyruvate dehydrogenase), then this imposes an upper limit to the lipid yield on glucose of 0.667 Cmol Cmol^{-1}. Secondly, product yields can be constrained by the need to balance oxidation and reduction of redox cofactors [e.g. NAD(P)$^+$ and NAD(P)(H)] (Weusthuis *et al.*, 2011). The impact of redox balances on product yields is illustrated by comparison of different metabolic pathways for anaerobic production of

ethanol from xylose and arabinose (van Maris *et al.*, 2006). This review focuses on a third important factor: conservation of free energy (ATP) during product formation.

Product pathways with a positive net ATP yield provide microorganisms with free energy for growth and maintenance processes. However, during industrial production of chemicals, excess microbial biomass constitutes an undesirable byproduct, whose formation goes at the expense of the product yield. Optimal product yields will therefore be reached at low, but positive yields of ATP in the product pathway, which are sufficient to maintain cellular performance but do not allow for unrestricted growth. The validity of this statement is illustrated by a comparison of the alcoholic fermentation of glucose to ethanol by wild-type strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis*. In *S. cerevisiae*, alcoholic fermentation via the Embden–Meyerhof–Parnas (EMP) pathway yields two ATP per glucose, whereas the Entner–Doudoroff (ED) pathway in *Z. mobilis* yields one ATP (Sprenger, 1996). Indeed, biomass yields of *Z. mobilis* in anaerobic cultures (0.03–0.04 g g glucose^{-1}) are much lower than those of *S. cerevisiae* (0.09–0.10 g g glucose^{-1}) and actively growing cultures of *Z. mobilis* exhibit a considerably higher ethanol yield (0.48–0.49 g g^{-1}) than *S. cerevisiae* (around 0.40 g g^{-1}) (Rogers *et al.*, 1979; Lee *et al.*, 1980; Kuyper *et al.*, 2005b). The option to increase ethanol yields in *S. cerevisiae* by replacing its EMP glycolytic pathway by an ED pathway has been patented (Lancashire *et al.*, 1998), but, to our knowledge, not further explored.

For some engineered pathways, the net ATP yield equals zero. An example is lactate production by engineered homolactic *S. cerevisiae* strains (van Maris *et al.*, 2004b). In contrast to lactic acid bacteria, which use lactate production as their primary catabolic pathway, an ATP requirement for export of lactic acid in *S. cerevisiae* cancels out the ATP production in the EMP pathway (van Maris *et al.*, 2004b). Stable, robust product formation via 'zero-ATP pathways' requires the simultaneous activity of other ATP forming reactions to meet free-energy demands for growth and maintenance. In the case of lactic acid–producing *S. cerevisiae*, this can be achieved through aerobic respiration (van Maris *et al.*, 2004b). However, aeration of industrial-scale fermentations is expensive and results in a partial conversion of sugar to CO_2 at the expense of product formation. Alternatively, alcoholic fermentation can provide the required ATP (Adachi *et al.*, 1998). This twin-product scenario, however, requires additional downstream processing costs and causes a dependency on two different markets.

The ideal scenario for production of commodity chemicals and biofuels involves fermentative, anaerobic pathways with a low, but positive ATP yield. Only such pathways enable low-cost anaerobic processes with a minimal production of excess biomass. Lactate production by lactic acid bacteria and bioethanol production by *S. cerevisiae* are classical examples of such processes. In addition, several processes that are the result of metabolic engineering fit this description. For instance, the pathway from glucose to isobutanol by engineered *Escherichia coli* (Atsumi *et al.*, 2008) is a typical fermentation pathway with an ATP yield of 2 mol mol glucose^{-1}. In addition to enabling high product yields, such fermentative pathways exhibit a strict coupling of growth and product formation, which simplifies the use of evolutionary engineering to select for improved productivity. This possibility has been extensively exploited for improvement of engineered *S. cerevisiae* strains that express heterologous pathways for pentose fermentation (Becker & Boles, 2003; Sonderegger *et al.*, 2004; Kuyper *et al.*, 2005a; Wisselink *et al.*, 2009).

Its impact on process economy makes the ATP stoichiometry of product pathways and precursor supply a highly relevant target in the design of metabolic engineering strategies. This mini-review does not aim to provide a full, in depth analysis of possible strategies. Instead, it discusses possibilities and challenges involved in modifying energy coupling in product pathways by discussing selected examples in *S. cerevisiae*. This discussion will be focused on four selected reaction steps that occur in many industrially relevant product pathways that use glucose or other sugars as carbon and energy source: Uptake and phosphorylation of sugars, Stoichiometries of oxidative phosphorylation and H^+-ATPase, $C_3 \rightarrow C_4$ carboxylation reactions, and Product export under industrial conditions. The final section provides a brief outlook.

Uptake and phosphorylation of sugars

Uptake, phosphorylation and, in the case of oligosaccharides, cleavage to yield monosaccharides are, not necessarily in this order, among the initial steps in microbial sugar metabolism. As will be discussed later, these reactions provide attractive opportunities to modulate the efficiency of free-energy (ATP) conservation by metabolic engineering.

In *S. cerevisiae*, the monosaccharides glucose, fructose, and galactose are imported via facilitated diffusion (Fig. 1), a process that does not require an input of free energy and is mediated by members of the Hxt (hexose transporter) family (Lagunas, 1993; Boles & Hollenberg, 1997). Anaerobic fermentation of glucose to ethanol via the EMP pathway yields two ATP. In contrast, maltose transport in *S. cerevisiae* occurs via symport with a proton (van Leeuwen *et al.*, 1992). At typical values of the

proton motive force (PMF) of −150 to −200 mV, the free energy required for proton translocation across the yeast plasma membrane is +15 to +19 kJ $(\text{mol } H^+)^{-1}$ (Rigoulet *et al.*, 1987; Serrano, 1991). Symport of maltose with a proton therefore enables transport against concentration gradients of up to 1000-fold. To maintain PMF and intracellular pH, proton import via the maltose symporter is balanced by proton export via the plasma membrane H^+-ATPase. In *S. cerevisiae*, this requires the hydrolysis of one ATP for export of a single proton (van Leeuwen *et al.*, 1992; Weusthuis *et al.*, 1993). Once inside the yeast cell, maltose is hydrolyzed by maltase and the resulting two glucose molecules are converted via the EMP pathway. The net requirement of one ATP for maltose uptake limits the ATP yield of maltose fermentation to three ATP, which is equivalent to 1.5 ATP per glucose. Consistent with this lower ATP yield, the anaerobic biomass yield of *S. cerevisiae* on maltose is 25% lower than the biomass yield on glucose and, because more maltose is fermented to ethanol to energize maltose-proton symport, the ethanol yield on maltose is 9% higher than on glucose (Weusthuis *et al.*, 1993).

A recent metabolic engineering study (Basso *et al.*, 2011) explored the possibility to use sugar-proton symport to increase the ethanol yield of *S. cerevisiae* on sucrose, the major sugar substrate for bioethanol production from cane sugar. Sucrose hydrolysis by wild-type *S. cerevisiae* is predominantly catalyzed by extracellular invertase (Burger *et al.*, 1961; Sutton & Lampen, 1962; Gascón & Lampen, 1968; Carlson & Botstein, 1982; Batista *et al.*, 2004; Basso *et al.*, 2011). When the subsequent uptake of the hydrolysis products, glucose and fructose, by Hxt transporters is followed by alcoholic fermentation via the EMP pathway, this yields four ATP per sucrose. *Saccharomyces cerevisiae* also harbors a low capacity sucrose-proton-symport mechanism and, in wild-type strains, a low fraction of the invertase is present intracellularly (Gascón & Lampen, 1968; Carlson & Botstein, 1982; Santos *et al.*, 1982; Stambuk *et al.*, 2000; Batista *et al.*, 2004). Complete rerouting of sucrose metabolism via proton symport and intracellular hydrolysis was achieved by a combination of metabolic and evolutionary engineering. This resulted in a 30% decrease in the biomass yield and an 11% increase in the ethanol yield relative to a reference strain (Basso *et al.*, 2011).

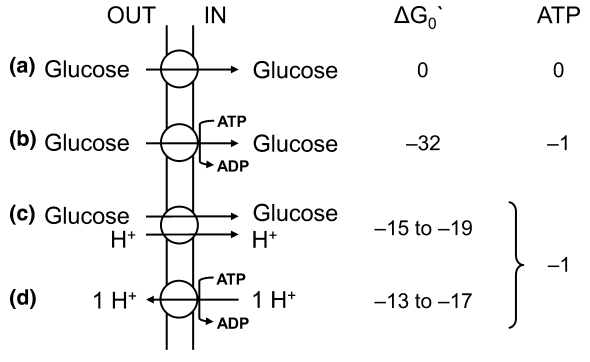

Fig. 1. Different mechanisms for glucose import, for which the standard Gibbs free-energy change ($\Delta G_0'$, in kJ mol^{-1}) and net ATP requirement for import are indicated. Mechanisms: (a) facilitated diffusion; (b) active transport; (c) proton symport; (d) plasma membrane H^+-ATPase.

In principle, replacement of facilitated diffusion systems by proton symporters or, alternatively, by ATP-driven transporters (Fig. 1), can be applied to other industrially relevant sugars, products and microorganisms to improve product yields of pathways with 'excess' ATP production. When applied to monosaccharides, the anticipated impact of this approach is even higher than for disaccharides. Theoretically, replacement of the Hxt transporters in *S. cerevisiae* by a hexose-proton symport mechanism should decrease the biomass yield of anaerobic yeast cultures on hexose sugars by 50%, with a concomitant increase in the ethanol yield of 14% (Basso *et al.*, 2011). Interestingly, in literature such proton symporters have been described for fructose [in *Saccharomyces pastorianus* and wine yeasts (Goncalves *et al.*, 2000)], glucose, arabinose, and xylose [in several non-*Saccharomyces* yeasts (Kilian & van Uden, 1988)]. Combined with the availability of strains in which all 20 genes of the Hxt transporter family have been deleted (Wieczorke *et al.*, 1999), this presents an opportunity to explore the energetic impact of the introduction of such heterologous transporters in *S. cerevisiae*.

The initial intracellular reactions in sugar metabolism provide interesting opportunities to improve energy coupling in product pathways with a negative or zero ATP yield. Hexose kinases conserve only a fraction of the free energy that is available from the cleavage of ATP to ADP [$\Delta G_0'$ −32 kJ mol^{-1}, but the *in vivo* ΔG can be estimated at −45 kJ mol^{-1} from previously published data (Canelas *et al.*, 2011)] in the resulting glucose–phosphate bond ($\Delta G_0'$ −14 kJ mol^{-1} for glucose-6-phosphate hydrolysis). Similarly, disaccharide hydrolases dissipate the free energy of disaccharide cleavage ($\Delta G_0'$ −16 kJ mol^{-1} for maltose hydrolysis by maltase). In contrast, the phosphorolytic cleavage of disaccharides with inorganic phosphate, as catalyzed by disaccharide phosphorylases, is coupled to phosphorylation of one of the monosaccharides and thereby converses free energy. For example, the maltose phosphorylase ($\Delta G_0'$ of +5 kJ mol^{-1}) reaction yields glucose and glucose-1-phosphate (whose formation from glucose and inorganic phosphate has a $\Delta G_0'$ of +21 kJ mol^{-1}) (Fig. 2). Glucose-1-phosphate can subsequently be converted with a phosphoglucomutase into

glucose-6-phosphate, the first intermediate of glycolysis. Although the maltose phosphorylase reaction, which does not naturally occur in *S. cerevisiae*, has a slightly positive $\Delta G'_0$ (+5 kJ mol^{-1}, Fig. 2), the actual ratio between the substrate and product concentrations enables a negative actual Gibbs free-energy change and thereby *in vivo* functioning of this reaction in several microorganisms (Stolz *et al.*, 1993; Ehrmann & Vogel, 1998).

Replacement of a disaccharide hydrolase by a disaccharide phosphorylase via metabolic engineering should, in theory, lead to a gain of one ATP per disaccharide molecule. This hypothesis was recently tested by replacing the native *S. cerevisiae* maltase by a maltose phosphorylase. Indeed, deletion of all maltase-encoding genes and introduction of a heterologous maltose phosphorylase gene in *S. cerevisiae* resulted in a 26% increase in the anaerobic biomass yield on maltose (de Kok *et al.*, 2011). This concept should, in principle, be applicable to other disaccharides, such as cellobiose (Sadie *et al.*, 2011), sucrose (Doudoroff, 1955), and lactose (De Groeve *et al.*, 2009). However, the ATP yield of the engineered *S. cerevisiae* strain expressing maltose phosphorylase (two ATP hexose molecule^{-1}) is still only equivalent to that of glucose metabolism (two ATP) or of extracellular maltose hydrolysis followed by monosaccharide uptake via facilitated diffusion [a mechanism that occurs, for example, in *Schizosaccharomyces pombe* (Jansen *et al.*, 2006)].

To enable conversion of 'zero ATP' pathways such as malic and lactic acid production by engineered *S. cerevisiae* strains (van Maris *et al.*, 2004b; Zelle *et al.*, 2010a, 2011) into pathways with a positive ATP yield, engineering of disaccharide metabolism needs to be taken one step further. One approach toward achieving this goal is to combine maltose phosphorylase expression with the replacement of the maltose transporter for a maltose facilitator, thereby eliminating the energy costs for maltose uptake. This strategy should yield five ATP for the anaerobic fermentation of maltose, equivalent to 2.5 ATP per hexose. To date, no disaccharide facilitators have been functionally expressed in *S. cerevisiae*. In *E. coli*, however, single amino acid changes have been shown to convert the lactose-proton symporter *lacY* of *E. coli* into a lactose facilitator (Guan & Kaback, 2006). When combined with the expression of a heterologous lactose phosphorylase (De Groeve *et al.*, 2009), these *lacY* alleles may be applicable for proof-of-principle experiments in *E. coli*.

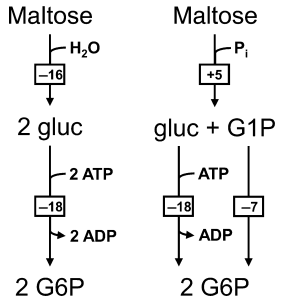

Fig. 2. The ATP yield on maltose can be increased by replacing maltose hydrolysis ($\Delta G'_0$ −16 kJ mol^{-1}) with maltose phosphorolysis ($\Delta G'_0$ +5 kJ mol^{-1}). Maltose phosphorylase cleaves maltose with inorganic phosphate and directly yields glucose-1-phosphate, which saves the hydrolysis of 1 ATP molecule during glucose phosphorylation by hexokinase. Standard Gibbs free-energy changes ($\Delta G'_0$, in kJ mol^{-1}) are indicated in boxes. gluc, glucose; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate.

A strategy to even further increase ATP yields on hexose equivalents is suggested by an elegant study on metabolism of cellodextrins in *Clostridium thermocellum* (Zhang & Lynd, 2005). In this anaerobic bacterium, cellodextrin oligosaccharides of up to six glucose units are transported via the same ATP-driven transport mechanism, followed by their intracellular phosphorolytic cleavage. As a result, the relative impact of the ATP-cost for transport decreases and the ATP yield per hexose equivalent increases with increasing chain length (Zhang & Lynd, 2005). Phosphorolytic cleavage of oligosaccharides with more than two hexose units therefore offers the potential to increase ATP yields per hexose unit above 2.5. For example, a scenario in which the trisaccharide maltotriose is taken up by facilitated diffusion and cleaved by a heterologous maltotriose phosphorylase, yields eight ATP per maltotriose, or 2.67 ATP per hexose unit (de Kok *et al.*, 2011). Obviously, any industrial implementation of such strategies is dependent on cost effective strategies to produce feedstocks with a high content of the relevant oligosaccharides.

Stoichiometries of oxidative phosphorylation and H$^+$-ATPase

When redox constraints or ATP requirements preclude the use of anaerobic process conditions, aerobic respiration provides a means to reoxidize reduced cofactors and yield additional ATP. Aerobic respiration of redox cofactors is a highly exergonic process ($\Delta G'_0$ −220 kJ mol^{-1} for NADH and −200 kJ mol^{-1} for $FADH_2$). During respiration, free energy is conserved as a proton motive force across the mitochondrial inner membrane. This proton motive force can subsequently be used to energize ATP production via the mitochondrial proton-translocating ATPase complex. The H$^+$/ATP ratio of ATP synthetases in respiring membranes of microorganisms is consistently found to be close to 3 (Watt *et al.*, 2010). Conversely, the number of protons translocated during respiration of, for example, NADH strongly varies between microorganisms and cultivation conditions,

depending on the composition of the respiratory chain and the proton coupling of individual components. The combined efficiency of proton pumping by respiratory chains and ATP synthesis is represented by the P/O ratio (ATP formed per electron pair transferred to oxygen) (Kalckar, 1974).

When respiration of reduced cofactors yields more ATP than is required in the product pathway, this may contribute to the formation of excess biomass and reduce the rate of product formation via respiratory coupling (Chance & Williams, 1955). In such cases, decreasing the P/O ratio provides an attractive strategy to increase product yields. Two metabolic engineering strategies have been described that reduce the P/O ratio of respiration in *S. cerevisiae*. The first strategy is based on the occurrence, in many fungi, of alternative oxidases that transfer electrons from cytochrome c to molecular oxygen without proton translocation (Joseph-Horne *et al.*, 2001). Indeed, expression of alternative oxidases in *S. cerevisiae* led to a small but significant reduction in the biomass yield in aerobic cultures (Huh & Kang, 1999; Vemuri *et al.*, 2007; Hou *et al.*, 2009). A second strategy that completely bypasses the respiratory chain, is the expression of a bacterial water-forming NADH oxidase in *S. cerevisiae*. As this system does not donate electrons from NADH to the respiratory chain, but directly to oxygen, its expression results in a decreased aerobic biomass yield (Heux *et al.*, 2006; Vemuri *et al.*, 2007; Hou *et al.*, 2009). Expression of a soluble NADH oxidase only had a small impact on biomass yields in glucose-limited aerobic chemostat cultures [*c.* 10% reduction; (Vemuri *et al.*, 2007)]. This can be explained from its targeting to the cytosol, due to which the P/O ratio for respiration of intramitochondrial NADH, which makes up the majority of the NADH formed during respiratory metabolism of glucose, is likely to have been unaffected.

For product pathways that do not yield ATP, the ATP yield and P/O ratio should be maximized, to minimize the amount of carbon substrate that needs to respired to generate ATP for growth and cellular maintenance. In contrast to many other yeasts and fungi, the mitochondrial respiratory chain of *S. cerevisiae* does not contain a proton-translocating complex I-type NADH dehydrogenase. Instead, *S. cerevisiae* uses a single-subunit, non-proton-translocating NADH dehydrogenase (Ndi1p) to couple oxidation of intramitochondrial NADH to the respiratory chain (Bakker *et al.*, 2001). As a result, its effective P/O ratio is low (close to 1) and complete glucose oxidation in *S. cerevisiae* probably only yields 16 ATP (Van Gulik & Heijnen, 1995; Bakker *et al.*, 2001). As eukaryotic complex I-type NADH dehydrogenases consist of 40–45 different protein subunits (Kerscher *et al.*, 2008), a strategy to functionally express, assemble and integrate such a complex in the mitochondrial inner membrane of *S. cerevisiae* represents a formidable metabolic engineering challenge.

With a proton-ATP stoichiometry close to 3 (Watt *et al.*, 2010), the ATP synthetases in respiring microbial membranes are thermodynamically very efficient [at a proton motive force of −150 mV, inward translocation of a single proton represents a free energy change of −15 kJ mol^{-1}, while the *in vivo* ΔG for ATP hydrolysis in *S. cerevisiae* is around −45 kJ mol^{-1} (Canelas *et al.*, 2011)]. A completely different situation exists for the plasma membrane H^+-ATPase in *S. cerevisiae*, which couples the hydrolysis of an ATP molecule to the translocation of only a single proton and therefore appears to operate far from maximum thermodynamic efficiency (van Leeuwen *et al.*, 1992; Weusthuis *et al.*, 1993). Increasing the stoichiometry of the yeast plasma membrane H^+-ATPase from 1 to 2 H^+/ATP could decrease the amount of ATP for maintenance processes and increase the ATP yield of processes that involve proton-coupled transport of substrates (e.g. maltose and NH_4^+) (van Leeuwen *et al.*, 1992; Weusthuis *et al.*, 1993; Marini *et al.*, 1997) or products (e.g. organic acids) (van Maris *et al.*, 2004a; Sauer *et al.*, 2008; Abbott *et al.*, 2009). For example, alcoholic fermentation of a disaccharide, using a disaccharide-proton symporter and disaccharide phosphorylase, combined with an H^+-ATPase stoichiometry of 2 H^+/ATP, would yield 4.5 ATP per maltose (2.25 ATP per hexose). Moreover, an increased H^+-ATPase stoichiometry might improve tolerance to both low pH and weak organic acids (Verduyn *et al.*, 1992; Piper *et al.*, 1998; Abbott *et al.*, 2007). Altering the H^+/ATP stoichiometry of the plasma membrane H^+-ATPase of *S. cerevisiae* therefore presents an interesting metabolic engineering strategy. Interestingly, specific single amino acid changes in the *S. cerevisiae* plasma membrane H^+-ATPase Pma1p have been reported that led to increased *in vitro* H^+/ATP stoichiometries (Petrov *et al.*, 2000; Guerra *et al.*, 2007). Further studies into the *in vivo* proton coupling of eukaryotic plasma membrane H^+-ATPases therefore remain of great fundamental and applied interest.

$C_3 \rightarrow C_4$ carboxylation reactions

Carboxylation reactions couple inorganic carbon (CO_2 or bicarbonate) to organic acceptor molecules. Examples of industrially relevant processes that involve carboxylation steps include the production of C_4-dicarboxylic acids, lipids, polyketides, flavonoids, propionate and 3-hydroxypropionate (van Maris *et al.*, 2004a; Jiang *et al.*, 2009; Henry *et al.*, 2010). In some of these processes, such as lipid synthesis, carboxylation occurs as an intermediate

step and is followed by a loss of CO_2, in other cases, such as production of C_4-dicarboxylic acids, a net fixation of CO_2 can occur.

Production of malic and succinic acid is a key topic in metabolic engineering and strategies for high-yield production of these compounds from sugars depend on reactions that carboxylate the glycolytic C_3-intermediates pyruvate or phosphoenolpyruvate (PEP) (Zeikus *et al.*, 1999; Song & Lee, 2006; Abbott *et al.*, 2009; Beauprez *et al.*, 2010; Raab & Lang, 2011; Yu *et al.*, 2011). Energy coupling of these carboxylation reactions has a strong impact on the maximum yields of C_4-dicarboxylic acid production.

Carboxylation of pyruvate to oxaloacetate with CO_2 requires the input of free energy ($\Delta G'_0$ +32 kJ mol^{-1}). In the reaction catalyzed by pyruvate carboxylase, this free energy is provided by ATP hydrolysis, leading to a $\Delta G'_0$ of 0 kJ mol^{-1}. Subsequent reduction of oxaloacetate yields a redox-cofactor balanced, CO_2-fixing pathway from glucose to two molecules of malic acid. However, the zero ATP yield of this pathway precludes efficient production under anaerobic conditions, because another pathway – usually aerobic respiration – is required to supply the cells with ATP for growth and cellular maintenance (Zelle *et al.*, 2008, 2010b). Similarly, redox-neutral pathways for production of succinic acid via pyruvate carboxylase and either the glyoxylate shunt or the oxidative branch of the TCA-cycle only yield 0.33 ATP per succinic acid (Vemuri *et al.*, 2002; Jantama *et al.*, 2008). As export of C_4-dicarboxylic acids under industrially relevant conditions [high product titers and preferably a low pH to facilitate product recovery (Abbott *et al.*, 2009)] is likely to require an additional input of free energy (e.g. one ATP per C_4-acid, see Product export under industrial conditions) (van Maris *et al.*, 2004a; Abbott *et al.*, 2009), increasing the ATP yield of product formation is a key objective for metabolic engineering of the production of C_4-dicarboxylic acids.

Several opportunities are available to increase free-energy (ATP) conservation of $C_3 \rightarrow C_4$ carboxylation reactions in dicarboxylic acid production (Fig. 3). For instance, malic enzyme (pyruvate + CO_2 + NAD(P)H → malic acid + NAD(P)$^+$; $\Delta G'_0$ +3 kJ mol^{-1}) combines the free energy from the reduction of oxaloacetate to malic acid ($\Delta G'_0$ −29 kJ mol^{-1}) to the carboxylation of pyruvate ($\Delta G'_0$ +32 kJ mol^{-1}). Alternatively, the energy-rich intermediate PEP ($\Delta G'_0$ −62 kJ mol^{-1} upon hydrolysis) can be used as substrate instead of pyruvate. Direct carboxylation of PEP to oxaloacetate by PEP carboxylase ($\Delta G'_0$ −30 kJ mol^{-1}) has been investigated for succinic acid production by engineered *E. coli* strains (Millard *et al.*, 1996), but does not yield additional ATP compared to pyruvate carboxylase. The remaining free energy of the

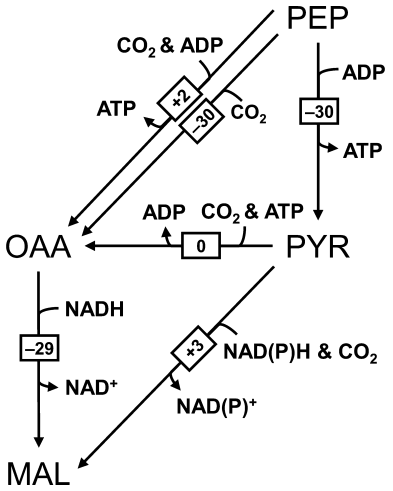

Fig. 3. Carboxylation of glycolytic C_3-intermediates to C_4-dicarboxylic acids (e.g. malic acid) can be mediated via several mechanisms with different efficiencies of free-energy (ATP) conservation. Standard Gibbs free-energy changes ($\Delta G'_0$, in kJ mol^{-1}) are indicated in boxes. PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; MAL, malic acid.

carboxylation of PEP to oxaloacetate ($\Delta G'_0$ −30 kJ mol^{-1}) can be conserved via phosphorylation of ADP to ATP, mediated by PEP carboxykinase ($\Delta G'_0$ +2 kJ mol^{-1}). Therefore, using either malic enzyme or PEP carboxykinase instead of pyruvate carboxylase increases the overall ATP yield by one ATP per $C_3 \rightarrow C_4$ carboxylation event. These replacements in theory enable anaerobic malic acid production and drastically increase the (marginal) ATP yield for redox-neutral succinic acid production from 0.33 to 1.17 ATP (together with the glyoxylate shunt) or 1.33 ATP (together with the oxidative TCA-cycle) per succinic acid (Stols & Donnelly, 1997; Kim *et al.*, 2004; Zhang *et al.*, 2009, 2011; Zelle *et al.*, 2010a, 2011).

Product export under industrial conditions

Product export across the plasma membrane is the final step in most yeast-based fermentation processes. Product export can be mediated via several mechanisms, whose different energy coupling strongly affects both the maximum achievable concentration gradients across the membrane and net ATP formation [for an overview, see (van Maris *et al.*, 2004a)]. The impact of the export mechanism on product formation is illustrated by succinic acid production under industrial conditions (i.e. high titer and low pH) with *S. cerevisiae*. Succinic acid is a dicarboxylic acid (pK_a's: 4.21 and 5.64) and therefore, depending on the pH, exists as undissociated acid (H_2Suc), monovalent

anion ($HSuc^-$) and divalent anion (Suc^{2-}). To minimize the addition of base during fermentation and acid during downstream processing, preferably the undissociated acid (H_2Suc) is produced at low extracellular pH. However, at the near-neutral pH values inside the cell, weak organic acids predominantly exist as divalent anions (Suc^{2-}), which has major consequences on the export energetics. Thermodynamic analysis of dicarboxylic acid production (van Maris *et al.*, 2004a; Abbott *et al.*, 2009; Jamalzadeh *et al.*, 2012) indicated that facilitated diffusion of the undissociated acid and the monovalent anion (or the divalent anion together with one proton) do not support the concentration gradients required for high titer (e.g. 1 M) succinic acid production at low pH (e.g. pH 3). In addition, facilitated diffusion of the divalent anion was only found thermodynamically feasible at relatively high intracellular succinic acid concentrations (around 0.1 M) and even then would still require the subsequent extrusion of two protons by the plasma membrane H^+-ATPase. An additional thermodynamic push through antiport of the divalent anion (Suc^{2-}) with one proton (or thermodynamically equivalent reaction) would allow export at low intracellular concentrations (< 0.01 M), but requires the simultaneous expulsion of three protons. As the plasma membrane H^+-ATPase in *S. cerevisiae* has a stoichiometry of one, this would respectively costs 2 and 3 ATP per succinic acid exported, unless the H^+/ATP stoichiometry of the plasma membrane H^+-ATPase can be increased as described earlier while maintaining the same proton motive force. Alternatively, it might be possible to directly couple succinic acid (H_2Suc) export to ATP hydrolysis via primary transport, thereby reducing the export costs to only one ATP. This illustrates that succinic acid export under industrially desirable conditions (high titer and low pH) thus always requires the investment of free energy (ATP) and thereby influences the overall ATP yield of succinic acid production from glucose.

Discussion and outlook

The examples discussed earlier illustrate how targeted modification of energy coupling of key reactions in product pathways can drive product yields of engineered yeast strains closer to their thermodynamic and stoichiometric maximum. Combined modification of several of these reactions may enable ATP-positive metabolic pathways to products of interest and thereby facilitate evolutionary engineering of strains through the coupling between product formation and growth (Sauer, 2001). In addition to their impact on product yield, such engineering strategies may also have a profound impact on process design when they enable a switch from aerobic to anaerobic process conditions, thereby eliminating aeration and associated costs of stirring, air compression and cooling.

Many other targets for metabolic engineering of energy coupling exist in addition to the ones discussed in this mini-review. One extensively studied strategy that has not been discussed is the introduction of ATP-hydrolyzing (sets of) reactions. Such futile cycles provide a powerful alternative means of modulating ATP stoichiometries (Navas *et al.*, 1993; Chao & Liao, 1994). However, optimization of product yields and cellular robustness requires a careful balancing of ATP-synthesizing and ATP-hydrolyzing reactions, which, in the case of futile cycles, may be difficult to maintain under dynamic industrial conditions. Another important metabolic node at which optimization of energy coupling can have a strong impact on product yields is the formation of Coenzyme A (CoA) esters. This is illustrated by the energy coupling of the synthesis of acetyl-CoA, a key precursor for a wide range of biotechnological products. In *S. cerevisiae*, formation of cytosolic acetyl-CoA via cytosolic acetyl-CoA synthetases costs two ATP equivalents. In contrast, synthesis of this precursor via the mitochondrial pyruvate dehydrogenase does not require a net input of ATP, thus illustrating the importance of metabolic compartmentation in the optimization of product pathways in yeast (Pronk *et al.*, 1996).

Microbial metabolic diversity provides an endless source of inspiration for novel metabolic engineering strategies to improve energy conservation in industrial microorganisms such as *S. cerevisiae*. An elegant and efficient example is provided by propionic acid bacteria (Schlegel, 1993). In a single pathway, these microorganisms combine three evolutionary 'innovations' to achieve a near-perfect conservation of free energy: (1) ATP-independent integration of a $C_3 \rightarrow C_4$ carboxylation with a $C_4 \rightarrow C_3$ decarboxylation by carboxytransferase, (2) an ATP-independent CoA transfer by CoA-transferase that circumvents ATP-dependent formation of a CoA-ester, and (3) ATP formation from fumarate respiration, which couples the reduction of fumarate with NADH to proton translocation and subsequent ATP synthesis (de Vries *et al.*, 1973; Kröger *et al.*, 1992).

Systematic exploration of metabolic biodiversity for the design of energy-efficient pathways can be intensified by mathematical models that enable a combinatorial, parallel evaluation of possible pathway configurations, by analyzing ATP yield as well as pathway thermodynamics (Jiang *et al.*, 2009; Henry *et al.*, 2010). Such a model-based analysis, which also included pathways based on predicted, but currently non-confirmed enzyme activities, has been successfully performed for 3-hydroxypropionic acid production (Jiang *et al.*, 2009; Henry *et al.*, 2010). Further functional analysis and exploration of hitherto

undiscovered metabolic processes remains essential, because model predictions are as only as good as the questions asked and the possibilities used as inputs (Vodovotz *et al.*, 2007).

As pathways are redesigned to increase free-energy conservation, the overall Gibbs free-energy change of the reaction by definition becomes less negative. In some cases, increased ratios of substrate and product concentrations or reactions elsewhere in the pathways can (partially) compensate for this decrease in the driving force for product formation. For example, replacing maltose hydrolysis ($\Delta G_0'$ −16 kJ mol^{-1}) with maltose phosphorolysis ($\Delta G_0'$ +5 kJ mol^{-1}) in *S. cerevisiae* coincided with an almost threefold higher residual maltose concentration in maltose-limited chemostat cultures (de Kok *et al.*, 2011). Similarly, when the native ATP consuming pyruvate carboxylase in *S. cerevisiae* was functionally replaced by the energetically more efficient malic enzyme or phosphoenolpyruvate carboxykinase reactions, readjustment of the intracellular metabolite pools, switching of cofactor specificity from NADH to NADPH, or even increased CO_2 concentrations could only partially overcome the decreased driving forces, as illustrated by the strongly decreased growth rates of these strains (Zelle *et al.*, 2010a, 2011).

Strategies for improving free-energy coupling should additionally consider the ATP demands for performance and robustness of the resulting strains under industrial conditions, such as high product concentrations, low pH, presence of weak organic acids, and other inhibitory compounds (Palmqvist & Hahn-Hägerdal, 2000). Trade-offs between stoichiometry, kinetics, and robustness should therefore be taken into account in the economic evaluations that ultimately decide whether modifications in free-energy conservation are beneficial to process development.

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