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How to make the reducing power of H_2 available for *in vivo* biosyntheses and biotransformations

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Solar-driven electrolysis enables sustainable production of molecular hydrogen (H_2), which represents a cheap and carbon-free reductant. Knallgas bacteria like *Ralstonia eutropha* are able to split H_2 to supply energy in form of ATP and NADH, which can be subsequently used to power reactions of interest. *R. eutropha* employs the Calvin-Benson-Bassham cycle for the fixation of CO_2, which is considered as an abundant and non-competing raw material. In this article, we summarize state-of-the-art approaches for H_2-driven biosyntheses using engineered *R. eutropha*. Furthermore, we describe strategies for synthetic H_2-driven NADH recycling. Major challenges for technical application and future perspectives are discussed.

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Introduction

Molecular hydrogen (H_2) is a carbon-free, clean and renewable alternative fuel in a world where sustainable energy carriers become increasingly important. Its energy density per weight is far higher than that of petrol, diesel or methane, and combustion of H_2 with O_2 results in the formation of only water [1]. Moreover, H_2 is widely used as reducing agent in chemical industry. In nature, a wide range of microorganisms exploit the reducing power of H_2 as an energy source — a trait that is of increasing importance for biotechnology. There is a particular interest in the utilization of (engineered) H_2-oxidizing biocatalysts for H_2-driven synthesis of biofuels as well as of fine chemicals and pharmaceutics, whose synthesis requires regioselective and stereroselective catalysts.

Basic metabolic configuration for microbial growth based on H_2, O_2, and CO_2

Knallgas bacteria are defined by their ability to gain energy from explosive H_2–O_2 gas mixtures [2]. While H_2 and O_2 serve as energy source and terminal electron acceptor, respectively, CO_2 is fixed via the Calvin-Benson-Bassham cycle to meet the cellular carbon requirements (Figure 1). The use of CO_2 as a raw material is attractive since it is abundant in the atmosphere and is fully detached from the food industry.

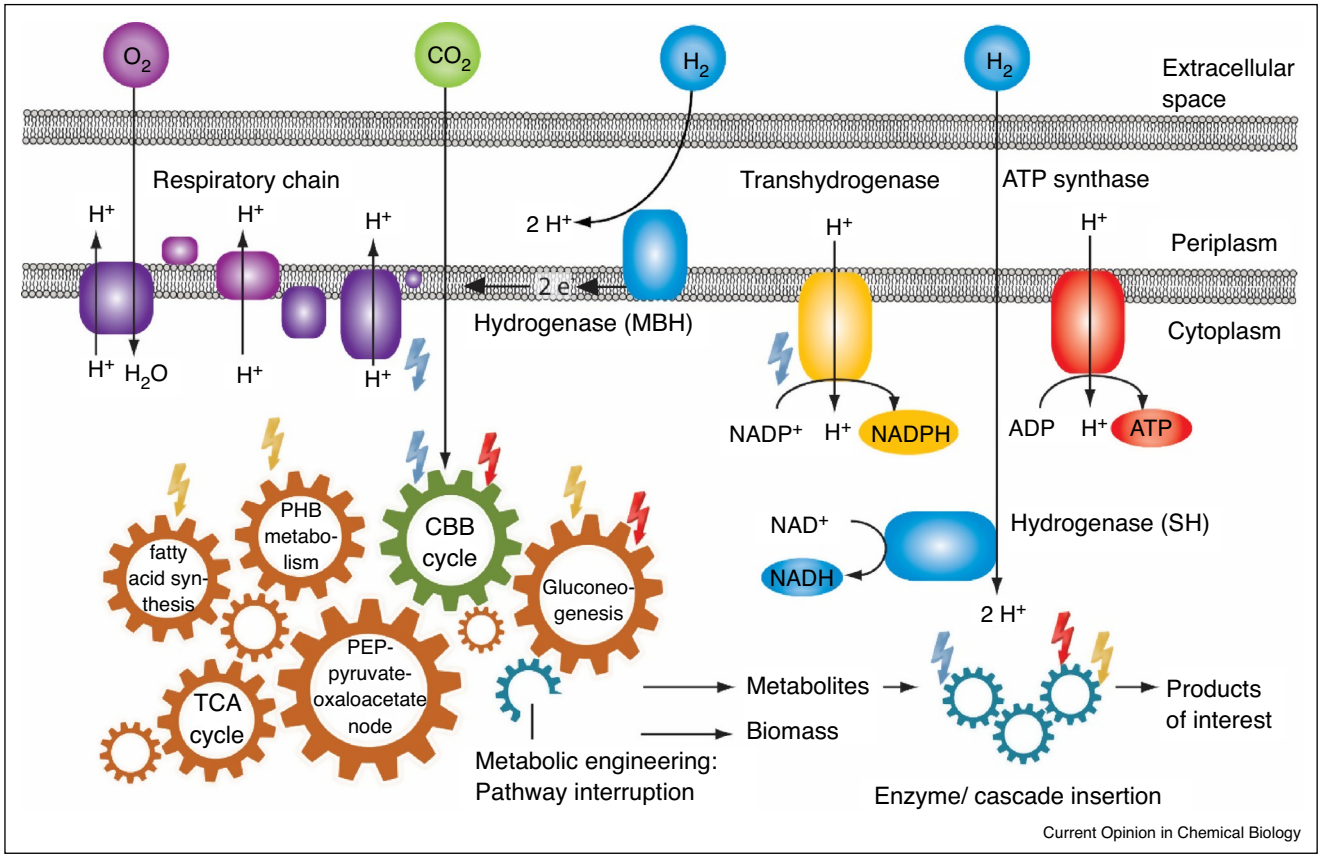
The most prominent Knallgas bacterium is probably *Ralstonia eutropha* H16 (also called *Cupriavidus necator*), which serves as model organism for the lithoautotrophic growth mode [2]. *R. eutropha* is able to grow to high cell densities and well known for its accumulation of polyhydroxyalkanoates, some of which are used as biodegradable bioplastics [3•,4•]. H_2 oxidation by *R. eutropha* is mediated by two independent energy-converting hydrogenases that split H_2 into two protons and two electrons [5]. A membrane-bound hydrogenase (MBH) delivers electrons from H_2 oxidation directly into the respiratory chain, and the resulting proton motive force is used for ATP synthesis [6]. A cytoplasmic, NAD^+-reducing hydrogenase (soluble hydrogenase, SH) couples H_2 oxidation directly with the reduction of the nicotinamide cofactor NAD^+ to NADH [7•,8]. The generated NADH is then used for CO_2 fixation and energy generation via NADH-quinone oxidoreductase (Complex I) and the other respiratory chain components (Figure 1) [9]. The phosphorylated congener of NAD(H), NAD(P)H is involved as redoxactive cofactor in mostly anabolic processes. During lithoautotrophic growth of *R. eutropha*, a transhydrogenase (PntAB) is upregulated, indicating that NADPH is generated from NADH under these conditions [10•].

A specialty of the *R. eutropha* hydrogenases is their ability to sustain catalytic activity in the presence of O_2 [5]. This is in contrast to most other hydrogenases, which are readily inactivated by O_2, and makes the Knallgas bacteria-derived hydrogenases particularly attractive for biotechnological applications.

H_2-driven regeneration of nicotinamide cofactors

In this chapter, we present the central concept for H_2-driven biocatalysis and summarize the progress that has been made with *R. eutropha* with regard to hydrogenase-supported *in vitro* syntheses of fine chemicals. For a more detailed review about H_2 driven *in vitro* cofactor regeneration we would like to refer to references [11•,12].

Figure 1



Energy conversion and main metabolic pathways that are running in *Ralstonia eutropha* during growth under lithoautotrophic conditions. The actual presence of the displayed enzymes and pathways is based on proteomic studies [10•]. Blue, yellow and red bolts indicate NADH, NADPH and ATP, respectively, which are necessary to drive the respective reactions. Blue gear-wheels indicate metabolic engineering requiring reducing equivalents in form of NAD(P)H and/or ATP for the reaction(s). Abbreviations: TCA, tricarboxylic acid cycle; CBB, Calvin-Benson-Bassham; PHB, polyhydroxybutyrate; PEP, 2-phosphoenolpyruvate.

Many industrial relevant oxidoreductases, such as P450 monooxygenases [13], require continuous supply of NAD(P)H, which serves as a hydride transfer agent resulting in the oxidized species, $NAD(P)^+$. To avoid stoichiometric addition of expensive NAD(P)H, an effective and reliable system to regenerate NAD(P)H from the oxidized species $NAD(P)^+$ is required. In general, hydrogenase-catalysed H_2 oxidation constitutes an elegant and atom-efficient approach for the regeneration of reduced cofactors in redox biocatalysis. In contrast to substrates generally used for NAD(P)H regeneration, which includes glucose, glycerol, isopropanol or organic acids [14], H_2 provides redox equivalents in a carbon-free and cheap manner, and no undesired by-products are formed upon oxidation [11•]. It is important to mention that upon ignition H_2 reacts efficiently with O_2, which can be a matter of concern. In air, H_2 has a flammability range of 4–74% and can be explosive at concentrations of 19–57% [15]. An undesired combustion, however, can be prevented when using either pure H_2 or gas mixtures with H_2 concentrations lower than 4%. Furthermore, H_2 has a low solubility in aqueous solutions. As the *R. eutropha* hydrogenases typically have a K_M for H_2 in the lower μM range [5], this usually does not present a problem.

The general applicability of SH for cofactor regeneration has already been demonstrated in several *in vitro* studies [16–18]. For example, the SH and a carbonyl reductase were coupled *in vitro* and enabled an almost quantitative conversion of acetophenone to phenylethanol using H_2 as reductant [17]. Future *in vitro* studies employing SH for cofactor regeneration in coupled reactions with purified monooxygenase or dioxygenase, which require O_2 as the co-substrate, will pave the way for a broader application of oxidoreductases in important industrial processes using H_2 as the reductant.

H_2-driven whole-cell biosyntheses and biotransformations

The central focus of this review lies on H_2-driven whole-cell biosyntheses including biotransformations. Whole-cell redox biosyntheses often represent an appealing

alternative to *in vitro* systems, as enzymes generally are more stable in the cellular context and undergo continuous renewal. In the following, we exclusively describe NAD(P)H-dependent enzymatic cascade reactions proceeding in H_2-grown *R. eutropha* cells. Already in 1961, Schlegel *et al.* demonstrated that under certain conditions, *R. eutropha* H16 accumulates the polyhydroxyalkanoate, polyhydroxybutyric acid (PHB) when growing on H_2, O_2, and CO_2 [19]. In the three-step PHB synthesis pathway, two acetyl-CoA molecules are condensed to form acetoacetyl-CoA by β-ketothiolase activity [20]. The intermediary product is subsequently reduced to (*R*)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase. (*R*)-3-hydroxybutyryl-CoA is then converted into PHB by the key enzyme, PHB synthase. Up to 56 g/l of PHB were accumulated during growth of *R. eutropha* on CO_2, H_2, and O_2 [21] (Table 1). Under these conditions, the required NAD(P)H is recycled through H_2 oxidation by SH and transhydrogenase activity (Figure 1).

At the time oxidoreductases became increasingly relevant for biocatalysis, new synthetic biological approaches were devised for H_2-driven *in vivo* biosyntheses. Within the last decade, *R. eutropha* was engineered to produce high-value fine chemicals, biofuels and biopolymers. New genetic tools, including the CRISPR-Cas9 technique for mutagenesis and stable broad-host-range plasmids with strong inducible promoters, facilitated this development [7•,22,23,24•,25–27]. One of these plasmids [27] was used by Oda *et al.* for construction of a recombinant *R. eutropha* strain overproducing a NADH-dependent alcohol dehydrogenase from *Kluyveromyces lactis*, with which they demonstrated the H_2-driven whole-cell biotransformation of hydroxyacetone to (*R*)-1,2-propanediol [28••]. The lithoautotrophy of *R. eutropha* was also exploited to isotopically label products of interest with either of ^{2}H, ^{18}O, ^{13}C, and ^{15}N [29]. For instance, a yield of 0.25–0.5 g/l of ^{13}C-labeled arginine has been achieved by expressing the cyanophycin synthetase gene (*cphA*) from *Synechocystis* sp. PCC6803 in *R. eutropha* [22] (Table 1).

Considerable effort was also put in the construction of recombinant *R. eutropha* strains to produce liquid fuels from CO_2, H_2, and O_2. Müller *et al.* engineered the fatty acid synthesis pathway in a way that allowed production of diesel-range methyl ketones, which are highly reduced and promising biofuels [30]. First, the indigenous thioesterase TesA was overproduced in order to enhance fatty acid production, while native β-oxidation was knocked out. Fatty acid synthesis from CO_2, that is under lithoautotrophic conditions, requires plenty of reducing equivalents in the form of NADH, which was recycled from NAD^+ by the SH in an H_2-dependent manner. Finally, heterologous overproduction of coenzyme A oxidase, FadB, from *Micrococcus luteus*, and long-chain acyl-CoA thioesterase, FadM, from *Escherichia coli* made the fatty acids available to synthesize methyl ketone. With this strategy, a total amount of methyl ketones of 50–180 mg/l of culture volume was achieved under lithoautotrophic growth conditions [30] (Table 1).

Electrosynthetic approaches for biotransformation with H_2 as a mediator

Recently, a *R. eutropha* strain was developed that produces terpene, a feedstock for the synthesis of pharmaceutics, fragrances, and advanced biofuels. Heterologous

Table 1

H_2-driven *in vivo* biosyntheses supported by the *R. eutropha* NAD^+-reducing hydrogenase in its native host and in *P. putida*

Product	Yield per liter of culture	Strain description (Genotype)	Reference
PHB	56 g	*Ralstonia eutropha* H16	[21]
Arginine (^{13}C-labeled)	0.25–0.5 g	*R. eutropha* H16 (*cphA*)	[22]
(*R*)-1,2-propanediol	68 g	*R. eutropha* H16 (*adh*)	[28••]
Methyl ketones	180 mg	*R. eutropha* H16 (Δ*fadE*, Δ*phaCAB, tesA*, *fadB*, *fadM*)	[30]
α-humulene	17 mg[a]	*R. eutropha* H16 $PHB^{-}4$ (*zssi*, *erg20*, *hmgs*, *hmgr*, *mvaK*, *mvaK2*, *mvaD*, *fni*)	[31••]
Isopropanol	216 mg	*R. eutropha* H16 (Δ*phaB1B2B3*, Δ*phaC, phaA*, *ctf, adc*, *adh*) [35•]	[34]
Isopropanol	600 mg	*R. eutropha* H16 (Δ*phaB1B2B3*, Δ*phaC, phaA*, *ctf, adc*, *adh*) [35•]	[36••]
Isobutanol and 3-methyl-1-butanol	220 mg	*R. eutropha* DJ21 (*adh*, Δ*phaCAB*, Δ*ilvE*, Δ*bkdAB*, Δ*aceE, ilvBHCDkivD*) [37]	[36••]
1-octanol	101 mg	*Pseudomonas putida* KT2440 (*CYP153A*, *fdx*, *fnr*, *hoxFUYHWIhypA2B2F2CDEX*)	[41•]

[a] Yield is given per gram of cell dry weight.

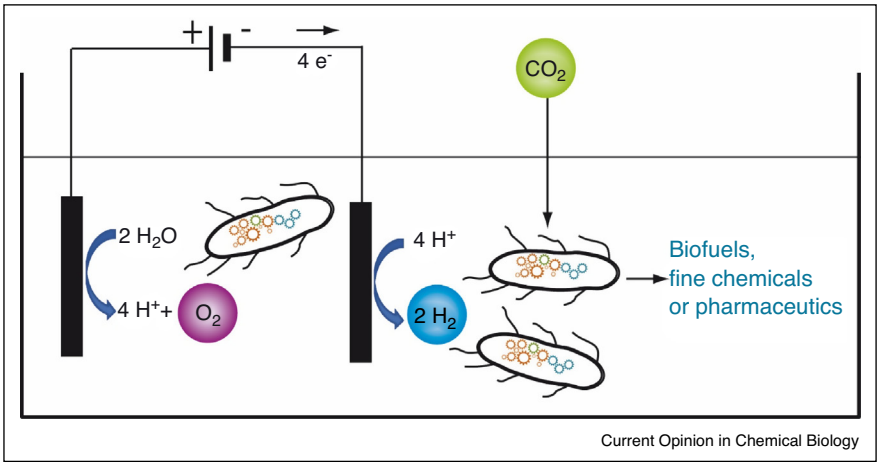
establishment of the mevalonate pathway from *Myxococcus xanthus* and α-humulene synthase from *Zingiber zerumbet* in *R. eutropha* lead to the production of 17 mg α-humulene per gram cell dry mass under lithoautotrophic conditions [31••] (Table 1). In this particular case, H_2 and O_2 were produced *in situ* by electrolysis within a microbial electrosynthesis (MES) reactor (Figure 2). In principle, the MES can be powered with solid-state photovoltaics allowing light-driven CO_2 fixation for synthesis of bioproducts [32]. A platinum electrode was used for H_2 production and an Ir-mixed metal oxide served as anode for O_2 formation [33]. This was the first reported electroautotrophic *de novo* production of a terpene from CO_2, H_2 and O_2. The study represents a promising starting point for production of different high-value terpene compounds [31••].

Further hybrid systems composed of inorganic electrocatalysts in combination with a lithoautotrophic organism have been constructed and promise a significant increase of the efficiency of electricity-driven biotransformations. Torella *et al.* used a bioelectrochemical cell that employs, instead of rare platinum, earth-abundant metals for water splitting at low overpotential. A cobalt phosphate catalyst split water into O_2 and protons and the protons were reduced to H_2 at either a nickel-molybdenum-zinc (NiMoZn) or a stainless-steel cathode. The produced H_2/O_2 mixture was used as the energy source for isopropanol production by an engineered *R. eutropha* strain [34]. The recombinant strain was deficient in PHB synthesis and previously engineered by Grousseau *et al.* to channel acetyl-CoA into an isopropanol synthesis pathway [35•]. This included the overproduction of ketothiolase (PhaA) and acetoacetyl-CoA transferase (Ctf) from *R. eutropha* as well as an acetoacetate decarboxylase (Adc) and NADPH-dependent alcohol dehydrogenase (Adh) from *Clostridium* species. Again, NAD(P)H required for CO_2 fixation and ADH function was mainly regenerated from H_2 by SH and transhydrogenase (Figure 1). The initially used MES by Torella *et al.* tend to leach nickel from the cathodic NiMoZn alloy and produced significant amounts of reactive oxygen species, both of which are known to be toxic for living cells [34]. This problem was overcome by employing an improved H_2-producing cathode composed of a Co-P alloy. With the new electrode design, which also reduced the overpotential required for water oxidation, the authors reported a CO_2 reduction energy efficiency of 40% for isopropanol/biomass production [36••]. With the same MES, another *R. eutropha* strain, engineered by Lu *et al.* for isobutanol and 3-methyl-1-butanol production [37], was fed with H_2 and O_2. In this strain, the NADPH-dependent isobutyraldehyde dehydrogenase was constitutively produced in a background where the native branched-chain amino acid biosynthesis pathway as well as the ketoisovalerate decarboxylase from *Lactococcus lactis* were overproduced. With the newly designed, NADPH-dependent pathway up to 220 mg/l isobutanol and 3-methyl-1-butanol were synthesized by the cells (Table 1), with a 24-hour maximum of 27% CO_2 reduction efficiency mediated by the MES-derived H_2 [36••].

Biotransformations supported by artificial, H_2-driven NADH recycling

Heterologous production of O_2-tolerant NAD^+-reducing hydrogenase from *R. eutropha* is challenging, as it requires the expression of at least 11 SH-related genes in the foreign host [7•]. Three independent studies reported the heterologous production of catalytically active SH in *E. coli* [38,39•,40], which, however, is already an H_2 oxidizer by nature. So far, there is only one report of heterologous SH production in a

Figure 2



Electroautotrophic cultivation and biosynthesis by *Ralstonia eutropha*. H_2 and O_2 are produced by electrosynthesis [33]. CO_2 is purged into the media. Alternatively, atmospheric CO_2 can be used. Metabolic engineering (indicated by blue gear wheels) enables biosynthesis of desired products.

non-H_2-oxidizing host, namely *Pseudomonas putida* KT2440. In this case SH activity was implemented to support H_2-driven biotransformation of *n*-octane to 1-octanol catalyzed by a P450 monooxygenase [41•]. The three genes required for synthesis and function of the monooxygenase were derived from *Polaromonas* sp. JS666 and set under the control of the pseudomonal, alkane-inducible *alkB* promoter. SH operon expression was controlled by the same promoter. In the engineered *P. putida* strain, the P450 monooxygenase oxidized *n*-octane to 1-octanol. The beneficial role of H_2-driven NADH cofactor regeneration by the SH was demonstrated by a significantly increased yield of 1-octanol production when the whole-cell biotransformation was performed in the presence of exogenous H_2 (Table 1). In this context, it is noteworthy that SH-mediated NADH regeneration took place even in the presence of O_2, the co-substrate of monooxygenase.

Conclusion and outlook

R. eutropha is a promising host for H_2-driven biotransformations. It can be easily genetically manipulated and engineered for production of valuable compounds. Applications and further optimizations of engineered O_2-tolerant SHs for, for example, efficient NADPH regeneration in coupled NADPH-dependent reactions is required in order to bypass energy-dependent transhydrogenases. Physiological implications of heterologously produced SHs, including the effect of altered NADH/NAD^+ ratios and the accumulation of storage compounds on the basic metabolism, could help to identify potential bottlenecks for synthetic H_2-driven *in vivo* biotransformations. By exploring heterologous production of SH in biotechnologically relevant bacteria such as pseudomonads and *Corynebacterium glutamicum* in combination with upscaling, we may make H_2-driven biotransformations compatible in a world where H_2 as an alternative energy carrier becomes more important. Finally, isolation and application of Knallgas bacterial SHs from, for example, rainforest soil and hot lakes [42,43], in addition to comparative genomics and physiological studies, may also enlarge the toolbox for H_2-driven biotransformations.

Conflict of interest statement

Nothing declared.

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