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MINI-REVIEW

Current state and perspectives in hydrogen production by *Escherichia coli*: roles of hydrogenases in glucose or glycerol metabolism

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Abstract
Escherichia coli has been a robust host strain for much biological research, in particular, research in metabolic engineering, protein engineering, and heterologous gene expression. In this mini review, to understand bacterial hydrogen production by *E. coli*, the effect of glucose and glycerol metabolism on hydrogen production is compared, and the current approaches to enhance hydrogen production from glycerol as a substrate are reviewed. In addition, the argument from past to present on the functions of *E. coli* hydrogenases, hydrogenase 1, hydrogenase 2, hydrogenase 3, and hydrogenase 4 is summarized. Furthermore, based on the literature that the *E. coli* formate-hydrogen lyase is essential for bacterial hydrogen production via recombinant hydrogenases, research achievements from the past regarding heterologous production of hydrogenase are rethought.

Keywords Hydrogen · Glycerol metabolism · Glucose metabolism · Heterologous expression · *Escherichia coli*

Introduction

Sustainable biogas production is an important goal; in particular, since hydrogen gas is renewable, efficient, and clean (Hansel and Lindblad 1998), it can be utilized as a possible gas for fuel cells (Dunn 2002), which may contribute to the global energy system by utilizing hydrogen in place of fossil fuels in the future (Dunn 2002). Biological means of hydrogen production has certain advantages over chemical ones because it does not require a high energy cost (e.g., extensive heating energy) (Das and Veziroglu 2001). Hydrogenases, which catalyze the reaction $2H^+ + 2e^- \leftrightarrow H_2$ (g), have a key role in hydrogen synthesis and uptake in photosynthetic and fermentative bacteria; based on a better understanding of the exact functions of bacterial hydrogenases, new strategies to enhance bacterial hydrogen production may be employed.

Theoretically, 2 mol of hydrogen from 1 mol of glucose can be biosynthesized in *Escherichia coli* (Yoshida et al. 2006), unlike other facultative bacteria, which have the potential to produce 4 mol of hydrogen from 1 mol of glucose (Vardar-Schara et al. 2008); however, *E. coli* is one of the best microorganisms for hydrogen production because genetic manipulation (including transcriptomics, P1 phage transduction, and use of the KEIO/ASKA library) is developed as well as the biochemistry of many metabolic pathways for enhanced hydrogen production is understood (Blattner et al. 1997). Therefore, to date, many researchers have used *E. coli* strains as a robust model for bacterial hydrogen production via metabolic engineering, protein engineering, and heterologous gene expression. For example, the isogenic *E. coli* K-12 KEIO mutant library (mutants of the 3985 non-lethal genes) (Baba et al. 2006) has been a powerful tool for enhanced hydrogen production by allowing the introduction of multiple mutations through successive P1 phage transductions after removing the kanamycin antibiotic resistance gene (Datsenko and Wanner 2000; Maeda et al. 2007a, 2008a). In addition, the *E. coli*

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ASKA library, which is an expression system of each *E. coli* gene, is also useful for characterizing the function of the genes cloned into plasmid pCA24N (Kitagawa et al. 2005). Furthermore, *E. coli* genes related to hydrogen production and glucose metabolism have been well characterized; in *E. coli*, hydrogen is produced from formate by the formate hydrogen lyase system (FHL) consisting of hydrogenase 3 (*hycABCDEFGHI*) (Bagramyan and Trchounian 2003) and formate dehydrogenase-H (*fdhF*) (Axley et al. 1990). HycA represses FHL activity (Bagramyan and Trchounian 2003) whereas FhlA activates the FHL function (Schlensog et al. 1994). Hydrogen is consumed by *E. coli* hydrogenase 1 (*hyaABCDEF*) (Forzi and Sawers 2007) and hydrogenase 2 (*hybOABCDEFG*) (Forzi and Sawers 2007). In addition, *E. coli* also consumes formate by using two additional formate dehydrogenases, formate dehydrogenase-N and formate dehydrogenase-O (Rossmann et al. 1991), and exports/imports formate via FocA (Suppmann and Sawers 1994) and FocB (Andrews et al. 1997). During glucose metabolism, pyruvate from glucose is converted by pyruvate dehydrogenase and pyruvate oxidase (Abdel-Hamid et al. 2001; Angelides et al. 1979). Also, fumarate reductase and lactate dehydrogenase are key enzymes for converting phosphoenolpyruvate into succinate and pyruvate into lactate (Olajuyin et al. 2016). In addition, although pseudogenes have been reported as a junk gene which does not have any biological function (Gil and Latorre 2012); *E. coli* pseudogenes *ydfW*, *ylcE*, *ypdJ*, and *yqiG* have a role in hydrogen metabolism (Mohd Yusoff et al. 2013). Also, *ydjA* and *yhjY*, which were previously uncharacterized, are related to hydrogen production in *E. coli* (Mohd Yusoff et al. 2012).

Thus, based on the above knowledge of anaerobic fermentation by *E. coli*, to date, metabolic engineering (Maeda et al. 2007a, 2008a), protein engineering (Maeda et al. 2008b; Sanchez-Torres et al. 2009), and heterologous expression (Akhtar and Jones 2008b; Penfold and Macaskie 2004) using *E. coli* strains have been pursued to enhance bacterial hydrogen production. Achievements in the research field have been summarized previously (Maeda et al. 2012); however, during the past 6 years, some new achievements and interesting research reports have been published. In this mini review, we focus on the current status of hydrogen production in *E. coli* by addressing (1) glucose versus glycerol metabolism for hydrogen production, (2) enhanced hydrogen production/yield from glycerol, (3) roles of native *E. coli* hydrogenases, and (4) rethinking heterologous hydrogenase expression in *E. coli*.

Glucose versus glycerol metabolism for hydrogen production

It has been long thought that *E. coli* is not able to consume glycerol due to the absence of an electron acceptor which is used for disposing the reducing equivalents caused by glycerol (Bouvet et al. 1995; Bouvet et al. 1994; Quastel and Stephenson 1925). However, *E. coli* is able to produce hydrogen from glycerol when the fermentation is conducted under an alkaline pH and with high concentrations of potassium and phosphate (Dharmadi et al. 2006; Gonzalez et al. 2008). Table 1 shows the difference in anaerobic metabolism with glucose and glycerol by *E. coli*. Anaerobic specific growth rates and hydrogen productivities with glycerol were ten times lower than those with glucose. In addition, glycerol is converted to metabolites through the 1,3-propanediol pathway (Gonzalez et al. 2008) whereas the glycolytic pathway is used for glucose (Lim and Jung 2017). Thus, it is somewhat different when glycerol is used as a carbon source due to the repression or the expression of some genes which are highly dependent on the presence of glucose (Holtman et al. 2001). Moreover, the transport mechanism of H^+ in glycerol is also different from that of glucose (Trchounian et al. 2013a). Therefore, hydrogen metabolism in *E. coli* from glycerol is far from understood compared to that from glucose.

Some proteins related to hydrogen production utilized with glucose or glycerol function differently. For example, formate channels (FocA and FocB) maintain the intracellular pH at neutral or slightly alkaline conditions by forcing out surplus formate inside the cells (Suppmann and Sawers 1994). Trchounian and Trchounian found that under glycerol metabolism, FocB is responsible for formate import at pH 7.5, while FocA is used preferentially to export formate (Trchounian and Trchounian 2014a). On the other hand, under glucose metabolism, FocA plays a role as formate import (Suppmann and Sawers 1994). The different behaviors of FocA and FocB might explain why formate is accumulated in the fermentation medium when glycerol is used (Sanchez-Torres et al. 2013; Tran et al. 2014, 2015), while it is not in the case of glucose (Mohd Yusoff et al. 2012). Although the inactivation of formate dehydrogenase-O for growth on glucose resulted in an increase in hydrogen production (Maeda et al. 2008a), the effect was not observed when glycerol was used as a substrate (Tran et al. 2014). This difference also suggests that the carbon source controls the regulation of some genes, and in turn, it affects the production of metabolite products like hydrogen.

Enhanced hydrogen production/yield from glycerol

Due to the advantages of its high reduced state and low cost (Ma et al. 2014; Maeda et al. 2009; Mohd Yasin et al. 2013; Taifor et al. 2017), various attempts have been conducted to produce value added chemicals and biofuels from glycerol; in particular, hydrogen gas (Clomburg and Gonzalez 2013; Dharmadi et al. 2006; Mazumdar et al. 2010; Murarka et al.

Table 1 Comparison of anaerobic fermentations of glucose and glycerol by *Escherichia coli*

Condition	Glucose	Glycerol
Anaerobic specific growth rate (1/h)	0.5 (Peterson et al. 2017)	0.05 (Sanchez-Torres et al. 2013; Tran et al. 2014)
Hydrogen productivity (μmol/mg-protein/h)	15 to 30 (Maeda et al. 2007a; Mohd Yusoff et al. 2012)	1.2 to 1.9 (Sanchez-Torres et al. 2013; Tran et al. 2014)
Anaerobic metabolism	Glycolytic pathway	1,3-Propanediol pathway
Formate export	FocA (Suppmann and Sawers 1994)	FocA (Trchounian and Trchounian 2014a)
Formate import	FocA (Suppmann and Sawers 1994)	FocB (Trchounian and Trchounian 2014a)
Deleterious formate dehydrogenase to enhance hydrogen production	Formate dehydrogenase-O (Maeda et al. 2007a)	Formate dehydrogenase-N (Tran et al. 2014)
Hydrogenase required for hydrogen production[a]	Hydrogenase 3 (Maeda et al. 2007b)	Hydrogenase 3 (Sanchez-Torres et al. 2013)
Hydrogenase associated with growth[a]	No hydrogenases	Hydrogenase 2 (Sanchez-Torres et al. 2013)

[a] The hydrogenases required during the fermentation may be changeable by different pH conditions

2008; Tran et al. 2014; Yazdani and Gonzalez 2007). Among these approaches, metabolic engineering has been shown to be a powerful approach for enhancing hydrogen production in *E. coli*. Recently, Tran et al. created an engineered strain that was able to improve hydrogen production from minimal glycerol medium by 5-fold while it sustains cell growth (Tran et al. 2014). This success was based on multiple disruptions of fumarate reductase (encoded by *frdC*), lactate dehydrogenase (*ldhA*), a formate dehydrogenase (*fdnG*), phosphoenolpyruvate (*ppc*), nitrate reductase (*narG*), methylglyoxal synthase (*mgsA*), and the regulator of the transcriptional regulator FhlA (*hycA*). Interestingly, blocking the pathway to the production of methylglyoxal (encode by *mgsA*) was reported to be beneficial for hydrogen production (Tran et al. 2014). This engineered strain was able to achieve the theoretical maximum yield of 1 mol of H_2 formed per 1 mol of glycerol consumed. In another attempt, by disrupting the succinate pathway together with co-overexpressing two key enzymes for the conversion of glycerol to glycolytic intermediates, glycerol dehydrogenase (*gldA*) and dihydroxyacetone kinase (*dhaKLM*), the modified strain, SY03, was capable of producing over 95% of the theoretical maximum yield of ethanol and hydrogen from glycerol (Shams Yazdani and Gonzalez 2008). Hence, by redirecting the metabolic pathway of *E. coli* or by enhancing the conversion efficiency of glycerol, hydrogen production and yield can be improved significantly.

Furthermore, by performing random mutagenesis and screening for high hydrogen producing mutant strains, Tran et al. found four new genes that are critical to hydrogen production in *E. coli* under glycerol metabolism. A single disruption of *aroM*, *gatZ*, *ycgR*, and *yfgI* resulted in 1.3–1.6-fold higher hydrogen productivity (μmol H_2/mg protein) (Tran et al. 2015). Interestingly, the inactivation of these individual genes not only improves hydrogen production, but also enhances cell growth and glycerol consumption under anaerobic conditions. AroM is predicted to be involved in the shikimate pathway; YcgR acts as the motility brake; GatZ is required for the synthesis of 1,6-biphosphate; and the function of YfgI remains unknown. In addition, by using adaptive evolution and chemical mutagenesis, Hu and Wood created a mutated strain, HW2, that produced 20-fold more hydrogen and grew 5-fold faster than the BW25113 Δ*frdC* strain (Hu and Wood 2010). The whole transcriptome analysis revealed that this strain was repressed in fructose-1,6-bisphosphatase (encoded by *fbp*), formate transportation (*focA*), and tagatose-1,6-bisphosphate aldolase (*gatYZ*). Taken together, these new findings in regard of metabolic pathways in the glycerol metabolism obviously contribute to a better understanding of previously unknown pathways involving in the hydrogen metabolism in *E. coli*. As a result, they can be used to improve hydrogen production via further metabolic engineering.

On the other hand, the culture conditions significantly affect hydrogen production. The concentrations of glycerol, phosphate, CO_2, H_2, and pH are the key factors which affect the efficiency of hydrogen formation and glycerol conversion in *E. coli*. Chaudhary found that an optimal condition for hydrogen production is at 20 g/L glycerol together with sparging the headspace of the fermentation reactor with argon (Chaudhary 2011). Hydrogen itself adversely impacts hydrogen production and cell growth (Kim et al. 2006; Mizuno et al. 2000; Murarka et al. 2008). Thus, a reduction of hydrogen accumulation in the headspace of the reactor can improve hydrogen production and cell growth, and this method has been often applied to enhance hydrogen production (Chaudhary 2011; Dharmadi et al. 2006; Kim et al. 2006; Maeda et al. 2008a; Mizuno et al. 2000; Tran et al. 2014). For instance, *E. coli* can easily reach the exponential phase just after 24 h of fermentation under a low partial pressure condition where hydrogen generated through the fermentation is transported out of the fermentation vial (Tran et al. 2014). Additionally, CO_2 is also a critical factor that positively affects

hydrogen production and cell growth (Dharmadi et al. 2006; Kim et al. 2006). Because the formate hydrogen lyase (FHL) complex is an acidic dependent enzyme which catalyzes formate to CO_2 and H_2 (Sawers 1994), CO_2 can freely be converted into bicarbonate, which lowers the pH of the cytosol. Therefore, the presence of CO_2 in the headspace improves the metabolism of glycerol and the CO_2 is produced by the FHL activity (Dharmadi et al. 2006).

Roles of native *Escherichia coli* hydrogenases

In *E. coli*, four membrane-bound proteins, namely hydrogenase 1, 2, 3, and 4 (Hyd), play critical roles in hydrogen metabolism. Under glucose metabolism, Hyd-1 and Hyd-2 generally act as hydrogen uptake (Menon et al. 1994; Menon et al. 1991), while Hyd-3 is involved in hydrogen synthesis (Maeda et al. 2007b; Maeda and Wood 2008). In contrast, under glycerol metabolism, Hyd-2 is a reversible enzyme and is most responsible for hydrogen production at a slightly alkaline pH condition (Trchounian and Trchounian 2009). To date, the functions of hydrogenases in *E. coli* create a considerable controversy because it seems the hydrogenases required in glucose or glycerol metabolism are different as well as some interesting but unusual reports that the expression of Hyd-1 triggers hydrogen production (Kim et al. 2010) and Hyd-2 and Hyd-4 are able to produce hydrogen (Mirzoyan et al. 2017; Pinske et al. 2015; Trchounian et al. 2013b). In addition, Hyd-4 activity may depend on glucose concentration (Trchounian and Trchounian 2014b). In fact, an engineered strain, BW25113 *hyaB hybC hycA fdoG frdC ldhA aceE* capable of producing 4.6 times higher hydrogen from glucose (Maeda et al. 2007a) could not produce a relatively high amount of hydrogen from glycerol (Tran et al. 2014). In contrast, Sanchez-Torres et al. claim that Hyd-3 rather than Hyd-2 is the most important enzyme for hydrogen synthesis in a medium containing yeast extract and tryptone (Sanchez-Torres et al. 2013) because a considerable amount of hydrogen gas produced from glycerol still relies on the Hyd-3 activity (Table 1). Thus, the dynamics of pH during the fermentation may be different in either a buffered medium or a normal medium; thereby, the different results/explanations can be proposed. Notwithstanding these different opinions, whether Hyd-2 has hydrogen synthetic activity or is required for optimum glycerol metabolism, Hyd-2 is crucial for hydrogen metabolism. Additionally, Hyd-1 and Hyd-4 are likely silent under glycerol metabolism at either slight alkaline pH or acidic conditions (Sanchez-Torres et al. 2013; Self et al. 2004) whereas Hyd-1 is partially functional to hydrogen synthesis at an alkaline pH and Hyd-4 has hydrogen uptake activity at a low pH condition (Trchounian et al. 2011, 2012; Trchounian and Trchounian 2009). These points of contention for *E. coli* hydrogenases are concisely summarized in Fig. 1 by comparing the literature regarding the functions of *E. coli* hydrogenases. However, the activity of hydrogenases is very much dependent on pH as well as the carbon sources tested (Trchounian and Trchounian 2014a) and then the main ideas are summarized in other review papers (Trchounian and Trchounian 2015; Trchounian et al. 2017). A multiple alignment analysis of hydrogenases shows that Hyd-1 and Hyd-2 are similar to bidirectional hydrogenases which not only produce but also consume hydrogen gas and that Hyd-4 is similar to Hyd-3 (Vardar-Schara et al. 2008); therefore, Hyd-1, Hyd-2, and Hyd-4 may be able to produce hydrogen.

Rethinking heterologous hydrogenase expression in *Escherichia coli*

Since there are many bacteria that produce hydrogen via several types of hydrogenases, which have been categorized as [NiFe], [FeFe], or [Fe] hydrogenases according to the type of iron and/or nickel sequestered inside the active site of the hydrogenases, an attempt to express such heterologous hydrogenases in *E. coli* may have great potential to enhance bacterial hydrogen production. To date, a considerable number of heterologous recombinants of *E. coli* have been generated and are summarized in Table 2. For example, a [Fe]-hydrogenase from *Enterobacter cloacae* (Chittibabu et al. 2006; Mishra et al. 2004), a [Fe-Fe]-hydrogenase derived from *Ethanoligenes harbinense* (Zhao et al. 2010), a HupSL consisting of small and large subunits of Hyd isolated from *Rhodobacter sphaeroides* (Lee et al. 2010), and HydF, HydE, HydG, and HydA of *Clostridium acetobutylicum* along with *E. coli* YdbK (a probable pyruvate-flavodoxin oxidoreductase) and *Clostridium pasteurianum* [4Fe-4S]-ferredoxin (Akhtar and Jones 2009), were expressed in *E. coli* BL21(DE3) derivatives which show a negligible hydrogen-producing activity. In addition, genes necessary for sucrose transport and metabolism (*scrK* encoding an ATP-dependent fructokinase, *scrY* encoding a sucrose-specific porin of the outer membrane, *scrA* encoding enzyme II_{scr} of the phosphotransferase system for sucrose uptake, *scrB* encoding an intracellular β-D-fructofuranoside fructohydrolase, which catalyzes the hydrolysis of sucrose 6-phosphate to β-D-fructose and α-D-glucose 6-phosphate, and *scrR* encoding the negative repressor of the *scr* regulon) were heterologously expressed into *E. coli* strains defective in the HycA FHL repressor and TAT system to promote hydrogen production from sucrose (Penfold and Macaskie 2004). Another modification was to express a heterologous alpha-type amylase derived from *Bacillus subtilis* (*amyE*) in an *E. coli iscR* mutant to produce hydrogen from starch as a substrate (Akhtar and Jones 2009) as well as the recent modification to construct an engineered *E. coli* strain capable of co-producing hydrogen and ethanol

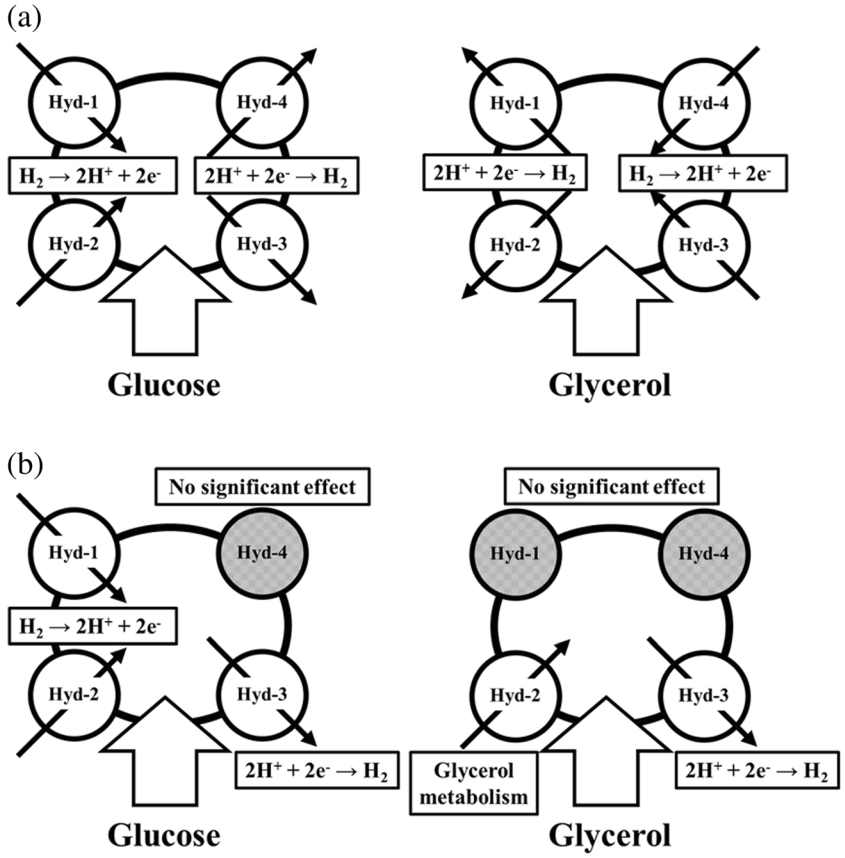

Fig. 1 Role of the *E. coli* four hydrogenases discussed from past to present. Hyd-1 hydrogenase 1, Hyd-2 hydrogenase 2, Hyd-3 hydrogenase 3, and Hyd-4 hydrogenase 4. **a** The concept of Trchounian group on the role of *E. coli* hydrogenases. In anaerobic fermentation using glucose, Hyd-1 and Hyd-2 have hydrogen uptake activity and Hyd-3 and Hyd-4 have hydrogen synthetic activity. In anaerobic fermentation using glycerol, Hyd-1 and Hyd-2 have hydrogen synthetic activity and Hyd-3 and Hyd-4 have hydrogen uptake activity (Mirzoyan et al. 2017; Pinske et al. 2015; Trchounian et al. 2011, 2012, 2013a, b; Trchounian and Trchounian 2009, 2014a). **b** The concept of Maeda and Wood group on the role of *E. coli* hydrogenases. In anaerobic fermentation using glucose, Hyd-1 and Hyd-2 have hydrogen uptake activity, Hyd-3 has hydrogen synthetic activity as well as a minor hydrogen uptake activity, and Hyd-4 has no significant effect on hydrogen metabolism. In anaerobic fermentation using glycerol, Hyd-1 and Hyd-4 have no significant effect on hydrogen metabolism, Hyd-2 is needed for bacterial growth using glycerol, and Hyd-3 has hydrogen uptake activity (Maeda et al. 2007a, b, 2008a; Sanchez-Torres et al. 2013). Note that the function of hydrogenases required during the fermentation may be changeable by different pH conditions

from glucose or gluconate by constructing a pentose phosphate pathway through expression of *zwf* and *gnd* (Seol et al. 2016).

Recently, a very interesting result has been reported by the group of Jo and Cha in that FHL activity is essential for the hydrogen production via the recombinant hydrogenases in *E. coli* BL21 (DE3) (Jo and Cha 2015). Therefore, in the heterologous systems using *E. coli*, the cloned hydrogenases themselves may not function to produce hydrogen gas directly and instead complement defects in hydrogen synthesis of *E. coli* BL21 (DE3). So far, there may be only two cases in which heterologous hydrogenases can have an enzyme function to produce hydrogen by themselves because in the two cases, an appropriate *E. coli* strain which does not have any function derived from all the native hydrogenases (Hyd-1, Hyd-2, Hyd-3, and Hyd-4, or Hyd-1, Hyd2, and Hyd-3) was used: (1) a cyanobacterial [Ni-Fe]-hydrogenase (HoxEFUYH) was cloned along with seven ORFs encoding the maturation proteins HypABCDEF and HoxW (Wells et al. 2011), and (2) the *Ralstonia eutropha* SH hydrogenase was cloned to increase the level of NADH (Ghosh et al. 2013).

Another mechanism to increase bacterial hydrogen production has been found in a previous study in which a cyanobacterial [Ni-Fe]-Hyd, HoxEFUYH derived from *Synechocystis* sp. PCC 6803 was heterologously expressed in *E. coli* TG1 (Maeda et al. 2007c). In this case, the heterologous hydrogenase inhibited hydrogen uptake activity through *E coli* Hyd 1 and 2 rather than producing hydrogen by itself.

Perspectives

The production of hydrogen using *E. coli* is still an active field of research for three reasons: (1) sustainable hydrogen production is still a hot topic due to the public interest in future energy

Table 2 Comparison of in vivo hydrogen production by heterologous recombinant *Escherichia coli* strains

System	H_2 production rate (reported units)	H_2 production rate (converted units)	Host strain	Substrate	pH used	Reference
Inactivation of HycA and TatC and expression of the genes encoding ScrKYABR invertase from *Bacillus subtilis*	1.38 mL H_2 (mg DCW)$^{-1}$ L^{-1}	3.9 μmol H_2 (mg protein)$^{-1}$ h^{-1}	MC4100 *hycA tatC*	Sucrose	pH 7.3	Penfold and Macaskie (Penfold and Macaskie 2004)
Expression of [Fe]-hydrogenase from *E. cloacae*	0.96 mmol h^{-1}	14.5 μmol H_2 (mg protein)$^{-1}$ h^{-1}[a]	BL21	Glucose	pH 6.0	Chittibabu et al. (Chittibabu et al. 2006)
Expression of HoxEFUYH hydrogenase from *Synechocystis sp.* PCC 6803	22 ± 3 μmol H_2 (mg protein)$^{-1}$	4 μmol H_2 (mg protein)$^{-1}$ h^{-1}	TG1	Glucose	pH 6.8	Maeda et al. (Maeda et al. 2007c)
Expression of HydFEGA	429.3 nmol H_2 min^{-1} L^{-1}	0.12 μmol H_2 (mg protein)$^{-1}$ h^{-1}[a]	BL21(DE3)	Glucose	–	Akhtar and Jones (Akhtar and Jones 2008b)
Expression of HydFEGA and inactivation of IscR	1257.5 nmol H_2 min^{-1} L^{-1}	0.34 μmol H_2 (mg protein)$^{-1}$ h^{-1}[a]	BL21(DE3)	Glucose	–	Akhtar and Jones (Akhtar and Jones 2008a)
Inactivation of IscR, expression of HydFEGA hydrogenase from *C. acetobutylicum*, CpFdX ferredoxin from *C. pasterianum*, and YdbK pyruvate-flavodoxin oxidoreductase from *E. coli*	9.6 mmol H_2 (g DCW)$^{-1}$ h^{-1}	19 μmol H_2 (mg protein)$^{-1}$ h^{-1}	BL21(DE3)	Glucose	pH 7.0	Akhtar and Jones (Akhtar and Jones 2009)
Inactivation of IscR, expression of HydFEGA hydrogenase from *C. acetobutylicum*, CpFdX ferredoxin from *C. pasterianum*, and YdbK pyruvate-flavodoxin oxidoreductase from *E. coli,* and amyE from *B. subtilis*	30 μmol H_2 culture^{-1}	0.65 μmol H_2 (mg protein)$^{-1}$ h^{-1}[a]	BL21(DE3)	Starch	pH 7.0	Akhtar and Jones (Akhtar and Jones 2009)
Expression of HupSL hydrogenase from *Rhodobacter sphaeroides*	19.68 μL H_2 (ml culture^{-1}) h^{-1}	1.1 μmol H_2 (mg protein)$^{-1}$ h^{-1}	BL21(DE3)	Glucose	–	Lee et al. (Lee et al. 2010)
Expression of *E. coli* hydrogenase	12. 5 mL H_2 L^{-1} h^{-1}	2.5 μmol H_2 (mg protein)$^{-1}$ h^{-1}[a]	BL21(DE3)	Glucose	pH 7[c]	Kim et al. (Kim et al. 2010)
Expression of HxEFUYH hydrogenase and the maturation proteins HypABCDEF and HoxW from *Synechocystis sp.* PCC 6803	8.4 μmol H_2 L^{-1}	0.004 μmol H_2 (mg protein)$^{-1}$ h^{-1}	BL21Star (DE3) *hyaB hybC hycE hyfG*	Glucose	–	Wells et al. (Wells et al. 2011)
Expression of the *Ralstonia eutropha* SH operon	63 nmol H_2 h^{-1}	0.3 μmol H_2 (mg protein)$^{-1}$ h^{-1}[b]	FTD147 (*hyaB hybC hycE*)	Glucose	pH 7[c]	Ghosh et al. (Ghosh et al. 2013)
Expression of [NiFe]-hydrogenase 2 from *Citrobacter* sp. SG	360 ppm H_2 h^{-1}	2.6 μmol H_2 (mg protein)$^{-1}$ h^{-1}[a]	BL21CodonPlus(DE3)	Glucose	pH 8.0	Maier et al. (Maier et al. 2015)

[a] Assuming that cell turbidity is 1 for hydrogen production

[b] Assuming that cell turbidity is 1 and 1 mL culture for hydrogen production

[c] Not described clearly, but estimated pH was indicated because the authors used M9 minimal medium

sources to replace scarce fossil fuels as well as the concerns related to global warming (Armaroli and Balzani 2011; Kessel 2000); (2) *E. coli* is still not completely characterized for bacterial hydrogen production, for example, because of the influence of uncharacterized genes and pseudogenes on hydrogen production (Mohd Yusoff et al. 2013; Mohd Yusoff et al. 2012) and because glycerol metabolism remains a matter of debate during hydrogen production; and (3) the true biological function of *E. coli* hydrogenases is still a highly controversial issue. In particular, it seems that the function of hydrogenases may be changeable according to the pH conditions and the substrates in an anaerobic fermentation (Trchounian et al. 2011; Trchounian and Trchounian 2014a). Understanding the evolution of hydrogenases should be pursued to understand the different biological functions of bi-directional hydrogenases and apparently unidirectional hydrogenases such as *E. coli* Hyd-1, Hyd-2, Hyd-3, and Hyd-4, as to which one may be older evolutionally. Also, how formate dehydrogenase-H is integrated into the Hyd-3 components to form the FHL should be discerned. In a recent interesting report, the gene for formate dehydrogenase-H (*fdhF*) was placed in the upstream locus of the *hybB* gene (McDowall et al. 2015) because FdhF works together with Hyd-3 to make the FHL complex. The resulting FHL is more stable than the native FHL. Thus, deeper understanding of the biological functions of the *E. coli* 4 hydrogenases is required to facilitate further development of an engineered *E. coli* strain capable of enhancing bacterial hydrogen production as well as producing value-added compounds.

Since *E. coli* consumes glycerol but grows in a slow manner, it is critical for hydrogen production to improve glycerol metabolism and growth rates. These can be achieved by (i) overexpressing glycerol dehydrogenase (*gldA*) and dihydroxyacetone kinase (*dhaKLM*), which helps boost the conversion efficiency; (ii) introducing beneficial knockouts directing the metabolic route toward hydrogen production; and (iii) optimizing the fermentation conditions such as acidic pH, high CO_2 concentration, a low partial pressure reactor, and a proper glycerol concentration. With all these improvements, a high hydrogen yield and productivity along with sustained cell growth on glycerol would create better strains for industrial applications. In addition, for the study of heterologous hydrogenase recombinants, an appropriate host strain in which all the hydrogenases and maturation system are totally deleted (no hydrogen can be produced) should be used and a synthetic biological approach using synthetic genes may be able to achieve a breakthrough in the heterologous recombinant system not only for hydrogenases but also for other useful enzymes.

Taken together, all the approaches, metabolic engineering, protein engineering, and heterologous gene expression hold great promise for making better engineered strains with the highest hydrogen productivity and the highest hydrogen yield by integrating each beneficial genetic and biochemical tool. In addition, biologically derived hydrogen gas is necessary for other important processes such as for CO_2 sequestration with seawater (Mohd Yasin et al. 2017; Mohd Yasin et al. 2015).

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

Ethical approval This article does not contain any studies performed with human participants or with animals by any of the authors.

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