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Escherichia coli and its application to biohydrogen production

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Abstract

Hydrogen is an attractive energy carrier because its high energy density, and used as a raw material in various chemical processes. Nowadays, hydrogen demand is supplied from non-renewable sources, and alternative sources are becoming mandatory. Hydrogen production by biological methods uses renewable resources as substrate and its production occurs at ambient temperature and atmospheric pressure. Thus, it is less energy intensive than the chemical and thermochemical methods used to produce hydrogen. This review is focused on fermentative hydrogen production by *Escherichia coli*. The hydrogen production pathway, the genetic manipulations, and expression of non-native pathways into this microorganism are reviewed. The hydrogen production using alternative substrates is a critical point to develop sustainable process by this reason the principal substrates for hydrogen production using *E. coli* are revised. Other strategies like two stages processes and immobilized cells are also discussed.

Keywords: Biofuels, Biohydrogen, Hydrogen yield, formate regulon, mixed acid fermentation, metabolic engineering

1. Introduction

Fossil fuels are the primary source of energy used to satisfy world´s energy demand, and their intensive use has caused an accelerated consumption of non-renewable resources (Davila-Vazquez et al. 2008). It has been suggested that depletion of fossil resources will lead to an energy crisis in the near future (Kapdan and Kargi 2006). In addition, there is now a general scientific consensus that observed trends in global warming are being caused by fossil fuel combustion and anthropogenic emissions of greenhouse gases (Luque et al. 2008). These issues have lead to explore new energy sources that could substitute fossil fuels, and be environmentally friendly and renewable.

Hydrogen is a promising fuel as it has a higher energy content than oil (142 MJ/kg for hydrogen versus 42 MJ/kg for oil) (Nurul Islam et al. 2005) and its combustion results only in water and energy. Hydrogen is not only used as a fuel carrier; it is widely used by the chemical industry for the production of ammonia and methanol as well for the hydrogenation of fats and oils in the food industry, production of electronic devices, steel processing and re-formulation of gasoline in refineries (Ramachandran and Menon 1998; Kapdan and Kargi 2006; Fonseca et al. 2008).

At present, 40% hydrogen is produced from natural gases, 30% from heavy oil and naphtha, 18% from coal, 4% from electrolysis and only about 1% from biomass (Sinha and Pandey 2011). These processes require high temperatures or pressures or both. If hydrogen production is based on fossil products or the processes to obtain this gas require high energy, then hydrogen is not the solution to solve the growing energy requirements. In the present scenario, biological hydrogen production processes are becoming important.

2. Biological hydrogen production

The main advantages of biological production are the use of renewable resources as substrate and its operation at ambient temperature and atmospheric pressure. Besides, it is less energy intensive than chemical and thermochemical methods used to produce hydrogen. Biological hydrogen production processes can be classified into three major categories: biophotolysis of water using algae and cyanobacteria, photofermentation of organic compounds by photosynthetic bacteria and dark fermentative production (Hallenbeck 2005).

In biophotolysis, photosynthetic organisms use solar energy to split water, producing O_2 and reduced ferredoxin, the latter can reduce a hydrogenase or nitrogenase, producing hydrogen (Hallenbeck and Ghosh 2009). The main advantage of this process is the abundance of substrate and simple products, whereas the disadvantages are low conversion efficiencies, sensibility of hydrogenase to oxygen and light dependence (Hallenbeck and Ghosh 2009). The anaerobic photosynthesis carried out by non-sulfur purple bacteria is called photofermentation. In this process the solar energy is used to produce ATP and high-energy electrons that reduce ferredoxin. ATP and reduced ferredoxin drive proton reduction to hydrogen by nitrogenase enzyme. These organisms cannot obtain electrons from water and therefore use organic compounds. The main disadvantages are low conversion efficiencies and the expensive photo-bioreactors required. The advantage of this process is the use of organic acid wastes as substrate allowing the use of residues of dark fermentation as substrate (see below) and increasing the overall hydrogen yield.

3. The Dark fermentation pathway

A variety of different microorganisms can be used anaerobically to break down mainly carbohydrate-rich substrates into hydrogen and other products, principally organic acids (lactic, acetic, butyric, etc.) and alcohols (ethanol, butanol, etc.). Final products depend of type of microorganism, oxidation state of the substrate and environmental conditions (Hallenbeck and Ghosh 2009). For hydrogen production by dark fermentation both, axenic or non-axenic cultures could be used.

Hypothetically up to 12 mol of hydrogen can be obtained per mole of glucose, but there are no single metabolic pathways in nature that would allow this reaction. The theoretical yields of hydrogen from dark fermentations depend on the type of organisms that are used in fermentation (Mathews and Wang 2009). Facultative anaerobes such as *Escherichia coli* produce a maximum yield of 2 moles of hydrogen from each mole of glucose consumed, whereas other enterobacteria such as *Enterobacter cloacae* produce 4 moles (Redwood et al. 2009). Both of these microorganisms produce hydrogen from formate. Sequences analysis of hydrogenase 3 (Hyd 3) large subunit from *E. coli* and hydrogenases of *E. cloacae* (hydrogenase 3 large subunit and Fe-hydrogenase) is shown in Figure 1. As noted, these large-subunit sequences of Hyd 3 of *E. coli* and *E. cloacae* show high identity. The presence of other hydrogenases in *E. cloacae* and the higher hydrogen yield of this microorganism implicate the simultaneous activity of NADH pathway in which the regeneration of NAD is coupled to the reduction of ferredoxin by NADH: ferredoxin oxidoreductase (NFOR), and the formate pathway to achieve this yield.

Dark fermentation seems to be the best promise for biohydrogen production due its low cost, rapid production rates, no direct solar input needed, and stable hydrogen-producing

enzymes. Also, organic wastes from agriculture or sewage can be used into anaerobic bioreactors, achieving the dual goals of waste management and hydrogen production (Chittibabu et al. 2006). Dark fermentations also solve the problem of expensive photo-bioreactors, which are necessary for direct biophotolysis and photofermentations, whereas the weaknesses are the low hydrogen yields and the large quantities of side products formed (ethanol and organic acids such as acetate, lactate, succinate and butyrate).

4. Hydrogen production by *Escherichia coli*

E. coli can grow in the presence or absence of oxygen. In both conditions, glucose is transported and catabolized to pyruvate, but the further metabolism of pyruvate is different. In aerobic condition, the glycolysis and the Krebs cycle generate NADH, which it is reoxidized by the respiratory chain. Under anaerobic condition, NADH is still produced by glycolysis, but the respiratory chain is not working and NADH must be reoxidized to continue the glycolysis process. Thus the key issue of fermentation is to recycle the NADH by the conversion of pyruvate to fermentation products (Clark 1989).

Figure 2 shows the fermentative pathway in *E. coli*. Carbohydrates are catabolized to phosphoenolpyruvate, which can be converted to oxaloacetate, by incorporation of CO_2 by phosphoenolpyruvate carboxylase (PPC). Oxaloacetate is further converted to malate, fumarate and finally to succinate. A pathway from decarboxylate succinate to propionate was proposed (Haller et al. 2000; Froese et al. 2009), and some works reported propionate in *E. coli* fermentations (Jian et al. 2010; Zhang et al. 2010; Rosales-Colunga et al. 2010a; Redwood et al. 2012). Nevertheless, the metabolic function of this pathway remains

uncertain. Most of the phosphoenolpyruvate is transformed to pyruvate, which is broken down into formate and acetyl-CoA by the pyruvate formate lyase (PFL). The formate is converted to hydrogen and CO_2 by the formate-hydrogen-lyase complex (FHL), whereas acetyl-CoA yields acetate or ethanol. There is evidence for a pathway that uses acetyl-CoA to butyrate formation via crotonyl-CoA (Lugg et al. 2008), and butyrate production has been reported in *E. coli* fermentations under particular culture conditions (Blackwood et al. 1956; Redwood et al. 2012).

Under circumstances of high pyruvate accumulation, this may be converted to lactate by lactate dehydrogenase enzyme (LDH) (Clark 1989). As mentioned above the pathway to produce hydrogen involves formate production. Thus formate metabolism is important for hydrogen production. There are three formate dehydrogenases (FDH) in *E. coli,* FDH-N, which are active when cells are growing anaerobically in the presence of nitrate, and are encoded by the *fdnGHI* operon. FDH-O is induced under anaerobic growth and is encoded by the *fdoGHI* operon. The fdhF gene encodes FDH-H and it is only active in fermentative conditions. It forms part of the FHL complex and is responsible for the catabolism of formate in the hydrogen production pathway. Hydrogen is produced by the Hyd-3 enzyme, which also forms the FHL complex. Besides Hyd-3, *E. coli* has another 3 hydrogenases, Hyd-1, 2 and 4. Hyd-1 and Hyd-2 are considered as up-take hydrogenases; however, Kim et al (2010) reported hydrogen production ability for Hyd-1 even under micro-aerobic conditions and Trchounian et al (2012) found that Hyd-1, Hyd-2 and Hyd-3 can operate in reverse mode depending on pH and substrate type Hyd-4 has not been biochemically characterized (Redwood et al. 2008).

FHL complex is shown in Figure 3. This complex comprises seven proteins; six of them are encoded in the *hyc* operon. HycB, C, D, F and G are membrane electron transfer proteins, while HycE is the hydrogenase Hyd-3. FocA is not part of the complex, but is related to the formate metabolism because this is the formate channel and it exports the formate to prevent the acidification of the cytoplasm and then re-imports the formate when the pH is low in the culture medium. The formate regulon comprises genes that are involved in the formate metabolism. Besides the operon *hyc* and *fdhf*, the regulon also includes the *hypA-E* operon, *fhlA*, and *hydN-hypF* operon. The HypA-E, HypF, and HycI proteins are required for assembly of the Ni-Fe cofactor and the maturation of the three hydrogenases. FhlA is the transcriptional activator of the regulon, whereas HycA is the negative regulator. A detailed description of the formate regulon has been published by Leonhartsberger et al (2002) and Sawers (2005).

5. Improvement of hydrogen production and yield by genetic manipulations of *E. coli* metabolic pathways

Among the genetically modified microorganisms reported for biohydrogen production, *E. coli* is one of the most widely used because its genome sequence is known, and the metabolic pathways and metabolism are the best understood of all bacteria. Also, there are molecular tools for its manipulation. Some example mutant strains of *E. coli* used for biohydrogen production are presented in Table 1.

Since HycA is the negative regulator of the formate regulon, the activity of FHL complex is increased when *hycA* gen is mutated (Leonhartsberger et al. 2002), thus HycA defective

161 strains are hydrogen overproducer (Penfold et al. 2003; Yoshida et al. 2005). Yoshida et al
162 (2005) overexpressed *fhlA* and performed the *hycA* inactivation. With these genetic
163 modifications, the transcription of *fdhF* and *hycE* increased 6.5- and 7-fold, respectively,
164 and hydrogen production increased 2.8-fold compared with the wild-type strain.
165 Hydrogenases 1 and 2 and formate dehydrogenase N and O are located in the periplasmic
166 space (Figure 3), whereas hydrogenase 3 and FDH-H are located in cytoplasm. The
167 transport of these proteins to the periplasmic is performed by the Twin arginine
168 translocation (Tat) protein system. Therefore, Tat mutant strains do not take formate up
169 needed for hydrogen production. Penfold et al (2006) reported that defective mutant strains
170 of Tat transport (Δ*tatC* and Δ*tatA-E*) showed a hydrogen production comparable to *E. coli*
171 strain carrying a Δ*hycA*. However, double mutant strain Δ*tatC* Δ*hycA* did not increase
172 hydrogen production. Thus, it is possible that discarding activities of the uptake
173 hydrogenases, which recycle a portion of hydrogen, and the formate hydrogenases N and O,
174 which oxidize the formate without hydrogen production, could increase the hydrogen
175 production by *E. coli*. Indeed, the effect of mutations in uptake hydrogenases, in lactate
176 dehydrogenase gene (*ldhA*) and *fhlA* was studied by Bisaillon et al (2006). They reported
177 that each mutation contributed to a slight increase in hydrogen production, and the effect
178 was synergistic. This same strain was used by Turcot et al (2008) and gave the highest
179 hydrogen production and yield in continuous cultures. The highest yields (at, or somewhat
180 higher than 2 mol H_2/mol glucose) were obtained with cultures limited for glucose (22 mM
181 glucose); in a posterior work (Ghosh and Hallenbeck 2009b), a yield of 1.69 mol H_2/mol
182 glucose was achieved under 75 mM glucose.

183 Maeda et al (2007a) performed multiple stable mutations to direct the metabolic flux
184 toward hydrogen production. The best strain involves mutations on *hyaB, hybC, hycA,*
185 *fdoG, frdC, ldhA* and *aceE* genes. The *hyaB* and *hybC* were deleted to abolish the uptake
186 activity of hydrogenases 1 and 2. The *fdoG* and *aceE* genes code for the α subunit of
187 formate dehydrogenase O and the pyruvate dehydrogenase respectively. The inactivation of
188 *frdC* abolishes the succinate synthesis pathway. The same group reached the theoretical
189 yield from formate with a strain with deletions of *hyaB, hybC, hycA, fdoG,* and
190 overexpression of *fhlA* (Maeda et al. 2008). Yoshida et al (2006) enhanced the hydrogen
191 yield from 1.08 with the wild type strain to 1.82 mol H_2/mol glucose using a *ΔldhA, ΔfrdBC*
192 strain. The same yield was obtained by Mathews et al (2010) using a strain with deletions
193 on uptake hydrogenases (*hyaAB, hybABC*), *hycA*, lactate dehydrogenase (*ldh*) and fumarate
194 reductase (*frdBC),* whereas Kim et al (2009) reached 2.11 mol H_2/mol glucose with a
195 similar strain under low hydrogen partial pressure.

196 The synthesis of PFL, FHL, and FHLA is activated by the global transcriptional factor Fnr
197 (Sawers 2005; Salmon et al. 2003; Perrenoud and Sauer 2005; Constantinidou et al. 2006),
198 whereas the dual transcriptional regulator NarL repressed the synthesis of PFL and FHL
199 (Overton et al. 2006). Fan et al (2009) described increases in specific and molar yields of
200 hydrogen achieved by the modification of *focA, ppc, narL,* and *fnr* genes. The strain ZF1
201 (*ΔfocA*) and ZF3 (*ΔnarL*) produced 14.9 and 14.4 µmol hydrogen/mg dry cell weight,
202 respectively, compared to 9.8 µmol hydrogen/mg dry cell weight produced by the wild type
203 strain. Strain ZF3 also displayed the best molar yield of 0.96 mol hydrogen/mol of glucose
204 compared to 0.54 for the wild type strain.
205

6. Improvement of hydrogen production by expression of heterologous pathways in *Escherichia coli*

As discussed above, the low hydrogen yield of *E. coli* pathway is the main disadvantage. To overcome this drawback, some efforts have been made focused on the heterologous expression of hydrogenase genes to enhance hydrogen production. The use of this strategy can be traced back more than 30 years ago to Karube et al (1983). The authors cloned and expressed the hydrogenase from *Clostridium butyricum* into *E. coli* HK16. Since this first attempt, some other efforts have been made (Table 2). The overexpression a Fe-hydrogenase from *Enterobacter cloacae* in a non-hydrogen-producing *E. coli* BL21 strain was made by Mishra et al (2004) using degenerate primers designed from the conserved zone of *hydA* gene. The resultant recombinant strain showed the ability to produce hydrogen. King et al (2006) reported the production in *E. coli* of active enzymes by the co-expression of proteins involved in maturation of hydrogenases from *Clostridium acetobutylicum* and Fe-Fe hydrogenases from *Clostridium acetobutylicum, C. pasteurianum*, and *Chlamydomonas reinhardtii.* The purified enzymes showed similar specific activities to those purified from native sources. Akhtar and Jones (2008b) constructed a functional synthetic operon with the Fe-Fe hydrogenase (*hydA*) and its maturation factors (*hydF*, *hydE* and *hydG*) from *Clostridium acetobutilicum* and demonstrated that the deletion of *iscR*, which codes for the transcriptional negative regulator of the iron-sulfur cluster, stimulated the recombinant Fe-Fe hydrogenase activity (Akhtar and Jones 2008a). Finally, they developed a synthetic hydrogen pathway by co-expression of a putative pyruvate flavodoxin/ferrodoxin oxidoreductase YdbK from *E. coli*, [4Fe–4S]-ferredoxin from *Clostridium pasteurianum* and *Clostridium acetobutylicum*

HydF, HydE, HydG, and HydA reached a maximum yield of 1.88 mol H_2/mol glucose consumed (Akhtar and Jones 2009). Kuchenreuther et al (2010) described the production of active Fe-Fe hydrogenases from *Chlamydomonas reinhardtii* or *Clostridium pasteurianum* using maturases from *Shewanella oneidensis.*

As discussed above, *E. coli* can perform the NADPH-dependent hydrogen production pathway if adequate hydrogenases from other microorganisms are expressed (King et al. 2006; Akhtar and Jones 2008b). Kim et al (2011) introduced *hydAEFG* from *C. acetobutiricum*, *fdxA* and *yumC* from *C. pasteurianum,* and *B. subtilis,* respectively, in an *E. coli* BL21 (DE3) strain. Since NADPH is generated mainly by the pentose phosphate pathway, and the activation of this pathway is accompanied by activation of gluconeogenesis, FBPase II (a key enzyme in gluconeogenic pathway which is less sensitive to regulation, encoded by *glpX*), and glucose 6 phosphate 1 dehydrogenase (a key enzyme activating pentose phosphate pathway, encoded by *zwf*) were overexpressed in that *E. coli* strain. Overexpression of *glpX* increased the hydrogen yield 1.48-fold whereas the co-expression of the two genes increased the yield further 2.32-fold.

Agapakis et al (2010) performed various hydrogen-producing electron circuits containing Fd-dependent hydrogenases from *C. acetobutylicum, C. saccharobutylicum, C. reinhardtii,* and *Shewanella oneidensis,* ferredoxins from *C. acetobutylicum, Spinacia olearcea,* and *Zea mays* and PFORs from *C. acetobutylicum, Desulfovibrio africanus,* and *E. coli.* The *E. coli* BL21 (DE3) strain had multiple deletions in uptake hydrogenases and competing carbon pathways. The resulting hydrogen production yield was 0.4 mol H_2/mol glucose.

Ni-Fe hydrogenases were also expressed in *E. coli* due to low oxygen sensivity. Maeda et al (2007b) cloned the bidirectional Ni-Fe hydrogenase (*hoxEFUYH*) from *Synechocystis sp*. in

E. coli. This strain yielded 41-times more hydrogen than the strain with the empty vector after 18 h. This effect was due to the inhibition of the uptake activity. Sun et al (2010) reported the co-expression of 4 structural genes for the NADP-dependent hydrogenase and 9 genes for its maturation from *Pyrococcus furiosus*. The recombinant enzyme showed to be as functional as the native enzyme. They also observed that the maturation machinery of *E. coli* produces a functional hydrogenase when it only expressed the structural genes for the hydrogenase and a protease from *P. furiosus*. However, the hydrogenase activity was only reported *in vitro*. Weyman et al (2011) cloned and expressed the structural gene for Ni-Fe hydrogenase, maturases and adjacent genes from *Alteromonas macleodii* "deep ecotype" in *E. coli* lacking native hydrogenases. The hydrogenase showed to be active *in vitro* in both aerobic and anaerobic conditions. They also demonstrated the activity of a Ni-Fe hydrogenase from *Thiocapsa roseopersicina* when co-expressed with the accessory proteins from *Alteromonas macleodii*. Wells et al (2011) expressed the *Synechocystis sp*. hydrogen production pathway and its maturation factors in an *E. coli* strain in which the hydrogenases and formate production pathway were abolished. They reported *in vivo* production of 20 µmol of hydrogen per liter of culture.

7. Hydrogen production with *E. coli* using alternative carbon sources

Most of the research to improve hydrogen production was conducted using glucose as substrate. However, to be competitive, biological hydrogen production must use carbohydrate rich wastes. Penfold and Macaskie (2004) transformed to *E. coli* HD701, a hydrogenase-upregulated strain and FTD701 (a derivative of HD701 that has a deletion of

274 the *tatC* gene), with the plasmid pUR400 carrying the *scr* regulon. This regulon contain the
275 genes of *Salmonella thompson* to metabolize sucrose. The resulting *E. coli* strain produced
276 hydrogen from sucrose. The parental strains did not produce hydrogen, whereas
277 recombinant strains produced 1.27 and 1.38 ml H_2/mg dry weight/L. Rosales-Colunga et al
278 (2010b) obtained a yield of 2.74 mol H_2/mol lactose consumed, using cheese whey as
279 substrate, and an *E. coli* Δ*hycA* Δ*lacI* strain (WDHL). In a subsequent work, hydrogen
280 production from lactose, glucose and galactose was reported; the maximum yield was
281 attained with galactose (Rosales-Colunga et al. 2012). Ghosh and Hallenbeck (2009a)
282 reported the hydrogen yields from arabinose, fructose, gluconate, glucose, lactose, maltose,
283 manitol, sorbitol, sucrose, trehalose, and xylose. The highest hydrogen yield obtained was
284 1.47 mol H_2/mol substrate using sorbitol. Morsy (2011) used hydrolyzed molasses as
285 substrate using the strain HD701. The highest hydrogen production of 570 mL of
286 hydrogen/L and a rate of 19 mL/L/h were obtained using a concentration of 10 g/L of
287 reducing sugars. However, the maximal yield (132 ml of hydrogen/g of reducing sugars)
288 was obtained from 2.5 g/L of reducing sugars.

289 Perego et al (1998) used a corn starch hydrolysate (85% glucose, dry basis) to produce
290 hydrogen with *E. coli* and *E. aerogenes*; with *E. coli* a maximum yield of 0.36 mol/mol
291 glucose was reached. In this study, *E. aerogenes* showed better production from this
292 substrate. Orozco et al (2012) performed the hydrothermal hydrolysis of starch with carbon
293 dioxide and detoxification of hydrolisate with activated coal. Hydrogen production using
294 this hydrolysis strategy was equal to glucose controls. Akhtar and Jones (2009) reported an
295 *E. coli* that expresses an amylase and it was used for hydrogen production from starch
296 without previous hydrolysis.

297 Glycerol has become an abundant and inexpensive carbon source due to its generation as
298 by-product from biodiesel fuel production. For this reason some efforts have been focused
299 on obtaining hydrogen from glycerol. Yazdani and Gonzalez (2008) created the strain
300 SY03 (pZSKLMgldA) in which the acetate and succinate pathways were minimized by
301 inactivation of phosphate acetyltransferase (*pta*) and fumarate reductase (*frdA*),
302 respectively. The enzymes responsible for the conversion of glycerol to
303 dihydroxyacetonephosphate, a glycolytic intermediate, were overexpressed. The yield of
304 ethanol and hydrogen reached was 95% of the theoretical maximum. Trchounian et al
305 (2011) studied the glycerol fermentation and hydrogen production, they found that at pH of
306 5.5 the hydrogen production was 1.5-fold higher than at a pH 6.5. Starting with *E. coli*
307 BW25113 *frdC* that lacks fumarate-reductase and by using both adaptive evolution and
308 chemical mutagenesis combined with a selection method based on increased growth in
309 glycerol. Hu and Wood (2010) obtained the strain HW2, that produced 20-fold more
310 hydrogen in glycerol medium.

311

312 ## 8. Fermentative approaches used to improve hydrogen production using
313 ## *E. coli*

314 The main disadvantage of fermentative hydrogen production is the low yield due to the
315 production of other metabolites, mainly organic acids. To improve the net yield of
316 hydrogen a two-stage system can be used. In the first stage, a dark fermentation is used to
317 produce hydrogen, and in the second stage the organic acids produced in the first step are
318 used as substrate for photofermentation, increasing the total hydrogen yield. For example,
319 Salih (1989) used cheese whey pretreated with *E. coli* to produce hydrogen by a

photosynthetic *Rhodospirillum rubrum*. *E. coli* was only used to pretreat of cheese whey and not for hydrogen production; however, hydrogen production increased when pretreated whey was used. Redwood and Macaskie (2006) tried to produce hydrogen in two stages, first, by fermentation of glucose by *E. coli* HD701 and then by photofermentation of the residual medium by *R. sphaeroides*. Nevertheless, hydrogen production did not occur during photofermentation of the residual liquor per se due to the presence of fixed nitrogen compounds. This issue was further solved by electroseparation of ammonium ion and the authors reported a continuous *E. coli* reactor and a continuous *R. sphaeroides* photobioreactor integrated by anion-selective electrodialysis, simultaneously transferring anionic fermentation products, while retaining repressive ammonium ion, *E. coli* cells and suspended solids (Redwood et al. 2009). This approach resulted in sustained hydrogen production by *E. coli* with a yield of 1.6 mol hydrogen/mol hexose and sustained hydrogen photoproduction by *R. sphaeroides*. The overall yield was 2.4 mol H_2/mol glucose. This electro-extractive strategy was also used to enhance continuous hydrogen and organic acid production by *E. coli* FTD67 (Redwood et al. 2012). The pH was controlled by separation of organic acids, which can be used in a further hydrogen production step by photofermentation. The maximal rate was 4.7 L/d/L of culture and yield of 0.7 mol/mol glucose.

Waks and Silver (2009) combined the industrial advantages of yeast with *E. coli* hydrogen production. They proposed biomass conversion to formate by *S. cerevisiae* and the subsequent conversion of formate to hydrogen by *E. coli*. The endogenous formate dehydrogenases of *S. cerevisiae* were deleted and the pyruvate formate lyase and alcohol dehydrogenase from *E. coli* were expressed; galactose was used in this first stage to

produce formate. The formate-enriched medium was further used to produce hydrogen by *E. coli.* Abd-Alla et al (2011) proposed the use of rotten dates to produce hydrogen in a 3-stage process. In the first stage, *E. coli* EGY was used to consume oxygen and maintain the anaerobic condition. In the second, stage hydrogen was produced using *C. acetobutylicum* ATCC 824 and finally photofermentation by *R. capsulatus* was used. The maximal total yield of the process was 7.8 mol hydrogen/mol sucrose. Seppälä et al (2011) examined hydrogen production in a co-culture of *E. coli* and *C. butyricum*. They found that the total hydrogen production of the co-culture was higher compared to the monoculture of each strain. However, the co-culture yield (1.65 mol hydrogen/mol glucose) was lower than that obtained by the pure culture of *C. butyricum* (2.09 mol hydrogen/mol glucose).

Another strategy widely used to produce a variety of products is the use of immobilized cells. Its main advantages are increase in the biomass concentration, low risk of contamination, operational stability, and high productivity (Seol et al. 2011). Therefore, this strategy has been used in hydrogen production. For example, Ishikawa et al (2006) probed the encapsulation of *E. coli* MC13-4 in alginate gel beads, and hydrogen increased 3-fold compared to a free cell system; nonetheless, the gas remained as bubbles in the interspace of the gel. This system was connected to a fuel cell and can produce electricity. In a later work (Ishikawa et al. 2008), a compact stacked flatbed reactor (CSFR) was developed to extract the produced gas easily. This reactor comprises pieces of agar plates containing *E. coli* MC13-4 at high density and reached the yield of 1.2 mol H_2/mol glucose, and the production rate of 6.7 L of hydrogen/g dry cell/L of reactor/h. Seol et al (2011) examined 3 different matrices (agar, agarose, and sodium alginate) to immobilize a hydrogen over-producer strain of *E. coli* (SH5). Using agar as matrix and optimal conditions a maximum

production rate of 2.4 L of hydrogen/L/h and yield around 100% of the theoretical from formate (1 mol/mol) were attained. They also probed a sustained production in a feed-batch operation mode.

Escherichia coli is a valuable microorganism in the study of hydrogen production as discussed above and is still a model that can provide useful information to know the hydrogen producer pathways and to improve them.

Conclusions

Genetic manipulations, the use of a diversity of carbohydrates, and redirection of the carbon flux to favor hydrogen production have been used to increase hydrogen yield. However, until now *E. coli* has been just an excellent microorganism to study processes to produce hydrogen in lab-scale. Additional efforts should be conducted to obtain suitable processes feasible to scaling-up to produce hydrogen for commercial purposes, for instance those where metabolites such as succinate or recombinant protein are the main products and hydrogen is a by-product. Novel and new approaches such as using synthetic biology to improve hydrogen production are still needed in a new generation of overproducing hydrogen strains.

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587 **Figure Caption**

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589 Figure 1. Alignment of hydrogenases from *Escherichia coli* and *Enterobacter cloacae*.

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591 Figure 2. The fermentative pathways in *Escherichia coli*.

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593 Figure 3. Schematic representation of the FHL complex.

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595 **Table 1** Some *E. coli* mutants performed to improve hydrogen production

Strain	Mutation	Maximum yield (mol H_2/mol substrate)	Substrate	Ref.
SR13	Inactivation of *hycA* and overexpression of *fhlA*.	NR	Formic acid	(Yoshida et al. 2005)
FTD701	Inactivation of *hycA* and *tatC*	NR	Glucose	(Penfold et al. 2006)
DJT135	Deletion of uptake hydrogenases, mutation of *ldhA* and constitutive expression of *fhl*.	2	Glucose	(Bisaillon et al. 2006), (Turcot et al. 2008)
		1.51	Glucose	(Ghosh and Hallenbeck 2009a)
		1.69	Glucose	(Ghosh and Hallenbeck 2009b)
BW25113 (modified)	Deletion of *hyaB, hybC, hycA, fdoG, frdC, ldha and aceE*	1.35	Glucose	(Maeda et al. 2007a)
BW25113 (modified)	Deletion of *hyaB, hybC, hycA, fdoG*, and over expression of *fhlA*	1	Formate	(Maeda et al. 2008)
SR15	Deletion of *ldhA* and *frdBc*	1.82	Glucose	(Yoshida et al. 2006)
WDHL	Deletion of *lacI* and *hycA*	0.30	Glucose	(Rosales-Colunga et al. 2012)
GW16	Deletion of *hyaAB, hybABC, hycA, ldhA* and *frdBC*	1.82	Glucose	(Mathews et al. 2010)
SH5	Deletion of *hyaAB, hybBC, hycA, ldhA* and *frdAB*	2.11	Glucose	(Kim et al. 2009)
ZF3	Deletion of *narL*	0.96	Glucose	(Fan et al. 2009)

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597 **Table 2.** Heterologous expression of genes used to improve hydrogen production.

Strain	**Heterologous gene**	**Maximum yield (mol H_2/mol substrate)**	**Ref.**
BL-21(DE3)/ PGEX4T-1/*hydA*	Fe-hydrogenase from *Enterobacter cloacae*.	NR	(Mishra et al. 2004)
BL-21(DE3) (Transformed with various plasmids)	Fe-Fe hydrogenases from *Clostridium acetobutylicum, C. pasteurianum* and *Chlamydomonas reinhardtii* and maturation proteins from *Clostridium acetobutylicum*.	NR	(King et al. 2006)
BL21(DE3)Δ*iscR* pAF pYdbK	Construction of a synthetic hydrogen pathway with genes from *Clostridium pasteurianum* and *Clostridium acetobutylicum*.	1.88	(Akhtar and Jones 2009)
BL21(DE3)Δ*iscR* pACYCDuet-1 *hydGX-hydEF* (maturases) pET21(b) (hydrogenases)	Fe-Fe hydrogenase from *Chlamydomonas reinhardtii* or *Clostridium pasteurianum* and maturases from *Shewanella oneidensis*.	NR	(Kuchenreuther et al. 2010)
HFdYzg	*hydAEFG* from *C. acetobutiricum, fdxA* from *C. pasteurianum* and *yumC* from *B. subtilis*. Homologous overexpression of *zwf* and *fdx*.	NR	(Kim et al. 2011)
BL21(DE3)*ΔhycE, ΔhyaB, ΔhybC, Δfpr, ΔydbK, Δhcr, ΔyeaX, ΔhcaD, ΔfrdB* (Transformed with various plasmids)	Various hydrogen producing electron circuits containing Fd-dependent hydrogenases from *C. acetobutylicum, C. saccharobutylicum, C. reinhardtii,* and *Shewanella oneidensis*. Ferredoxins from *C. acetobutylicum, Spinacia olearcea,* and *Zea mays* and PFORs from *C. acetobutylicum, Desulfovibrio africanus*.	0.4	(Agapakis et al. 2010)
TG1 pBS(Kan)Synhox	Hydrogenase (*hoxEFUYH*) from *Synechocystis sp*. PCC6803.	NR	(Maeda et al. 2007b)
MW4W	Ni-Fe hydrogenase and maturation factors from *Pyrococcus furiosus*.	NR	(Sun et al. 2010)
FTD147 pRC41	Ni-Fe hydrogenase (*hynSL*), maturases (*hynD, hupH and*	NR	(Weyman et al. 2011)

	hypCABDFE), and adjacent genes (*orf2*, *cyt*, *orf1*) from *Alteromonas macleoodii*.		
BL21(DE3) *ΔhyaB, ΔhybC, ΔhycE, ΔhyfG, ΔpflB* pSynHox and/or pETHox	Hydrogenase (*hoxEFUYH*) and maturation factors (*hypA1B1CDEF* and *hoxW*) from *Synechocystis sp*. PCC6803.	NR	(Wells et al. 2011)

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602 **Table 3** Hydrogen production using alternative carbon sources.

Strain	Strategy	Maximum yield (mol H_2/mol substrate)	Substrate	Ref.
FTD701/ pUR400	Inactivation of *hycA* and *tatC* and expression of the genes necessary for sucrose transport and metabolism.	NR	Sucrose	(Penfold and Macaskie 2004)
WDHL	Inactivation of *hycA* and *lacI*	2.74	Cheese whey	(Rosales-Colunga et al. 2010b)
		1.12	Galactose	(Rosales-Colunga et al. 2012)
DJ135	Deletion of uptake hydrogenases, mutation of *ldhA* and constitutive expression of *fhl*.	1.47	Sorbitol	(Ghosh and Hallenbeck 2009a)
HD701	Acid hydrolysis	132 ml/g reducing sugar	Molasses (hydrolyzed)	(Morsy 2011)
NCIMB	Enzymatic hydrolysis	0.36	Corn starch	(Perego et al. 1998)
HD701	Hydrothermal hydrolysis	0.38	Starch	(Orozco et al. 2012)
BL21(DE3)Δ*iscR* pAF pYdbK	Expression of *Bacillus subtillis* AmyE	NR	Starch	(Akhtar and Jones 2009)
SY03(pZSKLMgldA)	Inactivation of *frdA*, *pta* and overexpression of *gldA* and *dhaKLM*	≈1	Glycerol	(Shams Yazdani and Gonzalez 2008)
BW25113	Low pH	NR	Glycerol	(Trchounian et al. 2011)
HW2	Adaptative evolution and chemical mutagenesis	21µmol/mg protein	Glycerol	(Hu and Wood)

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607 **Table 4** Other strategies used to improve hydrogen production.

Strategy	**Maximum yield (mol H_2/mol substrate)**[a]	**Ref.**
Pretreatment of cheese whey with *E. coli* and photoproduction by *Rhodospirillum rubrum*.	NR	(Salih 1989)
Two stages using *E. coli* and photoproduction by *R. sphaeroides*.	0.376(G)	(Redwood and Macaskie 2006)
Two stages using *E. coli* and photoproduction by *R. sphaeroides* in continuous mode.	2.4(G)	(Redwood et al. 2009).
Electro-extractive fermentation	0.7 (G)	(Redwood et al. 2012)
Dual organism system using *S. cerevisiae* to produce formate and used to produce hydrogen by *E. coli*.	NR	(Waks and Silver 2009)
Three stages using *E. coli* to maintain the anaerobic condition and hydrogen production by *C. acetobutylicum* and *R. capsulatus*.	7.8(S)	(Abd-Alla et al. 2011)
Co-culture of *E. coli* and *C. butyricum*.	1.65(G)	(Seppälä et al.)
Compact high density reactor.	NR	(Ishikawa et al. 2006)
Compact Stacked Flatbed Reactor (CSFR).	1.2(G)	(Ishikawa et al. 2008)
Strain SH5 immobilized in agar matrix.	1(F)	(Seol et al. 2011)

608 [a] G= Glucose, S=Sucrose, F=Formate

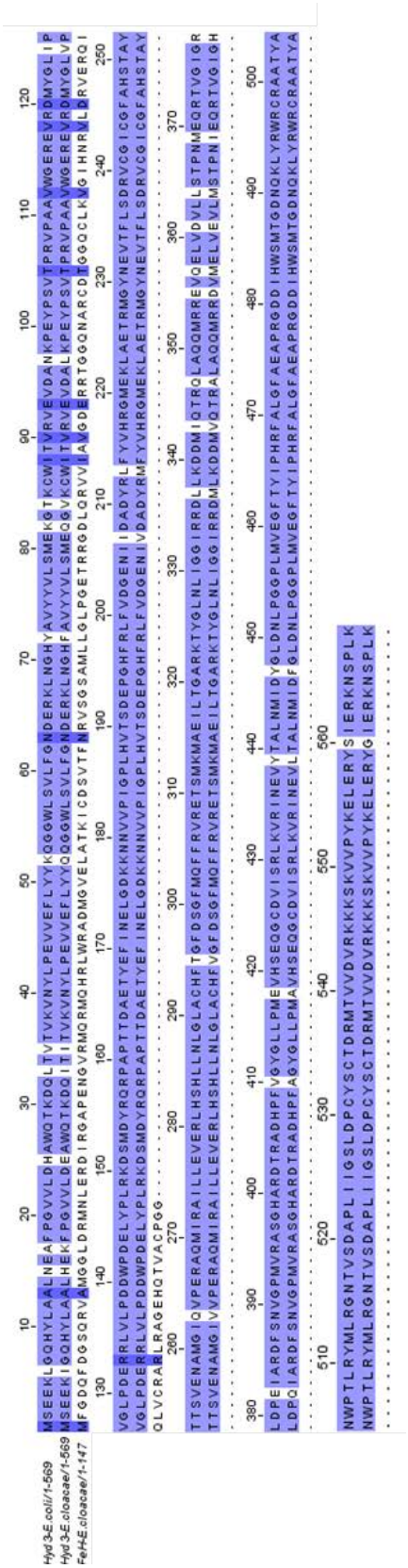

Fig. 1

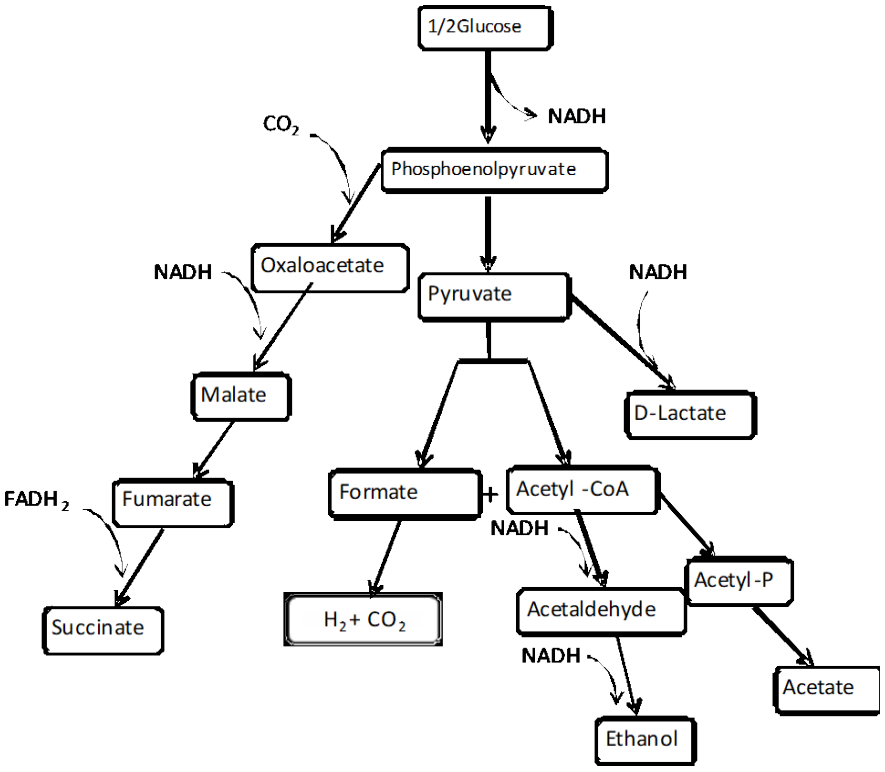
1/2Glucose
NADH
CO_2
Phosphoenolpyruvate
NADH
Oxaloacetate
Pyruvate
NADH
Malate
D-Lactate
$FADH_2$
Fumarate
Formate
+
Acetyl -CoA
NADH
Acetyl-P
Succinate
$H_2 + CO_2$
Acetaldehyde
NADH
Acetate
Ethanol

Fig. 2

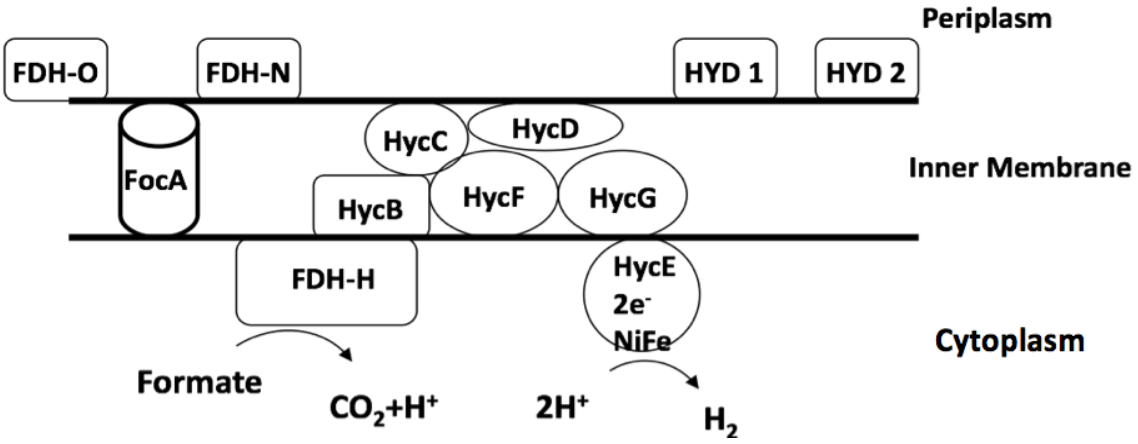
Periplasm
FDH-O
FDH-N
HYD 1
HYD 2
FocA
HycC
HycD
HycB
HycF
HycG
Inner Membrane
FDH-H
HycE
2e-
NiFe
Cytoplasm
Formate
CO2+H+
2H+
H2

Fig. 3.