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Minireview

Metabolic energy conservation for fermentative product formation

Pauline L. Folch, Markus M.M. Bisschops and Ruud A. Weusthuis*

Bioprocess Engineering, Wageningen University & Research, Post office box 16, Wageningen, 6700 AA, The Netherlands.

Summary

Microbial production of bulk chemicals and biofuels from carbohydrates competes with low-cost fossil-based production. To limit production costs, high titres, productivities and especially high yields are required. This necessitates metabolic networks involved in product formation to be redox-neutral and conserve metabolic energy to sustain growth and maintenance. Here, we review the mechanisms available to conserve energy and to prevent unnecessary energy expenditure. First, an overview of ATP production in existing sugar-based fermentation processes is presented. Substrate-level phosphorylation (SLP) and the involved kinase reactions are described. Based on the thermodynamics of these reactions, we explore whether other kinase-catalysed reactions can be applied for SLP. Generation of ion-motive force is another means to conserve metabolic energy. We provide examples how its generation is supported by carbon-carbon double bond reduction, decarboxylation and electron transfer between redox cofactors. In a wider perspective, the relationship between redox potential and energy conservation is discussed. We describe how the energy input required for coenzyme A (CoA) and CO_2 binding can be reduced by applying CoA-transferases and transcarboxylases. The transport of sugars and fermentation products may require metabolic energy input, but alternative transport systems can be used to minimize this. Finally, we show that energy contained in glycosidic bonds and the phosphate-phosphate bond of pyrophosphate can be conserved. This review can be used as a reference to design energetically efficient microbial cell factories and enhance product yield.

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*For correspondence. E-mail: ruud.weusthuis@wur.nl; Tel./Fax +31317484002.
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Introduction

Metabolic engineering has been extensively used in the past decades to improve the production of chemicals by microorganisms (Atsumi *et al.*, 2008; Keasling, 2010; Singh *et al.*, 2011; Zhao *et al.*, 2013; Vuoristo *et al.*, 2015). Recent advances in omics and genetic techniques have allowed fast and efficient modifications of microorganisms (Datsenko and Wanner, 2000; Mans *et al.*, 2015), broadening the spectrum of both substrates and products (Zhang *et al.*, 2008; Jung *et al.*, 2010; Lindberg *et al.*, 2010; Yim *et al.*, 2011).

Fermentation is a well-studied metabolic concept in which a substrate is oxidized to an intermediate – resulting in the reduction of redox cofactors – after which the intermediate is reduced – regenerating the oxidized cofactors. Microbial fermentation has been used to produc biofuels and bulk chemicals (Bennett and San, 2001; Bechthold *et al.*, 2008; Abdel-Rahman *et al.*, 2013; Wang *et al.*, 2016). These chemicals compete with petrochemical-derived compounds; therefore, their manufacture requires high targets for productivity, titre and – most importantly – substrate efficiency. In microbial processes, the carbon and energy source are generally used for maintenance, growth and product formation. To maximize product yield, growth must be minimized, and product formation should ideally conserve sufficient metabolic energy to fulfil the energy requirements of the cells (Fig. 1). If not, a part of the substrate is dissimilated to CO_2 and H_2O by respiration to fulfil the energy requirement of the cell. In addition, the yield of the metabolic pathway (Y^P) designed to convert substrate into product should be equal or very close to the maximum theoretical yield (Y^E). The Y^E can be determined based on the ratio of the degree of reduction of substrate and product (Cueto-Rojas *et al.*, 2015; Vuoristo *et al.*, 2016).

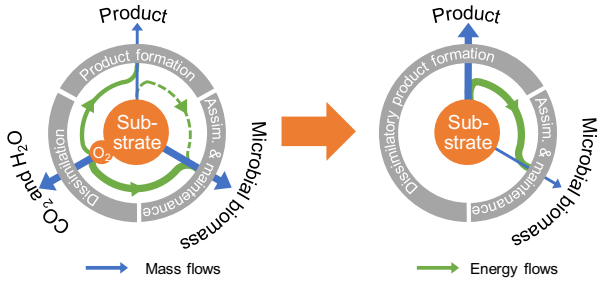


Fig. 1. Conservation of additional metabolic energy in the product pathway to improve product yield. On the left, classical aerobic bioconversion where part of the substrate is diverted away from product formation by dissimilation to fulfil the cells energy requirement. On the right, improved product formation by capturing metabolic energy in the product-forming pathways.

The degree of reduction represents the number of electrons in a molecule available for chemical reactions. To reach Y^E, metabolic pathways should be designed such that all electrons present in the substrate end up in the product and therefore be redox-neutral. The use of external electron acceptors, like in respiration, deviates electrons away from the product and is therefore a less preferred option because it decreases the network yield (Weusthuis *et al*., 2020).

Ethanol and lactic acid are synthesized from glucose by redox-neutral pathways that generate ATP, explaining why their practical yields approach Y^E. It is not straightforward to find such pathways for other substrate/product combinations. Obtaining redox balance often requires a metabolic network consisting of at least two pathways – one resulting in cofactor reduction, the other resulting in cofactor oxidation – that together act in a redox-balanced way. For instance, two parallel pathways combining oxidative and non-oxidative glycolysis have been implemented in *Corynebacterium glutamicum* to create a near redox-neutral metabolic network for the production of L-glutamate (Chinen *et al*., 2007). This approach allowed to reach a practical yield of 90% of the maximum theoretical yield on glucose. Another example is the redox-neutral combination of oxidative and reductive branches of TCA cycle for the production of succinate, citrate, itaconate (Sánchez *et al*., 2005; Vuoristo *et al*., 2016) and 1,4-butanediol (Yim *et al*., 2011).

These metabolic networks ideally should also provide energy for maintenance and growth. Table 1 shows the chemical conversion equations and thermodynamics of the previously described metabolic networks. The Gibbs free energy $\Delta G_0'$ of these reactions is comparable to the ones of ethanol and lactic acid production from glucose (Table 1). These negative $\Delta G_0'$ values show that – in principle – sufficient free energy is liberated to be conserved as metabolic energy (Cueto-Rojas *et al*., 2015). Metabolic reactions, however, in which energy can be conserved, are not common. Consequently, it is not straightforward to realize net ATP formation in these metabolic networks. In these cases, respiration is used to conserve metabolic energy, which has negative consequences for yield and productivity (Weusthuis *et al*., 2011).

This review focuses on the available mechanisms for the conservation of metabolic energy, – excluding those involved in respiration – and how unnecessary metabolic energy expenditure can be avoided, as well as how to implement them into product-forming metabolic networks based on carbohydrates.

Table 1. Thermodynamics of microbial processes using redox-neutral pathways. The $\Delta G_0'$ were calculated using eQuilibrator 2.2 with CO_2 as gas (g) and aqueous (aq) for all other compounds (Flamholz *et al*., 2012; Noor *et al*., 2012; Noor *et al*., 2013; Noor *et al*., 2014). For ATP formation, the EMP pathway and energy-neutral substrate uptake and product efflux were used.

Overall conversions	ATP[a]	$\Delta G_0'$ (kJ mol glucose^{-1})
Redox-neutral fermentation processes		
Glucose(aq) + 2 CO_2(g) = 4/3 Citrate (aq) + 2/3 H_2O	–2/3 to 0[b]	–175 ± 12
Glucose(aq) = 12/11 1,4-Butanediol(aq) + 18/11 CO_2(g) + 6/11 H_2O	–8/11[c]	–216 ± 10[d]
Glucose(aq) + 6/7 CO_2(g) = 12/7 succinate(ag) + 6/7 H_2O	4/7[e]	–257 ± 8
ATP generation in existing fermentation processes		
Glucose(aq) = 2 Ethanol(aq) + 2 CO_2(g)	2	–230 ± 13
Glucose(aq) = 2 Lactate(aq)	2	–187 ± 4
Glucose(aq) + H_2O(l) = Acetate(aq) + Ethanol(aq) + 2 Formate(aq)	3	–211 ± 6
Glucose(aq) = Butyrate(aq) + 2 CO_2(g) + 2 H_2(g)	3	–266 ± 18
Glucose(aq) + CO_2(g) = Succinate(aq) + Formate(aq) + Acetate(aq)	3	–249 ± 8
Glucose(aq) + 2 NH_3(aq) = 2 Alanine (aq) + 2 H_2O	2	–209 ± 4

a. Amount of ATP produced (positive sign) or consumed (negative sign).
b. Calculated as described by Vuoristo *et al*. (2016). The –2/3 ATP was obtained by using the reversed glyoxylate cycle, the 0 ATP was obtained by combining the reductive and oxidative TCA shunts to reach redox-neutral conversion.
c. Calculated using the pathway described by Yim *et al*. (2011) assuming that both reductive and oxidative TCA shunts were applied to obtain redox-neutral conversion and that the acetate formed was recovered to acetyl-CoA by means of an acetyl-CoA synthase at the expense of two ATP equivalents.
d. The eQuilibrator database does not contain data on 1,4-butanediol. The Gibbs free energy was estimated by using the value for (*S,S*)-butane-2,3-diol instead.
e. Calculated with a combined reductive and oxidative TCA shunts to reach redox-neutral conversion. The same result was obtained with a combined reductive TCA cycle and glyoxylate cycle.

Energy in a biological context

Organisms convert carbon and energy-sources into the desired products to conserve energy for growth and

maintenance purposes. This conversion has – by definition – a negative Gibbs free energy. A part of the Gibbs free energy can be conserved as metabolically available energy, and a part is dissipated to avoid chemical equilibrium.

The overall conversion is performed by a vast number of chemical reactions. Again, by definition, these reactions have negative Gibbs free energies under physiological conditions, with prevalent concentrations of substrates, products, intermediates and cofactors. The exact concentrations are mostly unknown, and the Gibbs free energy is therefore often expressed under normalized conditions. For product/substrate combinations ΔG_0' is most convenient, using 1 M concentrations for solutes, and 1 bar concentrations for gasses at pH 7.0. The actual concentrations of metabolic intermediates are however often much lower and therefore ΔG_m' is often applied for single reactions, using 1 mM concentrations for solutes instead (Flamholz *et al*., 2012; Noor *et al*., 2012; Noor *et al*., 2013; Noor *et al*., 2014). Expressing the Gibbs free energy as either ΔG_0' or ΔG_m' may result in positive values for reactions actually running under physiological conditions. Redox reactions can be expressed using either ΔG or the redox potential E'. The relationship between ΔG' and E' is 19.4 kJ 100 mV^{-1} when 2 electrons are transferred. The redox potential E' can be expressed as E_0' normalized for 1 M concentrations of solutes and 1 bar concentrations for gasses or as E_m' normalized for 1 mM concentrations of solutes.

Energy can be conserved in two interchangeable forms: as energy-rich bonds (for example via substrate-level phosphorylation, SLP) or as electrochemical gradients over membranes (via ion-motive force, IMF) (Decker *et al*., 1970).

Phosphate bonds used in, e.g. ATP, GTP and polyphosphates are an important class of energy-rich bonds available in cells. Table 2 lists the ΔG_m' values for the hydrolysis of the phosphate-phosphate bonds in a number of energy carriers.

The electrochemical gradient often exists in the form of a proton-motive force or a sodium ion-motive force. Its energy level is expressed as redox potential (E' (volt)). Typical values for the IMF of fermenting microorganisms are between − 40 and −170 mV (Kashket and Wilson, 1974; Marty-Teysset *et al*., 1996; Salema *et al*., 1996; Trchounian *et al*., 2013). Ion-motive force (IMF) is generated from the energy released by the difference in redox potentials of the compounds involved or the hydrolysis of phosphate-phosphate bonds. Bacteria use IMF for chemical conversions (e.g. drive endergonic ADP phosphorylation to ATP), for osmotic work (e.g. active transport of molecules across the membrane) and mechanical work (e.g. cell motility).

Table 2. Hydrolysis of phosphate-phosphate bonds of different energy carriers.

Reactions	ΔG_m' (kJ mol^{-1})
ATP + H_2O = ADP + Pi	–44 ± 1
ATP + H_2O = AMP + PPi	–52 ± 1
ATP + 2 H_2O = AMP + 2 Pi	–85 ± 1
ADP + H_2O = AMP + Pi	–41 ± 1
GTP + H_2O = GDP + Pi	–41 ± 3
PPi + H_2O = 2 Pi	–33 ± 0

IMF consists of a chemical gradient Δp_X (difference in intracellular and extracellular ion concentrations) and an electrical gradient $\Delta\Psi$ (membrane potential), and it involves the transfer of protons (proton-motive force, PMF) or sodium ions (Na^+ ion-motive force) across the membrane (Equation 1)

$$\Delta\mu_{X^+} = -\frac{2.3RT}{nF}\Delta p_X + \Delta\Psi \quad (1)$$

In this equation, $\Delta\mu$ represents the ion-motive force, X^+ the cation (H^+ or Na^+), Δp_X the chemical concentration gradient of cations over the membrane, $\Delta\Psi$ the membrane potential (V), n the charge of the species translocated (e.g. $n = 1$ for a proton), F the Faraday constant, T the temperature (K) and R the gas constant.

The energy contained in an IMF can be used to create phosphate-phosphate bonds by ATP synthase. This form of phosphorylation is called electron transport phosphorylation (ETP). Redox reactions with a redox potential larger than 43.5/19.4x100 = 224 mV have therefore sufficient Gibbs free energy to phosphorylate ADP to ATP.

Although all reactions are contributing to the overall ΔG', only a few reactions have a sufficiently negative Gibbs free energy to harvest it in a metabolically available form: more than ~ 40 kJ mol^{-1} for ATP synthesis and ~ 100–150 mV (equals ~ 20-30 kJ mol^{-1}) for ion-motive force.

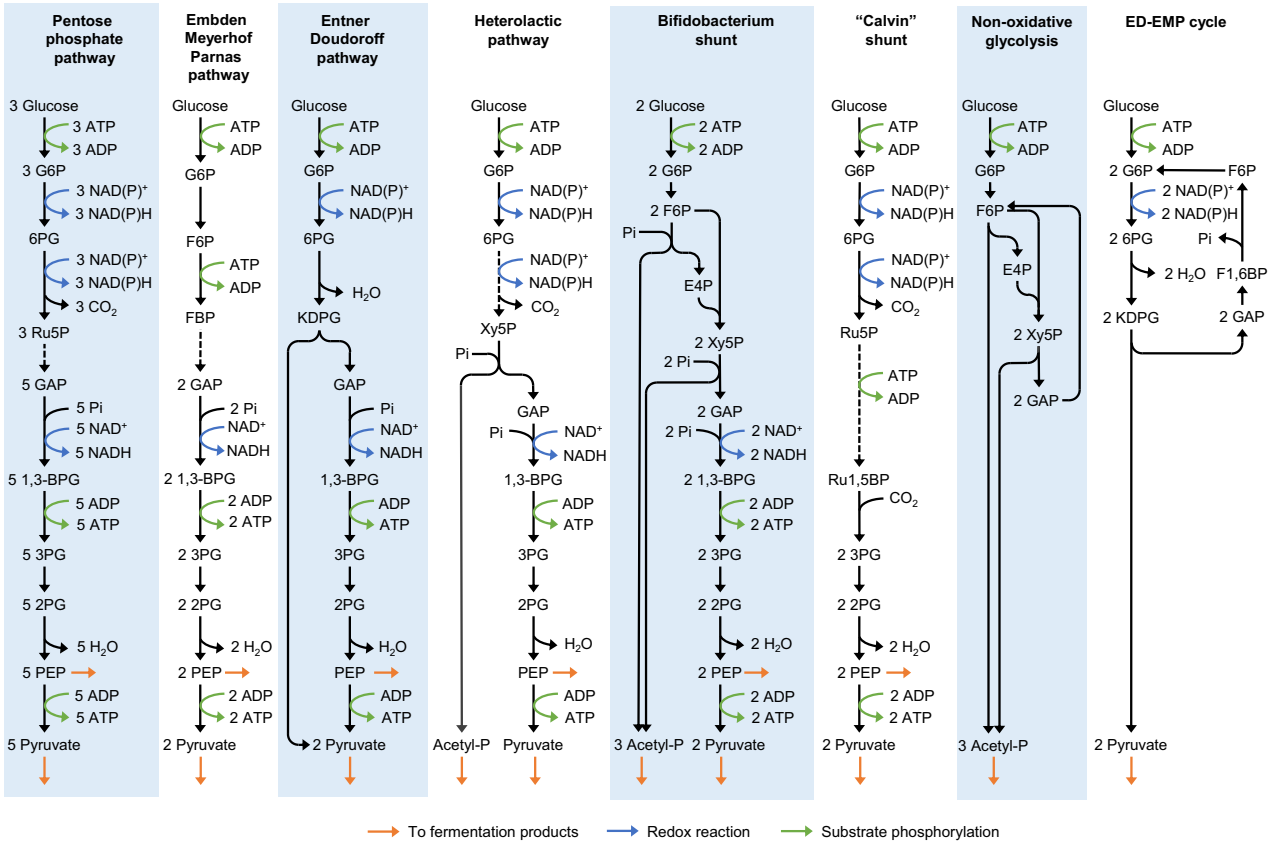
ATP generation in existing fermentation processes

Table 1 gives a non-exhaustive overview of product formation from glucose by fermentation processes and their ΔG_0' values. The ΔG_0' values are between −187 and −266 kJ mol $glucose^{-1}$, and sufficient to deliver the ΔG_m' necessary to create energy-rich phosphate-phosphate bonds once, twice or even three times.

Glycolytic pathways

The process in which C6 sugars are converted into oxidized intermediates is called glycolysis. Several different glycolytic pathways are used by fermenting microorganisms to conserve metabolic energy. The pathways can be characterized by the way they split sugar phosphates and differ with respect to the amount of energy

(A)



(B)

Pentose phosphate pathway:	Glucose + $^{7}/_{3}$ ADP + $^{7}/_{3}$ Pi + 2 NADP + $^{5}/_{3}$ NAD = $^{5}/_{3}$ Pyruvate + $^{7}/_{3}$ ATP + 2 NADPH + $^{5}/_{3}$ NADH + CO_2
Embden Meyerhof Parnas pathway:	Glucose + 2 ADP + 2 Pi + 2 NAD = 2 Pyruvate + 2 H_2O + 2 ATP + 2 NADH
Entner Doudoroff pathway:	Glucose + ADP + Pi + NAD + NAD(P) = 2 Pyruvate + H_2O + ATP + NAD + NAD(P)H
Heterolactic pathway:	Glucose + ADP + 2 Pi + 2 NAD(P) + NAD = Pyruvate + Acetyl-P + CO_2 + ATP + 2 NAD(P)H + NADH + H_2O
Bifidobacterium shunt:	Glucose + ADP + 2.5 Pi + NAD = Pyruvate + 1.5 Acetyl-P + ATP + NADH + 2.5 H_2O
"Calvin" shunt:	Glucose + 2 NAD(P) = 2 Pyruvate + 2 NAD(P)H
Non-oxidative glycolysis:	Glucose + ATP + 2 Pi = 3 Acetyl-P + ADP
ED-EMP cycle:	Glucose + ATP + 2 $NAD(P)^+$ = 2 Pyruvate + ADP + Pi + NAD(P)H

Fig. 2. Microbial glycolytic pathways (A) and their overall reaction equations (B).

harvested and reduced redox cofactors produced (Fig. 2). Thereby these pathways provide options for metabolic engineers to realize production of a certain compound. Below, we describe them in order of most to least ATP generation.

The pentose-phosphate pathway (PPP) is essentially an anabolic pathway able to generate NADPH and building blocks for biosynthetic purposes (Kruger and von Schaewen, 2003). In the PPP ribulose-5-phosphate is split and $^{7}/_{3}$ ATP generated per glucose. The co-production of CO_2 results in more reduced $NAD(P)^+$ than from other glycolytic pathways. This limits its function as glycolytic pathway for fermentation processes to reduced products.

The Embden–Meyerhof–Parnas (EMP) pathway (Kresge *et al.*, 2005) is characterized by the split of fructose-1,6-bisphosphate into dihydroxyacetone-phosphate and D-glyceraldehyde-3-phosphate. It generates 2 ATP per glucose with the concomitant reduction of 2 NAD^+.

The Entner–Doudoroff (ED) pathway is characterized by the cleavage of 2-keto-3-deoxy-6-phosphogluconate into pyruvate and D-glyceraldehyde-3-phosphate. It generates 1 ATP per glucose. Instead of reducing 2 NAD^+, it is also able to reduce 1 $NADP^+$ and 1 NAD^+ (Conway, 1992).

In the heterolactic pathway xylulose-5-phosphate is split into acetyl-phosphate and D-glyceraldehyde-3-

phosphate, which is subsequently oxidized to pyruvate (Burma and Horecker, 1958; Heath *et al*., 1958; Hurwitz, 1958). It produces 1 ATP per glucose and reduces 3 $NAD(P)^+$. The acetyl-phosphate can be converted into acetyl-CoA and – as such – be used for the synthesis of other compounds. This has, e.g. been applied to produce L-glutamate by *Corynebacterium glutamicum* (Chinen *et al*., 2007).

The Bifidobacterium shunt (de Vries *et al*., 1967) is hallmarked by splitting fructose-6-phosphate and xylulose-5-phosphate. The final products are acetyl-phosphate and pyruvate, with the concomitant synthesis of 1 ATP and 1 NADH per glucose.

A glycolytic pathway we dubbed 'the Calvin shunt' is a modification of the pentose-phosphate pathway in which two enzymes of the Calvin cycle are included: phosphoribulokinase and ribulose-1,5-bisphosphate carboxylase. It is not generating ATP and as such its application in fermentation processes is limited. It has been used to replace the pentose-phosphate cycle, to prevent glycerol production in *Saccharomyces cerevisiae*, resulting in increased ethanol formation (Guadalupe-Medina *et al*., 2013).

Non-oxidative glycolysis (NOG) is an artificial pathway based on the Bifidobacterium shunt but has only acetyl-phosphate as product (Bogorad *et al*., 2013). It has been developed especially for its excellent carbon yield (1 Cmol $Cmol^{-1}$). It however requires ATP input and can therefore only be used in combination with other ATP-generating glycolytic pathways or ATP-generating product pathways (Lin *et al*., 2018).

In *Pseudomonas putida* the D-glyceraldehyde-3-phosphate produced by the ED pathway can be converted back to glucose-6-phosphate by gluconeogenesis (Nikel *et al*., 2015). This ED-EMP cycle requires ATP input and generates NAD(P)H.

All the glycolytic pathways lead to the formation of the fermentation intermediates acetyl-phosphate, phospho-enol-pyruvate and/or pyruvate.

Fermentation pathways

Glycolysis is followed by reactions that convert phospho-enol-pyruvate (PEP), pyruvate and/or acetyl-phosphate into the final fermentation products (Fig. 3). Product formation has to regenerate the redox cofactors used in glycolysis. The redox reactions are either the reduction of oxo-groups to hydroxy groups (acetaldehyde to ethanol, pyruvate to lactate, acetoacetyl-CoA to 3-hydroxybutyryl-CoA, oxaloacetate to malate), the reduction of 2-oxoacids into amino acids (e.g. pyruvate into alanine) or the reduction of carbon-carbon double bonds (fumarate to succinate, crotonyl-CoA to butyryl-CoA).

In the fermentation pathways, two mechanisms are available to conserve additional metabolic energy. The reduction of carbon-carbon double bonds can be coupled to harvesting additional metabolic energy in the form of IMF (Kröger, 1978; Graf *et al*., 1985; Herrmann *et al*., 2008; Li *et al*., 2008) and the conversions of acetyl-phosphate and butyryl-phosphate into respectively acetate and butyrate are coupled to SLP and therefore generate ATP.

The harvesting of metabolic energy in these cases is connected to the production of a specific compound and therefore – as such – cannot be used for the production of other compounds. We therefore studied the mechanisms behind these cases of energy conservation to find out whether they can also be applied for harvesting metabolic energy in the production of other chemicals.

Substrate-level phosphorylation (SLP)

Reactions involved in substrate-level phosphorylation

1,3-bisphosphoglycerate + ADP = 3-phosphoglycerate + ATP, $\Delta G_m' = -19 \pm 1$ *kJ* mol^{-1}. This reaction is catalysed by phosphoglycerate kinase (EC 2.7.2.3). It harvests the energy released in the oxidation of D-glyceraldehyde-3-phosphate to 3-phosphoglycerate (Fig. 4A). This redox couple has an E_m' of −524 mV and its electrons are transferred to NAD^+/NADH with a redox potential E' of −300 mV. The difference in redox potential between this redox couple and NAD^+/NADH is 224 mV, just sufficient to produce ATP. Harvesting of ATP is realized in two sub reactions. In the oxidation reaction, D-glyceraldehyde-3-phosphate is converted into 1,3-bisphosphoglycerate by glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12). First, the aldehyde group of glyceraldehyde-3P is oxidized to a carboxyl group and NAD^+ is reduced to NADH. This reaction involves the formation of a high-energy thioester intermediate, which allows attachment of a phosphate group to D-glyceraldehyde-3-phosphate creating 1,3-bisphosphoglycerate. The latter is subsequently converted into 3P-glycerate, forming ATP by transferring a phosphate group to ADP.

This reaction is the only one that truly generates ATP in the glycolytic pathways. This reaction is upstream of pyruvate, PEP and acetyl-phosphate – and is therefore the main contributor to ATP production in existing and new fermentation processes.

PEP + ADP = pyruvate + ATP, $\Delta G_m' = -28 \pm 1$ *kJ* mol^{-1}. Although the conversion of PEP to pyruvate via pyruvate kinase (EC 2.7.1.40) is mentioned as part of SLP, this reaction does not actually lead to *de novo* ATP synthesis. In fact, the reaction enables the recovery of

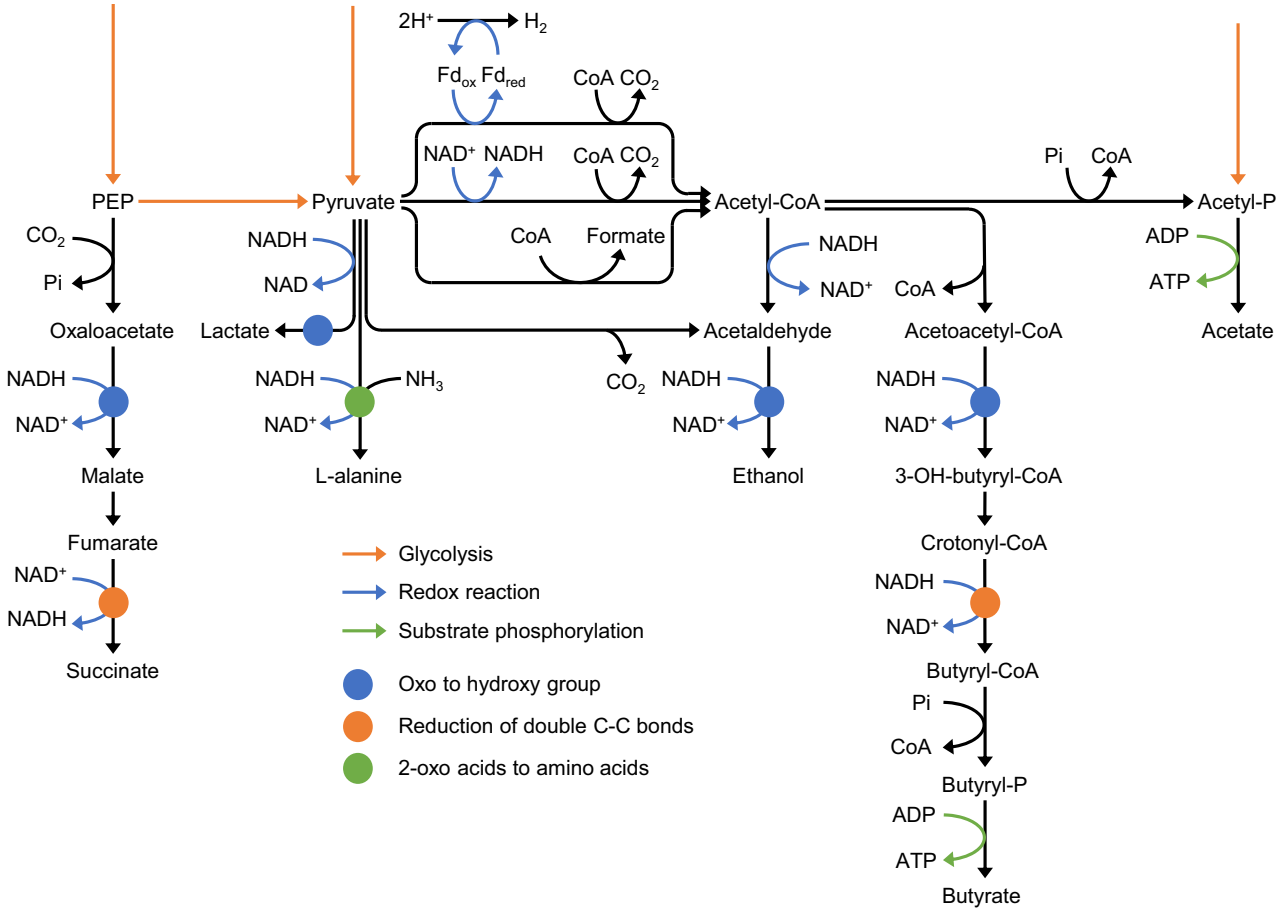


Fig. 3. Conversion of PEP, pyruvate and acetyl-phosphate into final products of fermentation processes.

previously invested energy during phosphorylation of sugar to fructose-1,6-bisphosphate (Fig. 4B).

Carbamoyl-phosphate + ADP = carbamate + ATP, $\Delta G_m' = -17 \pm 4$ *kJ mol*$^{-1}$. This reaction is catalysed by carbamate kinase (EC 2.7.2.2). It harvests the energy released during the degradation of L-arginine into L-ornithine, CO_2 and ammonium (Fig. 4C). Three sub reactions are used to harvest the energy.

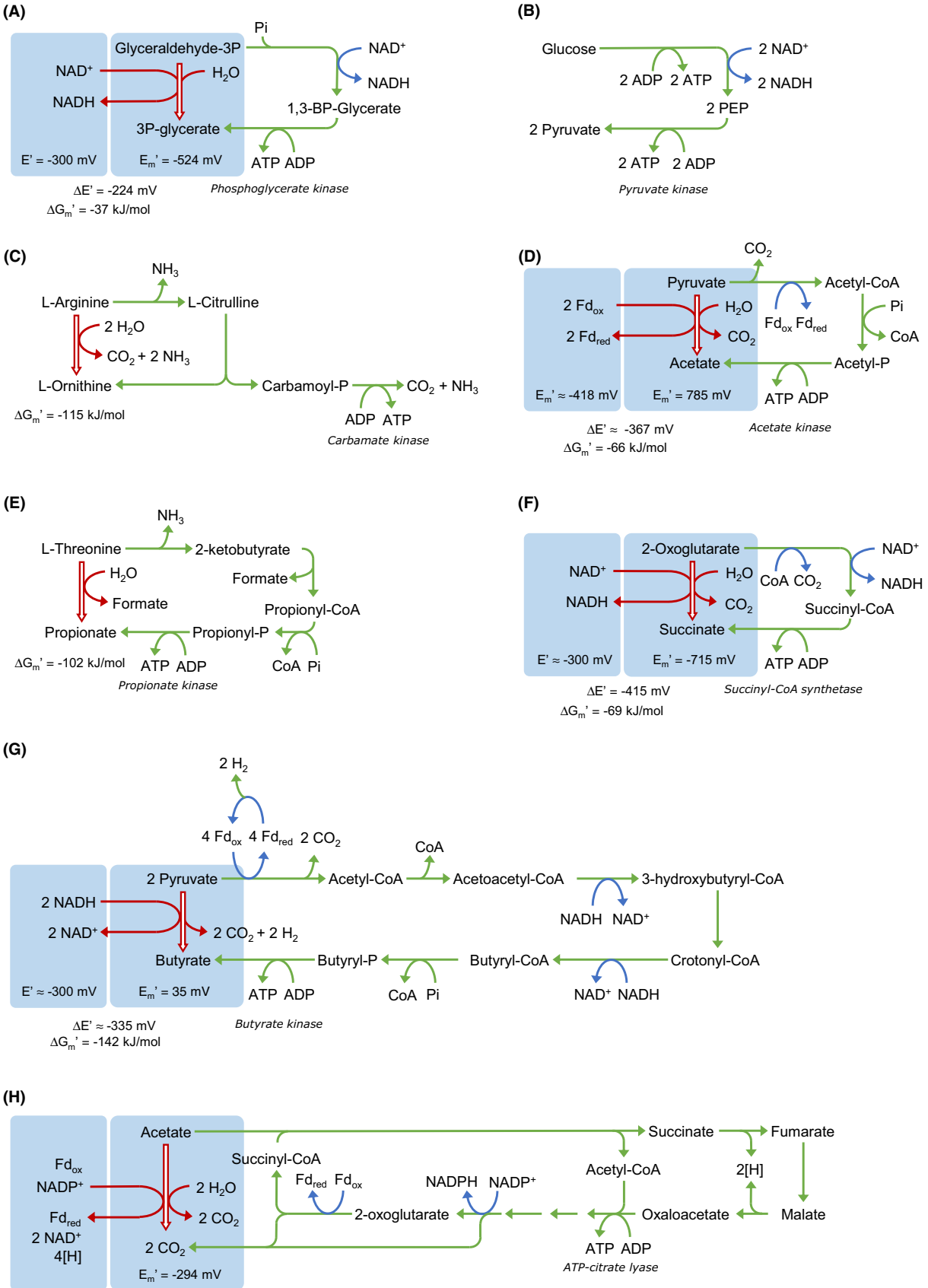
First, L-arginine is hydrolysed into L-citrulline and ammonia by arginine deiminase (EC 3.5.3.6). This reaction has a $\Delta G_m'$ of -52 ± 7 kJ mol^{-1} and the energy released is used to drive the following reaction. Ornithine carbamoyltransferase (EC 2.1.3.3) catalyses the phosphoroclastic cleavage of L-citrulline into L-ornithine and carbamoyl-phosphate ($\Delta G_m'$ of 29 ± 6 kJ mol^{-1}). Then, carbamate kinase cleaves the thioester bond in the energy-rich carbamoyl-phosphate and releases the energy necessary to form ATP from ADP. Carbamate is usually spontaneously converted into CO_2 and ammonium.

Using this reaction for ATP production aiming to synthesize products other than L-ornithine is limited as it leads to CO_2 and ammonium.

Carbamoyl-phosphate is – to our knowledge – also produced in two other reactions: the conversion of oxalureate into oxamate by carbamoyl-phosphate:oxamate carbamoyltransferase (EC 2.1.3.5) (Vander Wauven *et al.*, 1986) and the hydrolysis of glutamine to glutamate by carbamoyl-phosphate synthase (EC 6.3.5.5) (Thoden *et al.*, 1999). Both reactions seem not easily applicable for the production of chemicals or fuels.

N^{10}-formyl THF + ADP + Pi = formate + THF + ATP, $\Delta G_m' = +5 \pm 1$ *kJ mol*$^{-1}$. This reaction is catalysed by

Fig. 4. Reactions contributing to energy formation via substrate-level phosphorylation. Conversions of (A) 1,3-bisphophoglycerate to 3-phosphoglycerate; (B) phosphoenolpyruvate (PEP) to pyruvate; (C) carbamoyl-phosphate to carbamate; (D) acetyl-phosphate to acetate; (E) propionyl-phosphate to propionate; (F) succinyl-CoA to succinate; (G) butyryl-phosphate to butyrate and (H) acetyl-CoA and oxaloacetate to citrate. Red arrows: reactions with strong negative $\Delta G_0'$, green arrows: reaction sequences to harvest ATP; blue arrows: electron transfer. Blue boxes: overall conversions involving redox cofactors.



formyltetrahydrofolate synthetase (EC 6.3.4.3). This enzyme is found in numerous bacteria in which it however functions in the ATP-consuming direction, forming N^{10}-formyltetrahydrofolate. The positive $\Delta G_m'$ indicates that high substrate concentrations and low product concentrations are required to make SLP possible. To date, this reaction has only been mentioned to contribute to SLP in *Clostridium cylindrosporum* growing on purines (Curthoys *et al*., 1972). Consequently, it is not a suitable general option for conservation of metabolic energy using more conventional substrates.

Adenylyl sulfate + PPi = sulfate + ATP, $\Delta G_m'$ = −47 ± 2 kJ mol^{-1}. This reaction is catalysed by sulfate adenylyltransferase (EC 2.7.7.4). Energy is harvested during the oxidation of sulfite to sulfate.

First, the adenylyl sulfate reductase (EC 1.8.99.2) catalyses the AMP-dependent oxidation of sulfite to adenylyl sulfate. Electrons from sulfite are transferred to a cofactor; however, the nature of the cofactor remains elusive. Then, sulfate adenylyltransferase cleaves adenylyl sulfate forming sulfate and AMP. The energy released in the reaction is used to attach a PPi group to AMP and form ATP (Krämer and Cypionka, 1989). In most bacteria, this system is used in sulfate activation for sulfonation, using oxygen as electron acceptor and consuming ATP (Gregory and Robbins, 1960). This reaction should therefore not be considered as a strategy for energy conservation for microbial production processes.

Acyl-phosphate + ADP = fatty acid + ATP. The reactions listed in Table 3 fall in this category.

Acetate kinase.—The reaction catalysed by acetate kinase (EC 2.7.2.1) is member of a reaction sequence that harvests part of the energy released in the conversion of pyruvate into acetate (Fig. 4D). Two options are available in the cells: by oxidative decarboxylation or by co-production of formate. The latter is not discussed here because electrons end up in the by-product formate and not in the desired product.

The pyruvate/acetate redox couple has an E_m' of −785 mV. The electrons can be transferred to the NAD/NADH redox couple with E' around −300 mV or to the Fd_{ox}/Fd_{red} redox couple with E_m' around −418 mV. The difference in redox potential between pyruvate/acetate and NAD/NADH or Fd_{ox}/Fd_{red} is 485 mV and 367 mV, respectively, both of which are larger than the 224 mV required to phosphorylate ADP to ATP.

Harvesting energy is realized in three sub reactions. During the oxidation reaction (dehydrogenation reaction), electrons from pyruvate are transferred to either NAD^+ catalysed by the pyruvate dehydrogenase (PDH) complex, or $ferredoxin_{ox}$ catalysed by pyruvate:ferredoxin oxidoreductase (PFOR, EC 1.2.7.1). CO_2 is released, and CoA is bound to create acetyl-CoA. The energy released in this reaction is stored in the energy-rich compound acetyl-CoA. Then, phosphate acetyltransferase (PTA, EC 2.3.1.8) catalyses the exchange of CoA with a phosphate group to form acetyl-phosphate. Finally, acetyl-P is converted into acetate, transferring the phosphate group to ADP, forming ATP.

This reaction results in one specific fermentation product – acetate – and its contribution to SLP can therefore not be used to produce other compounds.

Propionate/acetate kinase.—The reaction catalysed by propionate/acetate kinase (EC 2.7.2.15) harvests the energy generated by the conversion of L-threonine into propionate ($\Delta G_m'$ of −102 ± 7 kJ mol^{-1}, Fig. 4E). L-threonine is deaminated to 2-ketobutyrate, which is concomitantly converted into propionyl-CoA and formate by a pyruvate formate-lyase type of enzyme. The propionyl-CoA is converted into propionyl-phosphate, which donates its phosphate group to convert ADP into ATP (Heßlinger *et al*., 1998).

Table 3. Reactions coupling the conversion of an acyl-phosphate to a fatty acid to ADP phosphorylation to ATP.

Reactions	Enzymes	$\Delta G_m'$ (kJ mol^{-1})
Enzyme activities contributing to SLP		
Acetyl-phosphate + ADP = acetate + ATP	Acetate kinase (EC 2.7.2.1) Propionate/acetate kinase	−13 ± 1
Propionyl-phosphate + ADP = propionate + ATP	Propionate/acetate kinase Butyrate kinase	−32 ± 6
Butyryl-phosphate + ADP = butyrate + ATP	Butyrate kinase	−29 ± 7
Isovaleryl-phosphate + ADP = isovalerate + ATP	Branched-chain fatty acid kinase Butyrate kinase	n.a.
2-methylbutyryl-phosphate + ADP = 2-methylbutyrate + ATP	Branched-chain fatty acid kinase	n.a.
Isobutyryl-phosphate + ADP = isobutyrate + ATP	Branched-chain fatty acid kinase Butyrate kinase	n.a.
Additional enzyme activities		
Valeryl-phosphate + ADP = valerate + ATP	Butyrate kinase	n.a.
Vinyl-acetyl-phosphate + ADP = vinyl-acetate + ATP	Butyrate kinase	n.a.
Isopropionyl-phosphate + ADP = isopropionate + ATP	Branched-chain fatty acid kinase	n.a.

n.a., not available.

This reaction depends on a non-conventional substrate – L-threonine – and can only be applied for the production of propionate. Application of this reaction to generate ATP in other fermentation processes is therefore unlikely.

Butyrate kinase.—The reaction catalysed by butyrate kinase (EC 2.7.2.7), harvests the energy released in the oxidation of 2 pyruvate into butyrate, 2 CO_2 and 2 H_2 (Fig. 4G). The E_m' of the 2 pyruvate + 4 e-/butyrate + 2 CO_2 + 2 H_2 redox couple is 35 ± 37 mV. The difference in redox potentials with NAD^+/NADH is sufficient to harvest energy in the form of ATP. The reduction reactions are performed by acetoacetyl-CoA reductase (EC 1.1.1.36) and butyryl-CoA dehydrogenase (EC 1.3.8.1). The energy released in the reduction reaction is harvested by a sequence of reactions in which butyryl-CoA is first converted into butyryl-phosphate, which is concomitantly used for the phosphorylation of ADP, producing butyrate.

Butyrate kinase is an example of an enzyme involved in SLP with broad substrate specificity. It is also able to produce isobutyrate, valerate, isovalerate, propionate or vinyl-acetate (Twarog and Wolfe, 1963; Hartmanis, 1987).

Branched-chain fatty acid kinase.—The reactions carried out in *Spirocheata sp* by the branched-chain fatty acid kinase (EC 2.7.2.14) harvest the energy generated by the oxidative decarboxylation of a 2-keto organic acid into an organic acid, e.g. 2-ketoisocaproic acid into isovaleric acid, similar to the case described below for succinyl-CoA. During starvation, this organism conserves the energy required for its maintenance by fermenting branched-chain amino acids, e.g. L-valine, L-leucine and L-isoleucine (although these compounds are not utilized as growth substrates) (Harwood and Canale-Parola, 1981a,b). ATP is formed via SLP using branched-chain fatty acid kinase. The branched-chain fatty acid kinase has been shown to accept a wide range of compounds such as 2-methylpropionyl-phosphate, 2-methylbutyryl-phosphate, butyryl-phosphate, valeryl-phosphate, propionyl-phosphate as well as different NTP (ATP, GTP, CTP) (Harwood and Canale-Parola, 1982).

Acyl-CoA + ADP + Pi = fatty acid + CoA + ATP. The reactions mentioned in Table 4 fall into this category.

Acetyl-CoA synthetase (6.2.1.13) combines the actions of phosphotransacetylase and acetate kinase (Labes and Schönheit, 2001). It also shows activity for propionate, (iso)butanoate, (iso)pentanoate, hexanoate, octanoate, imidazole-4-acetate, phenyl acetate, succinate and thioglycolate (Musfeldt *et al*., 1999; Jones and Ingram-Smith, 2014). The reaction catalysed by succinyl-CoA synthetase (EC 6.2.1.5) harvests the energy generated by the oxidative decarboxylation of 2-oxoglutarate to succinate (Fig. 4F). This redox couple has an E_m' of −715 mV. Electrons are either transferred to the NAD^+/NADH or Fd_{ox}/Fd_{red} redox couples. The differences in redox potential between the redox couples are large enough to allow ATP synthesis.

Table 4. Reactions coupling conversion of an acyl-CoA to a fatty acid to ADP phosphorylation to ATP.

Reactions	Enzymes	ΔG_m' (kJ mol^{-1})
Enzyme activities contributing to SLP		
Acetyl-CoA + ADP + Pi = acetate + CoA + ATP	Acetyl-CoA synthetase	–4 ± 1
Succinyl-CoA + ADP + Pi = succinate + CoA + ATP	Succinyl-CoA synthetase	–2 ± 3
Additional enzyme activities		
Itaconyl-CoA + ADP + Pi = itaconate + CoA + ATP	Succinyl-CoA synthetase	5 ± 15
3-sulfinopropionyl-CoA + ADP + Pi = 3-sulfinopropionate + CoA + ATP	Succinyl-CoA synthetase	n.a.
Oxalyl-CoA + ADP + Pi = oxalate + CoA + ATP	Succinyl-CoA synthetase	11 ± 7
Propionyl-CoA + ADP + Pi = propionate + CoA + ATP	Succinyl-CoA synthetase	–14 ± 6
Butyryl-CoA + ADP + Pi = butyrate + CoA + ATP	Succinyl-CoA synthetase	–8 ± 16
Adipyl-CoA + ADP + Pi = adipate + CoA + ATP	Succinyl-CoA synthetase	5 ± 15
Glutaryl-CoA + ADP + Pi = glutarate + CoA + ATP	Succinyl-CoA synthetase	5 ± 3

n.a., not available

Two sub reactions are used to harvest the energy. In an oxidation reaction, CoA is bound to form succinyl-CoA. This reaction is an oxidative decarboxylation that can be catalysed by either by the 2-oxoglutarate dehydrogenase complex (ODH) or the 2-oxoglutarate: ferredoxin oxidoreductase (OGOR). Succinyl-CoA is used as an energy-rich intermediate, and cleavage of the thioester bond releases the energy required to drive ADP phosphorylation to form ATP (catalysed by the succinyl-CoA synthetase).

This reaction is part of the citric acid cycle. Fermentative product formation relying on intermediates of the TCA cycle – between succinyl-CoA and oxaloacetate - can therefore benefit from this reaction to generate ATP.

Succinyl-CoA synthetase (EC 6.2.1.5) is able to catalyse the ADP-forming conversion of succinate analogues such as itaconate or 3-sulfinopropionate (Schürmann *et al*., 2011). Shikata *et al*. (2007) demonstrated that *Thermococcus kodakarensis* possesses an ADP-forming succinyl-CoA synthetase able to convert oxalate, propionate, butyrate, adipate and glutarate. It has not been proven that these additional reactions can contribute to SLP. The ΔG_m' values of the reactions involving itaconate, adipate and glutarate are relatively high

indicating that substrate concentrations and low product concentration are required to contribute to SLP.

Acetyl-CoA + oxaloacetate + H_2O + ADP + Pi = citrate + CoA + ATP, $\Delta G_m'$ = +9 ± 1 kJ mol^{-1}. This reaction is catalysed by ATP citrate synthase (EC 2.3.3.8 formerly EC 4.1.3.8). This enzyme usually works in the other direction: e.g. in oleaginous yeasts to make cytosolic acetyl-CoA available (Liu *et al*., 2013; Dulermo *et al*., 2015). Möller *et al*. (1987) have reported the ATP-harvesting action of this enzyme in *Desulfobacter postgatei*. The high $\Delta G_m'$ indicates that high substrate concentrations and low product concentrations are required to make SLP possible. The energy-conserving action is the oxidation of acetate to 2 CO_2 (acetate + 2 H_2O = 2 CO_2 + 8 e^-; E_0' = –272 ± 16 mV, Fig. 4H) and concomitant transfer of the electrons to $NADP^+$ and ferredoxin.

The conversion of oxaloacetate and acetyl-CoA into citrate is a metabolic step used in the production of citrate, itaconate, L-glutamate and other compounds that depend on the oxidative TCA cycle. Application of ATP citrate synthase instead of citrate synthase could enhance the ATP yield in these processes.

Thermodynamic constraints to SLP

As indicated above, some of the enzymes involved in SLP reactions are able to catalyse a range of additional reactions. It can be envisaged that this range can be increased further by protein engineering. The question remains whether these reactions can also contribute to SLP. Figure 5 shows the thermodynamic analysis of the hydrolysis of acyl-CoAs and carboxy-acyl-CoAs. It indicates that the hydrolysis of acetyl-CoA, propionyl-CoA and butyryl-CoA has a sufficiently low $\Delta G_m'$ to phosphorylate ADP and that the reaction catalysed by succinyl-CoA synthetase is on the verge of thermodynamic feasibility. The graph shows that SLP using ADP/ATP may not be feasible for other acyl-CoAs and dicarboxyl-CoAs, although it is difficult to draw a clear conclusion due to the large standard deviations of the $\Delta G_m'$ values. SLP based on pyrophosphate instead seems to be feasible for all carbon lengths and is worth investigating.

Generation of an ion-motive force

Reactions coupled to ion translocation over cellular membranes comprise decarboxylation reactions, reduction of carbon-carbon double bonds and transfer of electrons between redox cofactors.

Reduction of carbon-carbon double bonds

The redox potential of reactions in which carbon-carbon double bonds are reduced is between + 70 and –40 mV (Table 5). The redox potential difference with other redox couples as NADH is in most cases sufficiently large to

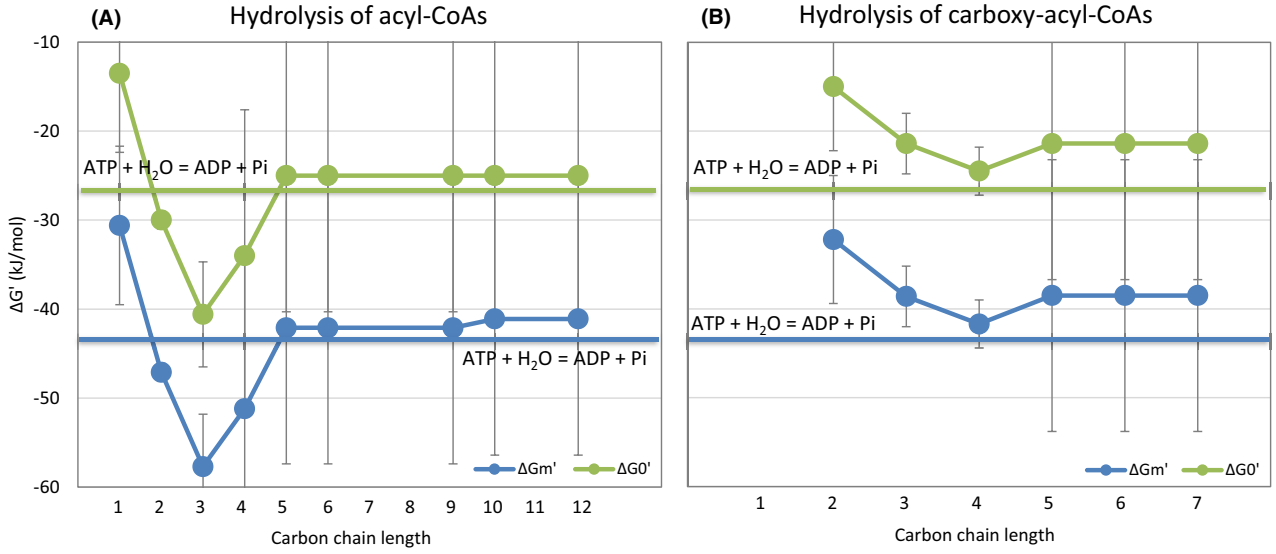


Fig. 5. Gibbs free energy ($\Delta G_m'$) of hydrolysis of (A) acyl-CoA and (B) carboxy-acyl-CoA molecules of different carbon lengths. (A) C1: formyl-CoA + H_2O = formate + CoA; C2: acetyl-CoA + H_2O = acetate + CoA; C3: propionyl-CoA + H_2O = propionate + CoA; C4: butyryl-CoA + H_2O = butyrate + CoA; C5: valeryl-CoA + H_2O = valerate + CoA; C6: hexanoyl-CoA + H_2O = hexanoate + CoA; C9: nonanoyl-CoA + H_2O = nonanoate + CoA; C10: decanoyl-CoA + H_2O = decanoate + CoA. (B) C2: oxalyl-CoA + H_2O = oxalate + CoA; C3: malonyl-CoA + H_2O = malonate + CoA; C4: Succinyl-CoA + H_2O = succinate + CoA; C5: glutaryl-CoA + H_2O = glutarate + CoA; C6: Adipyl-CoA + H_2O = adipate + CoA; C7: pimeloyl-CoA + H_2O = pimelate + CoA. The green lines show the $\Delta G_m'$ required to create phosphate-phosphate bonds to convert ADP into ATP and 2 Pi into PPi.

Table 5. Reactions involving the reduction of a carbon-carbon double bond and their redox potential. The non-referenced E_m' values are derived from eQuilibrator based on component contribution. The referenced values were determined experimentally.

Redox reaction	E_m' (mV)
Acrylyl-CoA + 2 e- = propionyl-CoA	−14 ± 87 +69 (Sato *et al.*, 1999)
Fumarate + 2 e- = succinate	−5 ± 21 +33 (Thauer *et al.*, 1977)
Crotonyl-CoA + 2 e- = butyryl-CoA	−37 ± 83 −13 (Sato *et al.*, 1999)
Caffeoyl-CoA + 2 e- = 1,3-dehydrocaffeoyl-CoA	n.a.

n.a. = not available

enable electron transport phosphorylation (ETP). Experimental values are also given in Table 5 as the values calculated using the group component contribution present large standard deviations.

Reduction of fumarate to succinate. Due to the relatively high redox potential of the fumarate/succinate couple (ca. +30 mV), several electron donors can be used to oxidize fumarate, e.g. H_2, NADH, lactate, formate, malate and glycerol-1-phosphate (Hirsch *et al.*, 1963; Thauer *et al.*, 1977; Kröger, 1978; Tran *et al.*, 1997). The two electrons released during these reactions are transferred to electron carriers, e.g. menaquinone or demethylmenaquinone, which are reduced to menaquinol or demethylmenaquinol, respectively (Spencer and Guest, 1973; Lambden and Guest, 1976; Kröger, 1978; Wissenbach *et al.*, 1990). The reduced electron carriers transfer electrons to fumarate reductase (EC 1.3.5.1; EC 1.3.5.4), allowing reduction of fumarate to succinate. *Escherichia coli* the electron transfer from NADH to fumarate is coupled to the formation of a transmembrane proton gradient by NADH dehydrogenase I (NDH-I or Complex I of the ETC), which is then used to synthetize ATP by ADP synthase (Tran *et al.*, 1997) (Fig. 6A).

The number of protons translocated per electron by NDH-I has been proposed to be between 1.5 and 2 (Bogachev *et al.*, 1996; Wikström and Hummer, 2012). Assuming that the ATP synthase requires an inward translocation of 4 protons per ATP, fumarate reduction using NDH-I allows the synthesis of 0.75 to 1 mol ATP per mol succinate formed.

This way of energy conservation can be applied for the production of succinate and succinate-derived chemicals such as 1,4-butanediol (Yim *et al.*, 2011).

Reduction of crotonyl-CoA to butyryl-CoA.—During butyrate fermentation, ATP is produced via SLP in a chain of reactions from pyruvate to butyrate using butyrate kinase (see SLP section). However, additional energy can be harvested in the reaction catalysed by butyryl-CoA dehydrogenase in which the carbon-carbon double bonds of crotonyl-CoA are reduced to form butyryl-CoA. This redox couple has an E_m' of −37 ± 83 mV. The difference with the redox potential of NAD^+/NADH is large enough to capture additional metabolic energy. In some bacteria such as Clostridia, cytoplasmic butyryl-CoA dehydrogenase/electron-transferring flavoprotein (Bcd/Etf; EC 1.3.1.109) couples the reduction of crotonyl-CoA and ferredoxin with the oxidation of NADH (Herrmann *et al.*, 2008; Li *et al.*, 2008; Seedorf *et al.*, 2008). In this reaction, the Bcd/Etf complex transfers electrons from NADH (E_0'= −320 mV) to crotonyl-CoA (E_0'= −10 mV), and the difference in redox potential is used to drive the reduction of ferredoxin (E_0'= ca −400 mV) by a second NADH. This coupled electron transfer reaction is an example of electron bifurcation (Herrmann *et al.*, 2008; Li *et al.*, 2008; Buckel and Thauer, 2013; Buckel and Thauer, 2018). The reduced ferredoxin is then used to reduce NAD^+ by a membrane-bound NAD^+:ferredoxin oxidoreductase (or Rnf complex) (Herrmann *et al.*, 2008) contributing to the generation of IMF (Fig. 6B). Reduction of crotonyl-CoA to butyryl-CoA via Bcd/Etf in combination with Rnf complex can lead up to 0.5 ATP formed per butyryl-CoA formed.

Reduction of caffeyl-CoA to dihydrocaffeyl-CoA.—The anaerobic acetogenic bacterium *Acetobacterium woodii* conserves energy during caffeate respiration via the reduction of caffeate to hydrocaffeate using H_2 as electron donor (Tschech and Pfennig, 1984; Hansen *et al.*, 1988). During caffeate respiration in *A. woodii*, caffeate is activated to caffeyl-CoA prior being reduced (Hess *et al.*, 2011). Once caffeate respiration reaches steady state, caffeate activation is replaced by caffeate CoA-transferase (CarA, EC 2.8.3.23) which transfers a CoA moiety from hydrocaffeyl-CoA to caffeate forming caffeyl-CoA (Hess *et al.*, 2013a). The electron-bifurcating caffeyl-CoA reductase (CarCDE) reduces caffeyl-CoA and ferredoxin with NADH (Bertsch *et al.*, 2013). The reduced ferredoxin is then used to reduce NAD^+ via the Rnf complex with the concomitant transfer of Na^+ ions across the membrane (Hess *et al.*, 2013b). In caffeate respiration, the electron-bifurcating hydrogenase HydABC uses H_2 as electron donor to reduce Fd_{ox} and NAD^+. The reduced ferredoxin produced in this reaction can in turn be used by the Na^+-dependent Rnf complex (Fig. 6C). The conversion of caffeate to hydrocaffeate leads to the production of 0.9 mol ATP per mol caffeate reduced (Bertsch *et al.*, 2013).

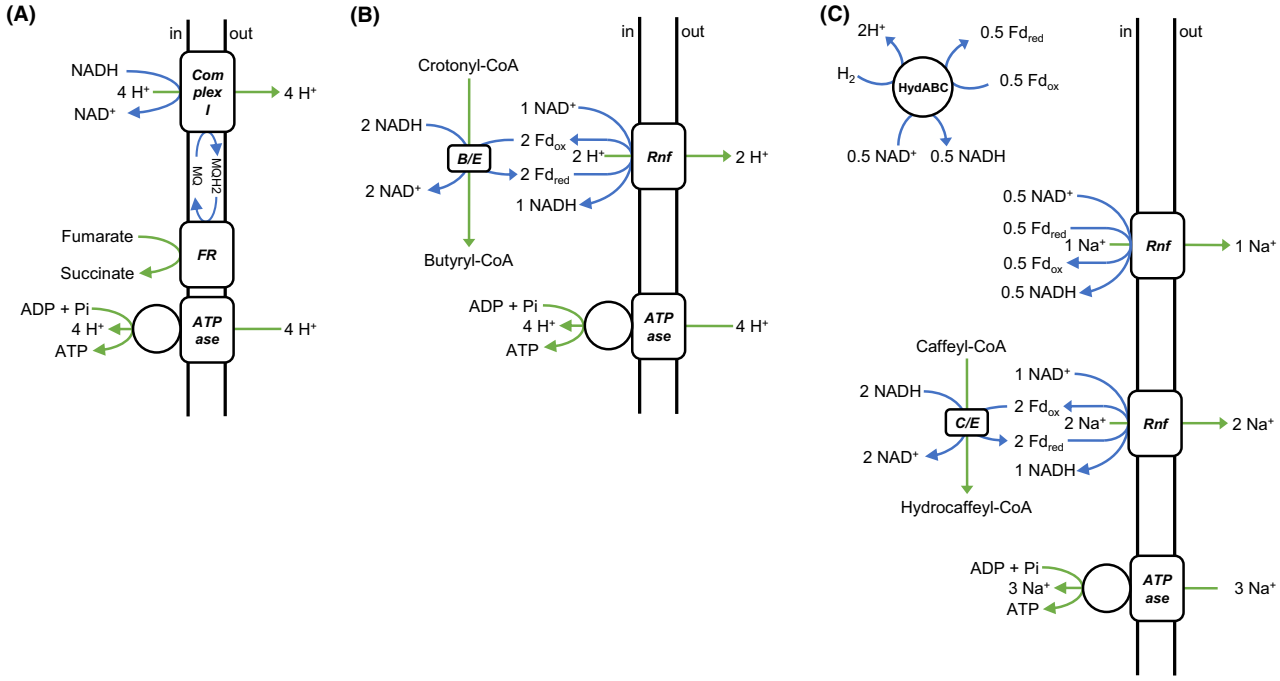


Fig. 6. (A) Fumarate reduction in *E. coli* using NADH dehydrogenase I as electron donor, (B) Reduction of crotonyl-CoA to butyryl-CoA in *Clostridium kluyveri* and (C) Reduction of caffeyl-CoA to hydrocaffeyl-CoA in *Acetobacterium woodii* using H_2 as electron donor.

Reduction of acrylyl-CoA to propionyl-CoA.—Not all carbon-carbon reduction reactions result in the creation of an IMF. An example is the reduction of acrylyl-CoA to propionyl-CoA (Baldwin and Milligan, 1964; Seeliger *et al*., 2002). Bacteria can ferment lactate to acetate and propionate via the succinate pathway (methylmalonyl-CoA) or via the acrylate pathway (acrylyl-CoA). The latter involves the reduction of acrylyl-CoA to propionyl-CoA catalysed by a non-bifurcating EtfAB-propionyl-CoA dehydrogenase (also called acrylyl-CoA reductase) (Hetzel *et al*., 2003). This reaction – which is present in anaerobic bacteria such as *Clostridium homopropionicum* – is not coupled to energy generation via IMF (Baldwin and Milligan, 1964; Seeliger *et al*., 2002). According to Sato *et al*. (1999), this is due to the relatively low redox potential of this redox couple (see Table 5). For more information, see Buckel and Thauer (2018) and Seeliger *et al*. (2002).

Table 6. Reactions involved in decarboxylation phosphorylation. The $\Delta G_m'$ values were calculated using eQuilibrator 2.2 with aqueous (aq) for all compounds and do not take into account the translocation of ions across the membrane.

Reactions	Enzymes	$\Delta G_m'$ (kJ mol^{-1})	Ions translocated
Oxaloacetate + H^+ = Pyruvate + CO_2	Oxaloacetate decarboxylase (EC 7.2.4.2)	–34 ± 6	2 Na^+
(S)-methylmalonyl-CoA + H^+ = Propionyl-CoA + CO_2	Methylmalonyl-CoA decarboxylase (EC 7.2.4.3)	–37 ± 12	1 Na^+
Glutaconyl-CoA + H^+ = Crotonyl-CoA + CO_2	Glutaconyl-CoA decarboxylase (EC 7.2.4.5)	–36 ± 17	1 Na^+
Malonate + H^+ = Acetate + CO_2	Malonate decarboxylase (EC 7.2.4.4)	–44 ± 7	1 Na^+

Decarboxylation phosphorylation

Oxidative decarboxylation reactions contribute to energy production via SLP; due to the large negative value of their $\Delta G_m'$. Non-oxidative decarboxylation reactions have a less negative $\Delta G_m'$ (Table 6). These reactions can however also be coupled to the generation of IMF as the energy released during such steps is comparable to the one required for generating an ion-motive force (~20–30 kJ mol, equivalent to 100–150 mV). This mechanism is called decarboxylation phosphorylation (Dimroth, 1997; Buckel, 2001; Dimroth and von Ballmoos, 2007) and can be used for ATP formation or transport of molecules across the membrane against concentration gradients. The transport of molecules across the membrane can be realized electrogenically, where a net charge is translocated, or electroneutrally, where no net charge is translocated, depending on the carrier.

Several anaerobic bacteria produce ATP exclusively through this mechanism such as *Propionigenium modestum* and *Malonomonas rubra* (Dimroth and Hilbi, 1997). In this process, the energy released during decarboxylation reactions is converted into a Na^+ electrochemical gradient, which is later used to drive ADP phosphorylation via ATP synthase. These Na^+-translocating decarboxylases are protein complexes consisting of soluble and membrane-bound subunits. They are biotin-dependent enzymes found in a limited number of microorganisms grown under anaerobic conditions (Galivan and Allen, 1968; Dimroth, 1981; Buckel and Semmler, 1982; Dimroth, 1982b; Hilpert and Dimroth, 1982). Decarboxylases shown to contribute to decarboxylation phosphorylation are oxaloacetate decarboxylase, methylmalonyl-CoA decarboxylase, glutaconyl-CoA decarboxylase and malonate decarboxylase (Table 6).

These decarboxylations are two-step processes. First, the carboxyl group from the substrate is transferred to biotin in an Na^+-independent manner, forming products and carboxybiotin. The latter is then decarboxylated to biotin, and Na^+ ions are translocated across the membrane. Malonate decarboxylation differs slightly from the three other decarboxylations because malonate needs to be activated prior decarboxylation under physiological conditions. Therefore, malonate decarboxylase carries a transferase that forms the thiol ester bond. For more details about the structures and mechanisms of Na^+-translocating decarboxylases, see Dimroth and Hilbi (1997), Buckel (2001), Dimroth *et al*. (2001), Dimroth and von Ballmoos (2007) and references therein.

Oxaloacetate decarboxylase. Oxaloacetate decarboxylase has been characterized in citrate-fermenting *Klebsiella aerogenes* and *Klebsiella pneumoniae* (Dimroth, 1980; Dimroth, 1982a,b; Schwarz *et al*., 1988) as well as in citrate- and tartrate-fermenting *Salmonella typhymurium* (Wifling and Dimroth, 1989; Woehlke *et al*., 1992; Woehlke and Dimroth, 1994). During citrate fermentation in *K. pneumoniae*, citrate uptake is realized by Na^+-dependent citrate carrier CitS. Citrate is subsequently cleaved into acetate and oxaloacetate by citrate lyase. Then, Na^+-pumping oxaloacetate decarboxylase converts oxaloacetate to pyruvate and CO_2, and the free energy from this decarboxylation reaction is used to translocate 2 Na^+ ions outside the cells. Pyruvate is cleaved into acetyl-CoA and formate by pyruvate formate-lyase. Formate hydrogen-lyase converts formate into H_2 and CO_2. Acetyl-CoA is further converted into acetate via phosphotransacetylase (PTA) and acetate kinase (AK). Citrate fermentation leads to the production of 1 mol ATP per mol citrate by SLP during acetate formation and to a Na^+ ion-motive force. The Na^+ ions gradient is used for the electroneutral uptake of citrate using CitS (Pos and Dimroth, 1996) while the electrical component of the Na^+ ion-motive force is presumed to contribute to ATP synthesis by ATP synthase.

Methylmalonyl-CoA decarboxylase. Methylmalonyl-CoA decarboxylase activity has been demonstrated in bacteria such as *E. coli* (Benning *et al*., 2000), lactate-fermenting *Veillonella parvula* (Hilpert and Dimroth, 1983; Hilpert *et al*., 1984) or succinate-fermenting *Propionigenium modestum* (Hilpert *et al*., 1984; Bott *et al*., 1997). Decarboxylation of methylmalonyl-CoA is a vital step in succinate fermentation for *P. modestum* as it allows the organism to conserve the energy required for growth (Schink and Pfennig, 1982; Dimroth and Schink, 1998). During succinate fermentation in *P. modestum* (Dimroth and Schink, 1998), succinate propionyl-CoA-transferase transfers a CoA group from propionyl-CoA to succinate leading to succinyl-CoA and propionate ($\Delta G_m' = -16 \pm 6$ kJ mol^{-1}). Succinyl-CoA is then converted into (*R*)-methylmalonyl-CoA by methylmalonyl-CoA mutase and further isomerized to (*S*)-methylmalonyl-CoA by methylmalonyl-CoA isomerase. Methylmalonyl-CoA decarboxylase catalyses the decarboxylation of (*S*)-methylmalonyl-CoA to propionyl-CoA and CO_2 and transfers 2 Na^+ ions across the membrane – one electrogenically and one electroneutrally (Hilpert and Dimroth, 1991; Di Berardino and Dimroth, 1996). The membrane potential of this Na^+ ion-motive force is then used by a Na^+-dependent ATP synthase to drive ADP phosphorylation (Laubinger and Dimroth, 1988; Dimroth *et al*., 2000). The Na^+-dependent F_0F_1 ATP synthase of *P. modestum* requires the inward translocation of 3.3 Na^+ ions to synthetize 1 molecule of ATP (Stahlberg *et al*., 2001; Dimroth and Cook, 2004).

Glutaconyl-CoA decarboxylase. Glutaconyl-CoA decarboxylase catalyses a key reaction for energy conservation during L-glutamate fermentation (via (*R*)-2-hydroxyglutarate) in several anaerobic bacteria including *Acidaminococcus fermentans* (Buckel and Semmler, 1982; Bendrat and Buckel, 1993; Braune *et al*., 1999), *Fusobacterium nucleatum* (Beatrix *et al*., 1990) and *Clostridium symbiosum* (Buckel and Semmler, 1983) and during glutarate degradation in *Pelospora glutarica* (Matthies and Schink, 1992a,b; Matthies *et al*., 2000).

During glutarate degradation, glutarate is first activated to glutaryl-CoA by glutaconate CoA-transferase which transfers CoA from acetyl-CoA to glutarate. Glutaryl-CoA is converted to glutaconyl-CoA by glutaryl-CoA dehydrogenase/Etf. Subsequently, glutaconyl-CoA decarboxylase catalyses the decarboxylation of glutaconyl-CoA to crotonyl-CoA with the concomitant transfer of Na^+ ions across the membrane. Crotonyl-CoA is further reduced to butyryl-CoA with NADH, allowing regeneration of the

NAD^+ used in the conversion of glutaryl-CoA to glutaconyl-CoA. Then, acetate CoA-transferase transfers the CoA moiety from butyryl-CoA to acetate thereby forming acetyl-CoA, which is required for glutarate activation.

In *A. fermentans*, 5 L-glutamate are converted to 5 ammonia, 5 CO_2, H_2, 6 acetate and 2 butyrate (Buckel and Thauer, 2013). L-glutamate is first converted to 2-oxoglutarate by NAD^+-dependent glutamate dehydrogenase. The latter is then reduced to (*R*)-2-hydroxyglutarate by 2-oxoglutarate reductase. (*R*)-2-hydroxyglutarate CoA-transferase transfers a CoA moiety from acetyl-CoA to (*R*)-2-hydroxyglutarate, forming (*R*)-2-hydroxyglutaryl-CoA and acetate. Then, (*R*)-2-hydroxyglutaryl-CoA dehydratase catalyses the conversion of (*R*)-2-hydroxyglutaryl-CoA to glutaconyl-CoA. Glutaconyl-CoA decarboxylase couples the decarboxylation of glutaconyl-CoA to crotonyl-CoA to the translocation of 2 Na^+ ions across the membrane (Buckel, 2001). Of each five crotonyl-CoA formed from five L-glutamate by *A. fermentans*, two are converted to butyrate via butyryl-CoA and three are converted to acetate via (*S*)-3-hydroxybutyryl-CoA, acetoacetyl-CoA and acetyl-CoA. In this latter pathway, the NADH formed during the conversion of (*S*)-3-hydroxybutyryl-CoA to acetoacetyl-CoA is used in the reduction of crotonyl-CoA to butyryl-CoA. Furthermore, 1 ATP is generated per crotonyl-CoA converted to acetate by the action of acetate CoA ligase (ADP-forming). In *A. fermentans*, Bcd/Etf catalyses the conversion of crotonyl-CoA to butyryl-CoA (See section 'Reduction of crotonyl-CoA to butyryl-CoA'). The reduced ferredoxin produced in this reaction is partly reoxidized by the Rnf complex and thereby contributing to the generation of an ion-motive force (Herrmann *et al*., 2008). The remaining reduced ferredoxin is converted to H_2 by the action of a hydrogenase. Finally, acetate CoA-transferase transfers the CoA moiety from butyryl-CoA to acetate thereby forming acetyl-CoA and butyrate. The Na^+ ions translocated outside the cell can either be used for ATP synthesis or to take up L-glutamate by a sodium-glutamate symporter (Chang *et al*., 2010). Buckel and Thauer (2013) calculated that 0.95 ATP can be formed per L-glutamate consumed in *A. fermentans*.

Malonate decarboxylase system. Malonate decarboxylation has been shown to be the sole energy-conserving route for anaerobic growth of some bacteria such as *Malonomonas rubra* and *Sporomusa malonica* (Dehning and Schink, 1989; Dehning *et al*., 1989). The malonate decarboxylase system consists of several enzymes catalysing distinct reactions to ultimately convert malonate into acetate and CO_2 with the concomitant generation of a sodium electrochemical gradient.

During malonate fermentation, malonate uptake is realized by a Na^+-dependent symporter (MadL-MadM) (Schaffitzel *et al*., 1998). Prior to decarboxylation, malonate is activated by one of the malonate decarboxylase system modules that transfers an ACP moiety from acetate to malonate thereby generating acetate and malonyl-ACP (Hilbi *et al*., 1992; Berg *et al*., 1996; Berg *et al*., 1997; Dimroth and Hilbi, 1997). The free carboxyl group of malonyl-ACP is then transferred to a biotin protein, allowing regeneration of acetyl-ACP for activation of malonate (Berg and Dimroth, 1998). Subsequently, a membrane-bound decarboxylase couples the decarboxylation of carboxybiotin to the outward translocation of 2 Na^+ ions. In this reaction, one Na^+ is transported electroneutrally and one Na^+ is translocated electrogenically. The electrogenic export of Na^+ ions can be used for ATP synthesis. During malonate fermentation in *M. rubra*, around three decarboxylation reactions are necessary to synthetize 1 mol of ATP (Dimroth and von Ballmoos, 2007).

Electron transfer between redox cofactors

Transfer of electrons between NAD^+/NADH and $ferredoxin_{ox}$/$ferredoxin_{red}$. The NAD^+/NADH redox couple is generally kept in an oxidized form, and therefore the E' is usually higher than the E_m'. The actual value depends on organism and growth conditions and may vary between −310 and −240 mV (20). Ferredoxins are a class of redox cofactor with a wide range of redox potentials. The ones used in fermentative metabolism have a lower redox potential than NAD^+/NADH. eQuilibrator uses an E_m' for $ferredoxin_{ox}$/$ferredoxin_{red}$ of −418 mV. The difference in redox potential between both redox cofactors is sufficient to translocate protons or sodium ions over the cell membrane and as such contribute to the formation of an IMF, which in turn can be used to drive ADP phosphorylation by ATP synthase. The Rnf complex most likely translocates one H^+ or Na^+ per electron (Buckel and Thauer, 2018). Association of Bcd/Etf complex and Rnf complex to produce ATP (see Fig. 6B) has been demonstrated during ethanol-acetate fermentation by *Clostridium kluyveri* (PMF) (Li *et al*., 2008; Seedorf *et al*., 2008) and during L-glutamate fermentation to butyrate and acetate in *Clostridium tetanomorphum* and *Acidaminococcus fermentans* (Na^+ ion-motive force) (Boiangiu *et al*., 2005; Herrmann *et al*., 2008; Jayamani and Buckel, 2008; Chowdhury *et al*., 2016). For more details, see Buckel and Thauer (2013) and Buckel and Thauer (2018) and references therein.

Transfer of electrons between NAD^+/NADH and $NADP^+$/NADPH. Although NAD^+/NADH and $NADP^+$/NADPH

have identical E_0' values, their actual redox potentials differ considerably. NAD^+/NADH is kept in the oxidized state to perform oxidation reactions for catabolic and anabolic purposes while $NADP^+$/NADPH is kept in the reduced state to perform reduction reactions (Spaans *et al*., 2015; Weusthuis *et al*., 2020) in biosynthetic pathways. Values reported for NAD^+/NADH ratios range from 3.74 to 1820, whereas $NADP^+$/NADPH ratios range from 0.017 to 0.95 (Spaans *et al*., 2015). At the mentioned extreme ratios, the redox potential difference between both cofactors is 149 mV (–240 mV for NAD^+/NADH and –389 mV for $NADP^+$/NADPH, which in principle is sufficient to contribute to the build-up of an IMF. Several reactions of central metabolism are able to generate NADPH (Table 7). Their redox potentials are lower than the 100–150 mV of the membrane potential, and therefore in principal low enough to contribute to the generation of IMF.

Many anaerobic bacteria such as *Clostridium kluyveri* and *Moorella thermoacetica* can transfer electrons from NADPH to oxidized ferredoxin and NAD^+ via an NAD-dependent ferredoxin NADPH oxidoreductase (Nfn). This enzyme couples the reversible reduction of 2 ferredoxin with 2 NADPH to the reduction of 1 NAD^+ (Wang *et al*., 2010). The reduced ferredoxin can subsequently be used by the Rnf complex to generate NADH and an electrochemical Na^+ ion gradient over the membrane. Such a mechanism leads to the overall conversion of 2 NADPH and 2 NAD^+ into 2 $NADP^+$, 2 NADH and the translocation of 2 Na^+ ions.

Microorganisms can use a membrane-bound proton-translocating transhydrogenase to transfer electrons from NADH to NADPH. The enzyme uses the electrochemical proton gradient across the membrane to drive the following reaction: $NADH + NADP^+ + H^+_{out} = NAD^+ + NADPH + H^+_{in}$. The reaction was shown to be reversible *in vitro* (Van de Stadt *et al*., 1971; Earle and Fisher, 1980; Vandock *et al*., 2011). The reversed *in vivo* action of the transhydrogenase would result in proton translocation over the cytoplasmic membrane which could in turn be used for ATP formation via ATP synthase. Nonetheless, this mechanism for energy conservation is purely theoretical, it has not been observed yet.

Table 7. Reactions involved in NADPH regeneration.

Reactions	Enzymes	E_0' (mV)
Pyruvate + CO_2 + 2 e^- = Malate	Malic enzyme	–379 ± 10
Ribose-5-phosphate + CO_2 + 2 e^- = 6-phosphogluconate	6-phosphogluconate dehydrogenase	–404 ± 12
2-oxoglutarate + CO_2 + 2 e^- = Isocitrate	Isocitrate dehydrogenase	–419 ± 10
6-phosphate	phosphogluconate + 2 e^- = Glucose-6-phosphate + H_2O dehydrogenase	Glucose-6- –458 ± 11
3-phosphoglycerate + 2 e^- = D-glyceraldehyde-3-phosphate	Glyceraldehyde-3-phosphate dehydrogenase (non-phosphorylating)	–425 ± 7
Pyruvate + CoA + 2 e^- = Acetyl-CoA + CO_2	Pyruvate dehydrogenase ($NADP^+$- dependent)	–541 ± 16

End-product efflux

The group of Konings (Otto *et al*., 1980; Otto *et al*., 1982; Konings, 1985; ten Brink *et al*., 1985) has shown that *Streptococcus cremoris* is able to generate an IMF by end-product efflux. The gradient of the end-product lactic acid over the plasma membrane was used as driving force by means of a lactate-proton symporter. Consequentially, the intracellular lactate concentration has to be larger than the extracellular concentration. Van Maris *et al*. (2004) calculated that the intracellular concentration has to be approximately thousand times higher at pH 7, and about a million times higher at pH 2, to drive the translocation of one proton per lactate ion. This shows that although build-up of an IMF by means of product efflux may have benefits in natural habitats with low product concentration, this mechanism seems irrelevant for industrial application at high product concentrations.

ATPase: converting IMF into ATP and vice versa

Microorganisms with a fermentative metabolism can convert ATP into IMF and vice versa by means of membrane-bound ATPases. Based on structure and physiological role, these ion-pumping ATPases can be divided into three categories, the rotary F-type and V-type ATPases that consist of multiple subunits and the much simpler P-type ATPases. Structurally, the F-type and V-type ATPases have several similarities and may share a common evolutionary origin with archaeal (A-type) ATPases (Grüber *et al*., 2001). Their function, in general, is however opposite. F-type ATPases, found in eukaryotes and prokaryotes, mostly produce ATP. In contrast, the V-type ATPases, mainly located in organellar membranes such as the vacuolar membrane, couple ATP hydrolysis to proton pumping across the membrane (Beyenbach and Wieczorek, 2006). Similarly, the P-type plasma membrane H^+/ATPase of plants and fungi is mostly involved in proton extrusion at the expense of ATP (recently reviewed by Palmgren and Morsomme (2019)). As such, the V-type and plasma membrane P-type H^+/ATPases are mostly involved in generation of IMF used to transport substrates or ions over the plasma or organellar membrane, respectively (Russnak *et al*.,

2001; Beyenbach and Wieczorek, 2006; Cyert and Philpott, 2013; Deprez *et al*., 2018). Together they are vital for the regulation of intracellular and intra-organellar pH (Deprez *et al*., 2018). Seen the importance of the V- and P-type ATPase activity for several biological processes in eukaryotes, there seems little room for engineering opportunities with respect to energy conservation. The biological role of the F-type ATPases is, as mentioned before, overall different and mostly in ATP synthesis from IMF. These ATPases are found in the plasma membrane of prokaryotes, the inner-mitochondrial membrane or thylakoid membranes. The F_0F_1-types H^+/ATPases of prokaryotes were recently reviewed by Neupane *et al*. (2019). The H^+/ATP ratio is of importance, as it shows how efficiently both forms of energy can be interconverted. The H^+/ATP stoichiometries of the F_0F_1-ATPase in *E. coli* are between 3 and 4 (Jiang *et al*., 2001; Arechaga *et al*., 2002). For yeasts and chloroplasts, the H^+/ATP rate was found to differ with nearly one proton per ATP: for yeast mitochondrial F-ATPase the ratio was 3, for spinach chloroplasts this ratio was 4 (Petersen *et al*., 2012). Such differences in ratio for F-type ATP synthases are mostly attributed to differences in subunit stoichiometry and may offer an opportunity to alter metabolic energy conservation from IMF (Tomashek and Brusilow, 2000; Petersen *et al*., 2012). For V-type ATPases, the H^+/ATP ratio is established at 2 protons extruded per ATP hydrolysed (Grabe *et al*., 2000; Tomashek and Brusilow, 2000) whereas the ratio for plasma membrane P-type H^+/ATPase is even lower at 1 (Serrano, 1991; Burgstaller, 1997). These lower H^+/ATPase ratios are directly coupled to their biological function as proton extrusion mechanisms against a chemical gradient.

Relationship between redox potential and energy generation

The redox potentials of most redox couples mentioned in this manuscript are plotted in Fig. 7 and – as such – offer the opportunity to reflect on SLP and IMF build-up based on redox potentials. The redox couples can be subdivided into several chemical categories based on their redox potential.

The oxidative decarboxylation of 2-oxoacids – pyruvate and 2-oxoglutarate to acetate and succinate respectively – represent the redox couples with the lowest redox potentials. The redox potential difference with NAD^+/NADH is larger than the 224 mV required for SLP. SLP is realized by binding CoA, the exchange of CoA with inorganic phosphate (Pi) and the concomitant phosphorylation of ADP (See Substrate-level phosphorylation (SLP). Even when the reactions are combined with binding CoA, for instance, oxidizing pyruvate and 2-oxoglutarate to acetyl-CoA and succinyl-CoA respectively, there is still a sufficient redox potential difference with NAD^+/NADH. The difference may not be enough to support SLP, but certainly sufficient to potentially contribute to the build-up of an IMF. An example is the fermentation of L-glutamate (Herrmann *et al*., 2008). The reduced ferredoxin formed during this process can be used to reduce NAD^+ and generate a Na^+ ion-motive force. Recently, Orsi *et al*. (2020) inferred that Rnf is involved at high substrate concentration while it is not necessary at concentrations below 1 mM as the reaction becomes more exergonic.

The next group represents the organic acid/aldehyde redox couples. The ones with the lowest redox potential – acetate/acetaldehyde, butyrate/butyraldehyde and 3-phosphoglycerate/glyceraldehyde-3-phosphate – support SLP. When these oxidations involve binding CoA or Pi, so resulting in the formation of acetyl-CoA, butyryl-CoA or 1,3-bisphosphoglycerate respectively, the redox potential difference with NAD^+/NADH becomes too small to contribute to the generation of additional IMF. The redox potentials of gluconate/glucose and 6-phosphogluconate/glucose-6-phosphate are relatively high for this class of redox couples and these therefore not contribute to either SLP or the build-up of IMF but can reduce $NADP^+$.

The next group represents the oxidative decarboxylation of organic acids without an oxo-group on the 2-carbon position. The redox potential difference with NAD^+/NADH is not enough to support SLP. The redox potential is however low enough to regenerate NADPH.

The redox cofactors NAD^+/NADH, $NADP^+$/NADPH and Fd_{ox}/Fd_{red} represent a group with intermediate redox potentials. The redox potential difference between Fd_{ox}/Fd_{red} and NAD^+/NADH is sufficient to contribute to generating an IMF. The redox potential difference between $NADP^+$/NADPH and NAD^+/NADH could in theory be sufficient to support IMF generation, but this has not been shown yet (See section 5).

The groups representing the reduction of aldehydes to alcohols and 2-oxoacids to amino acids are able to receive electrons from NAD^+/NADH. The redox potential difference E_m' of some redox couples with NAD^+/NADH seems to be large enough to support the build-up of an IMF but, in reality, this does – as far as we know – not occur. This may be caused by the fact that the alcohols and amino acids are often end products at high concentrations, which rises the redox potential.

The reduction of carbon-carbon double bonds is the class with the highest redox potential. The E_m' difference with the redox cofactors is sufficient to support SLP, but this is not described in literature. Instead, the transfer of electrons from NAD^+/NADH to this class of redox couples is used to contribute to IMF build-up.

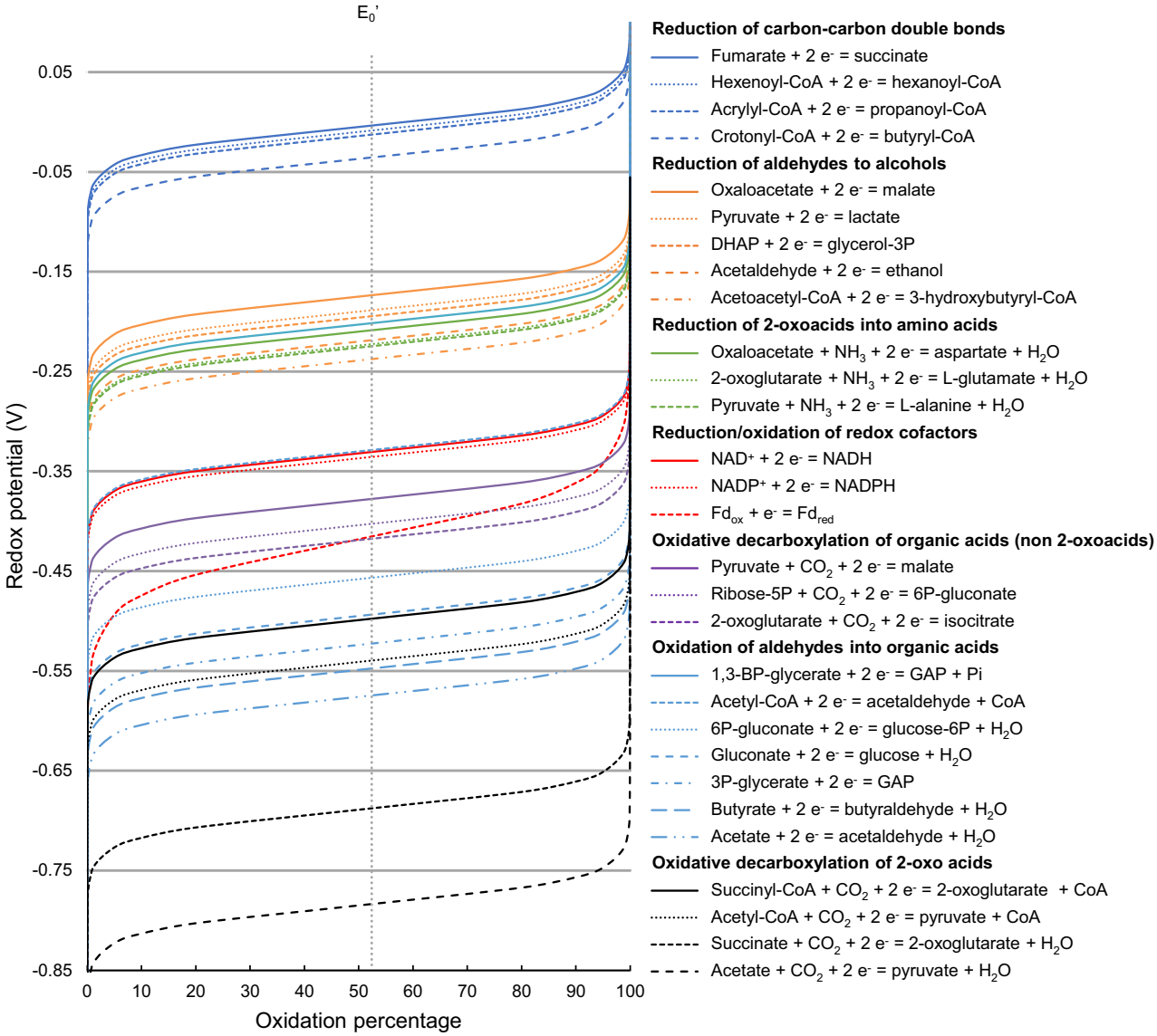


Fig. 7. Redox potential profile of various couples as a function of the oxidation percentage. The colours depict the type of chemical reaction in the redox couples. Dark blue: reduction of carbon-carbon double bonds, orange: reduction of aldehydes to alcohols, green: reduction of 2-oxo acids into amino acids, red: reduction and oxidation of redox cofactors, purple: oxidative decarboxylation of organic acids, light blue: oxidation of aldehydes into organic acids and black: oxidative decarboxylation of 2-oxo acids. The graph is limited to a maximum redox potential of 0.1 V since redox couples with higher potentials are involved in respiration. The values were calculated using eQuilibrator 2.2.

These observations with respect to redox potential of classes of redox couples and the mechanism of energy conservation they support are a valuable tool for the design of metabolic networks for product formation.

Energy-saving systems

Increasing energy efficiency of cells can be achieved by implementing energy-conserving reactions as mentioned in SLP and IMF sections. On the other hand, preventing loss of energy during energy-requiring processes is equally important. Energy-intensive reactions are, for instance, binding of CoA and Pi, carboxylation reactions and transport of solutes inside and outside the cells.

Preventing energy input for binding of CoA and CO_2

The energy released during hydrolysis of CoA and Pi bonds can be used for ATP formation by the action of a kinase. However, creation of such bonds requires the input of energy. For instance, conversions of acetate to acetyl-CoA and propionate to propionyl-CoA have a very

high ΔG_m' of 47 $\pm$ 1 and 58 $\pm$ 6 kJ mol^{-1}, respectively, and therefore require the input of ATP. Preventing energy input to bind CoA is a viable strategy to increase energy efficiency in cells. Enzymes such as CoA-transferases can be implemented in the product pathway to conserve energy.

Saving energy input for CoA binding using CoA-transferases. CoA-transferases can limit the energy input as they catalyse the reversible transfer of a CoA moiety from an acyl-CoA thioester to a free carboxylic acid. These reactions do not require any cofactor nor activation of the carboxylic acid. CoA-transferases are usually divided in three classes based on substrate specificity, acyl transfer mechanisms and sequences. The Class I CoA-transferases consist of enzymes found primarily in fatty acid metabolism that act on 3-oxo acids, short-chain fatty acids and (*E*)-gluconate and use succinyl-CoA and acetyl-CoA as primary CoA donors. The Class II only comprises two enzymes: acetyl-CoA: citrate CoA-transferase (EC 2.8.3.10) and acetyl-CoA: citramalate CoA-transferase (EC 2.8.3.11). These enzymes are the homodimeric α subunits of citrate and citramalate lyases (EC 4.1.3.6 and EC 4.1.3.22), respectively. The Class III enzymes transfer CoA in a highly substrate-, stereo-specific manner. They are found in the 3-hydroxypropionate cycle of CO_2 fixation and in the metabolism of oxalate, toluene, carnitine and other aromatic compounds. A non-exhaustive list of the reactions catalysed by CoA-transferases is given in Table 8. These enzymes are able to catalyse more reactions than given in the table with various levels of activities. More details can be found in the Brenda database based on the EC number of the enzymes. Protein engineering can be employed to increase activities of enzymes towards certain reactions and also low activities are therefore of interest for cell factory design.

Implementation of CoA-transferases in product pathways has been used to increase product titre, rate and yield (Yang *et al*., 2010; Lee *et al*., 2012; Deng *et al*., 2015; Wang *et al*., 2015b; Yu *et al*., 2015; Chen *et al*., 2018). Deng *et al*. (2015) replaced the native butyryl-CoA:acetate CoA-transferase of *Thermobifida fusca* by an exogenous one with higher activity to increase butyrate titres. Yang *et al*. (2010) successfully engineered *E. coli* to produce polylactic acid (PLA) by expressing a heterologous pathway containing propionyl-CoA-transferase from *Clostridium propionicum* and polyhydroxyalkanoate (PHA) synthase 1 from *Pseudomonas* sp. MBEL6-19. The enzyme activities were further enhanced by random and directed mutagenesis.

Some CoA-transferases have been shown to transfer CoA to a wide range of substrates from various CoA donors *in vitro*. These enzymes could therefore be used to increase pathway yield and conserve energy for other reactions than the ones they perform *in vivo*. Nevertheless, CoA-transferases show different levels of activity depending on the substrates used. Mutagenesis and adaptive laboratory evolution (ALE) could be used to increase their expression and activity.

Preventing energy input for binding CO_2 to increase energy efficiency of microorganisms – examples of fatty acid synthesis. Fatty acid synthesis (Fig. 8A) from sugars is thermodynamically feasible without energy input as indicated by a ΔG_0' of the conversion of glucose into dodecanoate of -856 ± 31 kJ mol^{-1} dodecanoate. However, at a metabolic level ATP input is required. Glycolysis yields one ATP per fatty acid elongation, but the activation of acetyl-CoA to malonyl-CoA and the conversion of NADH produced in glycolysis to NADPH used in fatty acids synthesis requires a total input of 2 ATP. The breakdown pathway of fatty acids, β-oxidation, is very similar to the fatty acid synthesis pathway (Fig. 8B). The algae *Euglena gracilis* is able to reverse this β-oxidation pathway to convert paramylon into wax esters, so in the fatty acid synthesis direction. It is able to do so because it uses an NADH-dependent enoyl-CoA reductase instead of a combination of acyl-CoA dehydrogenase and Etf. This reverse β-oxidation pathway is able to use acetyl-CoA instead of malonyl-CoA, bypassing an ATP-requiring carboxylation step (Inui *et al*., 1984) (Fig. 8C). Dellomonaco *et al*. (2011) have applied reverse β-oxidation successfully in *E. coli* and production of fatty acids has improved over the last years (Mehrer *et al*., 2018). In general, this shows that biosynthetic pathways, like the fatty acid synthesis, are not necessarily operating at the highest energetic efficiency, and that more efficient pathways can be designed for product formation.

Energy conservation by transcarboxylation

Carboxylation reactions are often considered bottlenecks in metabolic pathways as they require energy input. This is of particular importance in CO_2-fixing bacteria such as Clostridia. Most of these reactions are coupled – directly or indirectly – to ATP hydrolysis, which provides the energy necessary for the carboxylation step (Bar-Even *et al*., 2012). The energy loss during such conversions can be avoided using transcarboxylases.

Transcarboxylases or carboxyl transferases transfer a carboxyl group between compounds, allowing the reactions to run near equilibrium and preventing high-energy input. Replacing carboxylases by transcarboxylases would reduce the energetic cost of such reaction and allow microorganisms to dedicate a higher amount of

Table 8. Non-exhaustive list of reactions catalysed by CoA-transferases. More substrates have been tested for the different CoA-transferases with different level of activities. For more information about the substrates that have been tested with the different CoA-transferases, see Brenda database.

EC numbers	Enzymes	Reactions
2.8.3.1	Propionate CoA-transferase	Acetyl-CoA + Propionate = Acetate + Propionyl-CoA Propionyl-CoA + (*R*)-lactate = Propionate + (*R*)-lactoyl-CoA Propionyl-CoA + (*S*)-lactate = Propionate + (*S*)-lactoyl-CoA Butyryl-CoA + Acetate = Butyrate + Acetyl-CoA Acetyl-CoA + (*S*)-lactate = Acetate + (*S*)-lactoyl-CoA Acetyl-CoA + (*R*)-lactate = Acetate + (*R*)-lactoyl-CoA
2.8.3.10	Citrate CoA-transferase	Acetyl-CoA + Citrate = Acetate + (3*S*)-citryl-CoA Acetyl-dephospho-CoA + Citrate = Acetate + (3*S*)-citryl-dephospho-CoA Acetyl-[acyl-carrier protein] + Citrate = Acetate + (3*S*)-citryl-[acyl-carrier protein]
2.8.3.11	Citramalate CoA-transferase	Acetyl-CoA + (*S*)-citramalate = Acetate + (*S*)-citramalyl-CoA Acetyl-[acyl-carrier protein] + Citramalate = Acetate + Citramalyl-[acyl-carrier protein] Succinate + (3*S*)-citramalyl-CoA = Succinyl-CoA + citramalate
2.8.3.12	Glutaconate CoA-transferase	Acetyl-CoA + *trans*-gluconate = Acetate + (2*E*)-glutaconyl-CoA Acetyl-CoA + Glutarate = Acetate + Glutaryl-CoA Acetyl-CoA + (*R*)-2-hydroxyglutarate = Acetate + (*R*)-2-hydroxyglutaryl-CoA Acetyl-CoA + Propenoate = Acetate + Propenoyl-CoA Acetyl-CoA + Propionate = Acetate + Propionyl-CoA
2.8.3.13	Succinate-hydroxymethylglutarate CoA-transferase	Succinyl-CoA + 3-hydroxy-3-methylglutarate = Succinate + (3*S*)-hydroxy-3-methylglutaryl-CoA Malonyl-CoA + 3-hydroxy-3-methylglutarate = Malonate + (3*S*)-hydroxy-3-methylglutaryl-CoA
2.8.3.14	5-hydroxypentanoate CoA-transferase	Acetyl-CoA + 5-hydroxypentanoate = Acetate + 5-hydroxy-pentanoyl-CoA
2.8.3.15	Succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase	Succinyl-CoA + (*R*)-2-benzylsuccinate = Succinate + (*R*)-2-benzylsuccinyl-CoA
2.8.3.16	Formyl-CoA-transferase	Formyl-CoA + Oxalate = Formate + Oxalyl-CoA Formyl-CoA + Succinate = Formate + Succinyl-CoA
2.8.3.17	Cinnamoyl-CoA:phenyllactate CoA-transferase	(*E*)-cinnamoyl-CoA + (*R*)-3-phenyllactate = *trans*-cinnamate + (*R*)-3-phenoyllactoyl-CoA (2*R*)-2-hydroxy-3-(4-hydroxyphenyl)propionate + (*E*)-4-coumaroyl-CoA = *trans*-4-coumarate + (*R*)-3-(4-hydroxyphenyl)lactoyl-CoA (*E*)-3-(indol-3-yl)acryloyl-CoA + (*R*)-3-(indol-3-yl)lactate = (*E*)-3-(indol-3-yl)acrylate + (*R*)-3-(indol-3-yl)lactoyl-CoA (*E*)-cinnamoyl-CoA + 3-phenylpropionate = (*E*)-cinnamate + 3-phenoylpropionate
2.8.3.18	Succinyl-CoA:acetate CoA-transferase	Succinyl-CoA + Acetate = Succinate + Acetyl-CoA
2.8.3.19	CoA:oxalate CoA-transferase	Acetyl-CoA + Oxalate = Acetate + Oxalyl-CoA Formyl-CoA + Acetate = Formate + Acetyl-CoA Formyl-CoA + Oxalate = Formate + Oxalyl-CoA
2.8.3.2	Oxaloate CoA-transferase	Succinyl-CoA + Oxalate = Succinate + Oxalyl-CoA
2.8.3.20	Succinyl-CoA-D-citramalate CoA-transferase	Succinyl-CoA + (3*R*)-citramalate = Succinate + (3*R*)-citramalyl-CoA Succinyl-CoA + (*R*)-malate = Succinate + (*R*)-malyl-CoA Succinyl-CoA + Itaconate = Succinate + Itaconyl-CoA
2.8.3.21	L-carnitine CoA-transferase	γ-butyrobetainyl-CoA + (*R*)-carnitine = 4-(trimethylamino)butyrate + (*R*)-carnitinyl-CoA Crotonobetainyl-CoA + (*R*)-carnitine = Crotonobetaine + (*R*)-carnitinyl-CoA
2.8.3.22	Succinyl-CoA-L-malate CoA-transferase	Succinyl-CoA + (*S*)-malate = Succinate + (*S*)-malyl-CoA Succinyl-CoA + Itaconate = Succinate + Itaconate Succinyl-CoA + (3*S*)-citramalate = Succinate + (3*S*)-citramalyl-CoA
2.8.3.23	Caffeate CoA-transferase	Hydrocaffeyl-CoA + (*E*)-caffeate = 3-(3,4-dihydroxyphenyl)propionate + (*E*)-caffeyl-CoA Hydrocaffeyl-CoA + 4-coumarate = 3-(3,4-dihydroxyphenyl)propionate + 4-coumaroyl-CoA Hydrocaffeyl-CoA + Ferulate = 3-(3,4-dihydroxyphenyl)propionate + Feruloyl-CoA
2.8.3.24	(*R*)-2-hydroxy-4-methylpentanoate CoA-transferase	4-methylpentanoyl-CoA + (*R*)-2-hydroxy-4-methylpentanoate = 4-methylpentanoate + (*R*)-2-hydroxy-4-methylpentanoyl-CoA
2.8.3.25	Bile acid CoA-transferase	Lithocholoyl-CoA + Cholate = Litocholate + Choloyl-CoA Deoxycholoyl-CoA + Cholate = Deoxycholate + Choloyl-CoA
2.8.3.3	Malonate CoA-transferase	Acetyl-CoA + Malonate + Acetate + Malonyl-CoA

Table 8. (Continued)

EC numbers	Enzymes	Reactions
2.8.3.5	3-oxoacid CoA-transferase	Succinyl-CoA + a 3-oxoacid = Succinate + a 3-oxoacyl-CoA Succinyl-CoA + Acetoacetate = Succinate + Acetoacetyl-CoA Succinyl-CoA + 3-oxopropionate = Succinate + 3-oxopropionyl-CoA Succinyl-CoA + 3-oxopentanoate = Succinate + 3-oxopentanoyl-CoA Succinyl-CoA + 3-oxo-4-methylpentanoate = Succinate + 3-oxo-4-methylpentanoyl-CoA Succinyl-CoA + 3-oxohexanoate = Succinate + 3-oxohexanoyl-CoA
2.8.3.6	3-oxoadipate CoA-transferase	Succinyl-CoA + 3-oxoadipate = Succinate + 3-oxoadipyl-CoA
2.8.3.8	Acetate CoA-transferase	Acyl-CoA + Acetate = a fatty acid anion + Acetyl-CoA Butyryl-CoA + Acetate = Butyrate + Acetyl-CoA Pentanoyl-CoA + Acetate = Pentanoate + Acetyl-CoA Succinate + Acetyl-CoA = Succinyl-CoA + Acetate
2.8.3.9	Butyrate-acetoacetate CoA-transferase	Butyryl-CoA + Acetoacetate = Butyrate + Acetoacetyl-CoA
2.8.3.B1	(*R*)-2-hydroxyisocaproate CoA-transferase	4-methylpent-2-enoyl-CoA + (*R*)-2-hydroxy-4-methylpentanoate = 4-methylpent-2-enoate + (*R*)-2-hydroxy-4-methylpentanoyl-CoA
2.8.3.B3	Mesaconate CoA-transferase	Succinyl-CoA + Mesaconate = Succinate + 2-methylfumaryl-CoA

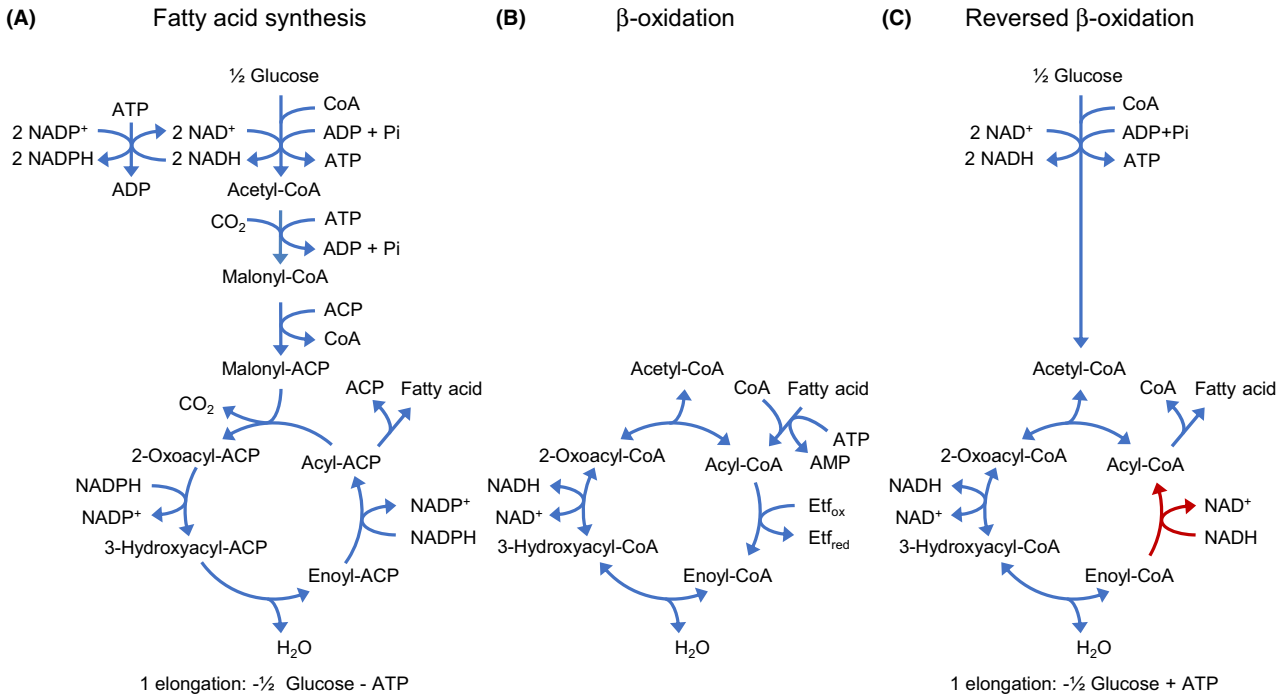


Fig. 8. (A) Fatty acid chain elongation by the fatty acid synthesis pathway requires ATP input in the conversion of acetyl-CoA into malonyl-CoA and for the upgrade of NADH produced in glycolysis to NADPH required for fatty acid synthesis. B. The β-oxidation pathway is chemically very similar. The acyl-CoA dehydrogenase/Etf determine the direction of the cycle towards fatty acid breakdown. C. Reversal of the β-oxidation pathway is possible by introducing an NADH-dependent trans-enoyl-CoA reductase (red arrow) and results in a pathway that generates 1 ATP per chain elongation.

ATP for other purposes. Such an example is the fermentation of lactate to propionate.

Propionibacteria convert lactate into propionate and acetate. The conversion of lactate into acetate yields one ATP, but also results in net reduction of NAD^+. The conversion of lactate into propionate is used to regenerate NAD^+. The pathway used can conserve metabolic energy via proton translocation at the reduction of succinate to fumarate (see paragraph 5.1). It also requires the carboxylation of pyruvate to oxaloacetate and the decarboxylation of S-methyl-malonyl-CoA to propionyl-CoA. If these reactions would be performed

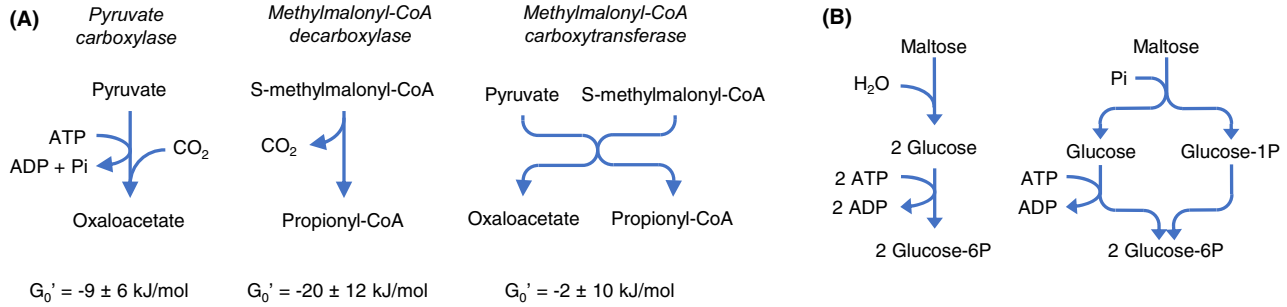


FIG. 9: *(A) Hydrolysis and phosphorylation of sugars. (B) Conversion of pyruvate to propionyl-CoA via either a combination of pyruvate decarboxylase and methylmalonyl-CoA decarboxylase or via methylmalonyl-CoA carboxytransferase.*

separately by pyruvate carboxylase (EC 6.4.1.1) and methylmalonyl-CoA decarboxylase (EC 7.2.4.3) this would require the input of ATP, severely reducing the overall energy yield (Fig. 9A). Instead, the carboxylation of pyruvate to oxaloacetate is coupled to the decarboxylation of S-methyl-malonyl-CoA to propionyl-CoA (Fig. 9 A). This transcarboxylation reaction, performed by methylmalonyl-CoA carboxytransferase (EC 2.1.3.1), has a ΔG_0' of -2 ± 10 kJ mol^{-1}, close to equilibrium. This enzyme has a relaxed specificity as it is also able to perform the following transcarboxylations: acetoacetyl-CoA + oxaloacetate = 3-oxoglutaryl-CoA + pyruvate; acetyl-CoA + oxaloacetate = malonyl-CoA + pyruvate and butyryl-CoA + oxaloacetate = ethylmalonyl-CoA + pyruvate (Swick and Wood, 1960). As far as we are aware this way of energy conservation has not been used to design efficient metabolic networks for product formation yet.

Preventing energy input for extracellular transport (nutrient uptake and product excretion)

Transport of substrates and products over the microbial plasma membrane is an integral part of fermentation processes. In many studies homologous and heterologous transporters have been expressed to modify substrate specificity, growth and product formation (Zaslavskaia *et al*., 2001; Hernández-Montalvo *et al*., 2003; Wieczorke *et al*., 2003; De Anda *et al*., 2006; Doebbe *et al*., 2007; Subtil and Boles, 2011; Young *et al*., 2011; Wang *et al*., 2015a; Shin *et al*., 2018). Several transport mechanisms are available which differ with respect to the requirement of metabolic energy input (Jahreis *et al*., 2008). Equipping the microbial cell factories with energy-independent transport systems is evidently important if fermentation processes have a negative ΔG', but the metabolic network is unable to harvest this energy.

Substrate influx. The substrate concentration in fermentation processes can be controlled by applying fed-batch or chemostat cultivation and by limiting concentrations of nutrients other than the carbon source. The substrate concentration can then be high enough to use its concentration gradient over the membrane as driving force. Input of metabolic energy is in such case not necessary.

Proton and Na^+ symporters use the ion-motive force to drive solute transport. Weusthuis *et al*. (1993) determined the ATP costs of proton symport of maltose in *S. cerevisiae*, in comparison with glucose transported by means of facilitated diffusion. Proton symport required the input of 1 ATP per maltose. ATP-binding-cassette transporters (ABC transporters) also require the input of ATP. ATP/substrate stoichiometries from 1 to 50 have been reported (Patzlaff *et al*., 2003). Both mechanisms are therefore able to drive solute translocation against the concentration gradient, but at the expense of metabolic energy.

Facilitated sugar transporters (Barrett *et al*., 1999; Jahreis *et al*., 2008; Leandro *et al*., 2011) use the concentration gradient of the solute over the plasma membrane as driving force and therefore do not require the input of metabolic energy. Phosphoenolpyruvate:sugar phosphotransferase systems (PTS) use the energy released in the conversion of phosphoenolpyruvate to pyruvate to phosphorylate sugars and simultaneously import the sugar (Jahreis *et al*., 2008). PTS systems are classified as active transport systems (Saier, 1977; Jeckelmann and Erni, 2019), but do not require energy that would be otherwise available for metabolism. Instead, energy that would be dissipated as heat is used to transport the sugar.

Facilitated sugar transporters and PTS are therefore the mechanisms of choice to engineer fermentation processes with net ATP output. Because PTS systems require the conversion of phosphoenolpyruvate into pyruvate, their application for products relying on phosphoenolpyruvate but not pyruvate is limited (Floras *et al*., 1996; Hernández-Montalvo *et al*., 2003; Nakamura and Whited, 2003; De Anda *et al*., 2006; Shin *et al*., 2018;

Yang *et al*., 2018). Sugar facilitator transporters have been identified in mammals, yeasts and bacteria (Wieczorke *et al*., 2003; Jahreis *et al*., 2008; Leandro *et al*., 2011). Several groups have successfully expressed the glucose facilitator of *Zymomonas mobilis* in *E. coli* strains (Snoep *et al*., 1994; Parker *et al*., 1995; Weisser *et al*., 1995) and human glucose facilitators in *S. cerevisiae* (Wieczorke *et al*., 2003).

PTS systems occur in eubacteria, a few archaebacteria but not in plants and animals (Jeckelmann and Erni, 2019). They consist of two cytoplasmic phosphotransferase proteins (EI and HPr) and a variable number of sugar specific enzyme II complexes. Thompson *et al*. (2001) and Pikis *et al*. (2006) have introduced homologous enzymes II proteins to change the substrate specificity of *Klebsiella pneumoniae* and *E. coli*, respectively.

Product efflux. Efflux systems in bacteria and their metabolic engineering applications have recently been reviewed by Jones *et al*. (2015) and Kell *et al*. (2015). They play a critical role in alleviating feedback inhibition and product toxicity. Overexpression of the transporters generally results in titre improvements (Jones *et al*., 2015), also in fungi (Steiger *et al*., 2019). Most of these studies however cover aerobic production systems in which sufficient metabolic energy is available to use active transport systems. The role of efflux systems in fermentative metabolism has received less attention.

The selling price of a product is inversely correlated with the final titre of a product (Hoek *et al*., 2003). Fermentation processes often aim at the production of low value, bulk products. The desired final titre is therefore high and is generally between 50 and 200 g l^{-1}. This implies that the intracellular concentration is even higher when facilitated diffusion is used. Active forms of transport will be able to maintain lower intracellular concentrations and as such could alleviate product inhibition and toxicity. To date however identification of all exporters involved in organic acids excretion remains challenging as illustrated by the work of Mans *et al*. (2017) on lactic acid excretion in yeasts.

Table 1 shows that in natural fermentation processes in general 1 ATP is generated per mole of product. Consequently, product efflux has to rely on mechanisms that require substantially less than 1 mol ATP to transport 1 mol of product. Diffusion of small uncharged molecules or facilitated diffusion and symporters are therefore the mechanisms of choice.

The relationship between the input of Gibbs free energy and the gradient of product that can be reached over the plasma membrane has been assessed by van Maris *et al*. (2004) for the production of lactic acid and 3-hydroxypropionic acid in *S. cerevisiae*.

Using the energy available in glycosidic bonds

Disaccharides and oligosaccharides belong to the main sugar sources used in biotechnological applications. The $\Delta G_0'$ of the hydrolysis of the glycosidic bonds connecting the constituent monosaccharides is about –22 to –38 kJ/mol. Microorganisms like *Saccharomyces cerevisiae* and *E. coli* typically apply sugar hydrolases in order to use these carbon sources, and the $\Delta G'$ is dissipated as heat (Fig. 9B). The monosaccharides are subsequently phosphorylated, e.g. by hexokinase, glucokinase or the PTS system, requiring the input of ATP. Other microorganisms are able to use sugar phosphorylases: e.g. maltose is converted into glucose-1-P and glucose by incorporation of inorganic phosphate. This reaction has a $\Delta G_0'$ of -8 ± 2 kJ mol^{-1}. The glucose-1-phosphate is subsequently isomerized to glucose-6-phosphate by phosphoglucomutase (Fig. 9B). Consequently, the $\Delta G_0'$ available in the glycosidic bond is used to phosphorylate the sugar and as such reduces the input of ATP. This has been realized for cellobiose (Sadie *et al*., 2011; Ha *et al*., 2013), maltose (de Kok *et al*., 2011), and sucrose (Marques *et al*., 2018) in *Saccharomyces cerevisiae* as well as for maltodextrin and cellodextrin (Puchart, 2015). The increased energetic efficiency was reflected by higher biomass yields obtained under anaerobic conditions in the maltose and sucrose cases.

Pyrophosphate

Pyrophosphate (PPi) is released in the production of DNA, RNA, proteins, membrane lipids, etc. (Gutiérrez-Luna *et al*., 2018). It is concomitantly hydrolysed to inorganic phosphate by inorganic pyrophosphatase, rendering the PPi-releasing reactions virtually irreversible (Kornberg, 1957). The phosphate-phosphate bond of PPi is energy rich (Table 2). If product formation involves the release of PPi, conservation of this energy may proof to be beneficial.

Two types of inorganic pyrophosphatases have been recognized: soluble and membrane-bound versions (Gutiérrez-Luna *et al*., 2018). The membrane-bound versions translocate protons or Na^+ ions over the membrane, generating an IMF, and could as such contribute to conserving energy (Fig. 10A). Such a step is present during caffeate respiration in *A. woodii*. Prior being reduced, caffeate is first activated to caffeyl-CoA by AMP-, PPi-forming caffeyl-CoA synthetase (Hess *et al*., 2011). Then, PPi is hydrolysed by a membrane-bound pyrophosphatase which couples the hydrolysis to Na^+ ions translocation (Biegel and Müller, 2011).

In some microorganisms, PPi is a central energy carrier (Bielen *et al*., 2010). They harbour a pyrophosphate-

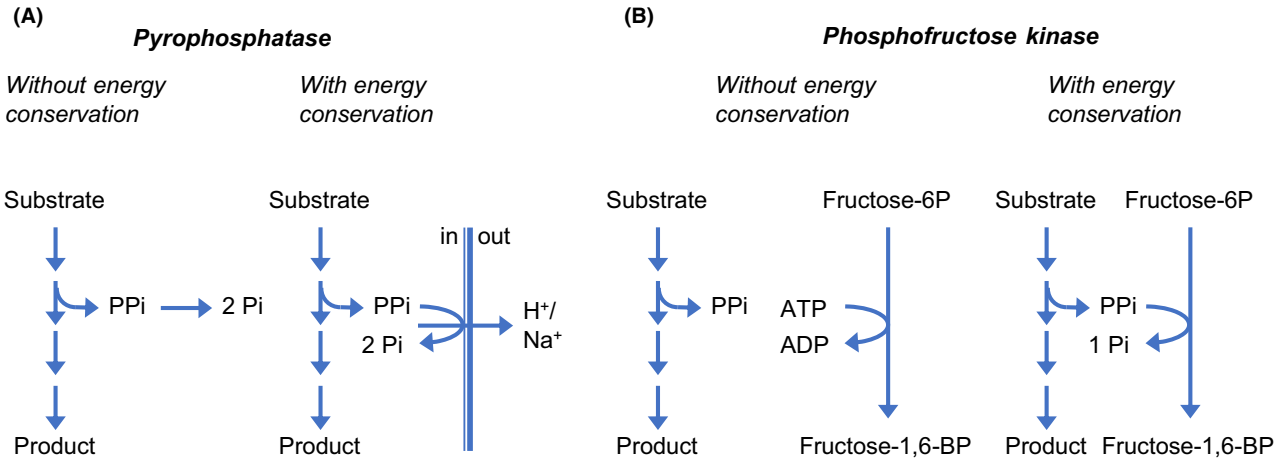


Fig. 10. Energy conservation using PPi hydrolysis by using (A) membrane-bound pyrophosphatases and (B) PPi-dependent phosphofructokinases.

dependent phosphofructokinase. This opens another energy-conserving option: if product formation involves PPi release, PPi can serve instead of ATP to phosphorylate fructose-6-phosphate (See Fig. 10B).

A third option is the reaction catalysed by pyruvate phosphate dikinase (PPDK), converting phosphoenolpyruvate and AMP with pyrophosphate into pyruvate and ATP (Cui *et al.*, 2020). Sufficient AMP should be present in the cell, in order to conserve the energy in PPi by the PPDK reaction. A way to generate this required amount of AMP is by employing the enzyme adenylate kinase (EC 2.7.4.3).

Consequently, the soluble pyrophosphatase activity must be low or absent in order for these two energy-conserving options to be successful.

Concluding remarks and future perspectives

Conservation of metabolic energy is one of the limiting factors during fermentative product formation. Here, we reviewed the mechanisms available in microorganisms to conserve energy as well as to reduce unnecessary energy expenditure. We distinguished general and product-specific methods to conserve metabolic energy. General methods like energy-independent transporters, mechanisms that use the difference in redox potential between redox couples or using disaccharides are the most interesting because they can be applied in many fermentation processes regardless the product of interest. Substrate-level phosphorylation is one of the main sources of energy under fermentative conditions. It is however usually a product-specific method (with the exception of the reaction catalysed by phosphorylating glyceraldehyde-3-phosphate dehydrogenase) and therefore cannot be applied in general. Some enzymes contributing to SLP have a broad substrate specificity *in vitro* and appear to be a good option. However, the thermodynamics of such reactions may not allow SLP to function under physiological conditions and should therefore be considered carefully.

The options to increase energy conservation described in this review should not be applied to maximize energy conservation *per se* but be used to optimize product formation. Just as too little energy conservation has detrimental effect on product formation, the same holds for too much energy conservation, as it moves the overall reaction to a thermodynamical equilibrium and may result in decreased product yield caused by excess biomass formation.

Nowadays, a lot of research is directed towards improving product titre, productivity and yield by either overexpressing enzymes involved in product pathways, knocking out competing pathways or using redox-neutral metabolic networks. However, little research is done on using metabolic energy in a more efficient way. Energy conservation is a means to achieve high titre, productivity and yield during microbial processes. Understanding and applying the various mechanisms available in microorganisms to conserve energy is therefore a key step towards improving fermentative product formation.

Conflict of interests

The authors declare that there are no conflicts of interest related to this work.

Author contributions

All authors contributed equally to the manuscript.

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