Title: Metabolic engineering for the optimization of hydrogen production in Escherichia coli: A review

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Corresponding Author: Dr. Antonio Valle, Ph.D.

Corresponding Author's Institution: University of Cadiz

First Author: Antonio Valle, Ph.D.

Order of Authors: Antonio Valle, Ph.D.; Domingo Cantero, Professor; Jorge Bolívar, Asistant Professor

Abstract: Hydrogen is a potential sustainable energy source and it could become an alternative to fossil fuel combustion, thus helping to reduce greenhouse gas emissions. The biological production of hydrogen, instead of its chemical synthesis, is a promising possibility since this process requires less energy and is more sustainable and eco-friendly. Several microorganisms have been used for this purpose, but Escherichia coli is the most widely used in this field. The literature in this area has increased exponentially in the last 10 years and several strategies have been reported in an effort to improve hydrogen production. These strategies include heterologous gene expression, adaptive evolution and metabolic and protein engineering. On the other hand, culture conditions, including the use of carbon sources such as glycerol, a waste product from the biodiesel industry, have also been considered. This review includes a discussion of the hydrogenase complexes responsible for the hydrogen synthesis in E. coli and the central carbon metabolism pathways connected to this process. The different strategies applied to obtain engineered strains in which the carbon and electron fluxes are redirected towards hydrogen synthesis are also discussed. Yields and productivities of the most relevant engineered strains reported using several carbon sources are also compared.

He is an expertise in bioprocess using raw material from crops and byproductsfrom industry. He is an eminence in this field, hundreds ofpublished articles, ten of plenary lecturers, books, etc..

Ramon González Ph. D.
Professor, Chemical and biomolecular engineering, University of Rice
ramon.gonzalez@rice.edu
He leads the Laboratory for Metabolic Engineering and Biomanufacturing with the goal of engineering biological platforms for the synthesis of organic molecules with applications in fuel, chemical and pharmaceutical production.

Alfredo Martínez Ph. D.
Professor, Cellular engineering and biocatalysis, Autonomous National University of México (UNAM)
alfredo@ibt.unam.mx
He leads studies in metabolic engineering en Escherichia coli for the production of ethanol as biofuel by using wastes from agro-industry.

Antonio Villaverde Ph. D.
Professor, Institute of Biotechnology and Biomedicine (IBB), Autonomous University of Barcelona
antonio.villaverde@uab.cat
He is an expertise in metabolic engineering and microbial biotechnology. He is the editor in chief of journal Microbial cell factories.

Luis Manuel Rosales-Colunga Ph. D.
Assistant Professor, Biotechnology and agrofood industry, Autonomous university of San Luis Potosi
luis.rosales@uaslp.mx
He has focused in the field of hydrogen production in Escherichia coli.

Nicholas J.W. Rattray Ph. D.
Associate research scientist, Environmental Health Sciences, Yale University
nicholas.rattray@yale.edu
He is a bioanalytical chemist and he has experience on metabolomics to inform and influce th emetabolic engineering of microbial/mould based scaffolds for new valuable chemicals.

Thomas K. Wood Ph. D.
Professor, Chemical Engineering, Pennsylvania State University
tuw14@psu.edu
He is an expertise in metabolic engineering in Escherichia coli to improve hydrogen production.

**Cover Letter**

Department of Biomedicine,
Biotechnology and Public Health
Biochemistry
Faculty of Science

Torre Sur, Planta 1ª
Campus Univ. Rio San Pedro
11510 Puerto Real (Cádiz)
Tel. 956012791.

30th, October 2018

Dear Editor
Biotechnology advances

Please, find enclosed the manuscript entitled **"Metabolic engineering for the optimization of hydrogen production in *Escherichia coli*: A review"**. The aim of this review is to study the mechanisms of hydrogen synthesis by *Escherichia coli* and the state of the art of the metabolic engineering strategies used to improve hydrogen yield and productivity in this microorganism. These strategies are summarized and illustrated in a metabolic diagram.

The authors of this work are: Antonio Valle, Domingo Cantero and Jorge Bolívar and all of them declare no financial or commercial conflict of interest and we proposed Jorge Bolívar Pérez and Antonio Valle Gallardo as corresponding authors for this paper. This is an original work and it has not been submitted previously to any journal.

Jorge Bolivar & Antonio Valle

**\*Conflict of Interest Form**

**UCA** | Universidad de Cádiz

Department of Biomedicine, Biotechnology and Public Health
Biochemistry
Faculty of Science

Torre Sur, Planta 1ª
Campus Univ. Rio San Pedro
11510 Puerto Real (Cádiz)
Tel. 956012791.

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Jorge Bolivar & Antonio Valle

1  **Metabolic engineering for the optimization of hydrogen production in**

2  ***Escherichia coli*: A review**

3

4  Antonio Valle[1a*], Domingo Cantero[2a] and Jorge Bolívar[1b*]

5

6  [1]Department of Biomedicine, Biotechnology and Public Health-Biochemistry and

7  Molecular Biology.

8  [2]Department of Chemical Engineering and Food Technologies.

9  [a]Instituto Universitario de Investigación Vitivinícola y Agroalimentario (IVAGRO),

10  [b]Instituto Universitario de Investigación de Biomoléculas (INBIO)

11  Faculty of Sciences, University of Cádiz, 11510 Puerto Real, Cádiz, Spain.

12

13  [*]Corresponding to: email: antonio.valle@uca.es and jorge.bolivar@uca.es

14  Phone: +34 956 012820 // 2045

15  Fax +34 956016411

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**Abstract**

Hydrogen is a potential sustainable energy source and it could become an alternative to fossil fuel combustion, thus helping to reduce greenhouse gas emissions. The biological production of hydrogen, instead of its chemical synthesis, is a promising possibility since this process requires less energy and is more sustainable and eco-friendly. Several microorganisms have been used for this purpose, but *Escherichia coli* is the most widely used in this field. The literature in this area has increased exponentially in the last 10 years and several strategies have been reported in an effort to improve hydrogen production. These strategies include heterologous gene expression, adaptive evolution and metabolic and protein engineering. On the other hand, culture conditions, including the use of carbon sources such as glycerol, a waste product from the biodiesel industry, have also been considered. This review includes a discussion of the hydrogenase complexes responsible for the hydrogen synthesis in *E. coli* and the central carbon metabolism pathways connected to this process. The different strategies applied to obtain engineered strains in which the carbon and electron fluxes are redirected towards hydrogen synthesis are also discussed. Yields and productivities of the most relevant engineered strains reported using several carbon sources are also compared.

## 1. Introduction

Global warming is caused by greenhouse gas emissions from fossil fuel combustion (Montzka et al., 2011) and this has been the primary source to satisfy the world's energy demand (Davila-Vazquez et al., 2008). The scientific community agree that it is necessary to mitigate the depletion of fossil fuel resources and to protect the environment against their negative effects by exploring new renewable and environmentally friendly energy sources that could replace fossil fuels (Dharmadi et al., 2006).

Of all of the renewable energy sources, which include ethanol or algal diesel, hydrogen ($H_2$) is an interesting alternative to be explored since it has several advantages when compared to fossil fuels. These advantages include the following: (i) molecular hydrogen has a higher energy bond by a factor of around 3 when compared to carbon fuels (Hallenbeck and Ghosh, 2009; Momirlan and Veziroglu, 2005; Mudhoo et al., 2011), (ii) nontoxic by-products are produced by breaking the $H_2$ covalent bond, (iii) hydrogen is a renewable, efficient and clean energy source (Hansel and Lindblad, 1998) and (iv) it is utilized for fuel cells in portable electronics, power plants and internal combustion engines (Dunn, 2002).

However, $H_2$ is currently considered as a clean energy carrier rather than an energy source because it does not come from natural sources. Indeed, $H_2$ is produced mainly by the electrolysis of water (Armaroli and Balzani, 2011), by the water-gas shift reaction (Yi and Harrison, 2005), and as a by-product from petroleum refining or chemical production (Das and Veziroglu, 2001; Westermann et al., 2007). These methods for $H_2$ production are neither ecological nor environmentally friendly because they require high energy input to generate $H_2$ gas (Das and Veziroglu, 2001). The commercial production of $H_2$ by electrolysis of water achieves an efficiency of 75%; however, the

cost of this process is currently several times higher than that of hydrogen produced from fossil fuels (Ewan and Allen, 2005). Moreover, most of the production depends on fossil resources and this is clearly unsustainable (Edwards et al., 2007). It is therefore necessary to improve this technology, not only by the development of $H_2$ storage and distribution, but also through an efficient and sustainable production method. In this respect, over the last few years the European Commission has developed several activities through the technological initiative 'Fuel Cells and Hydrogen Joint Undertaking' (FCH JU) under Horizon 2020 ((FCH)). This program is a unique public-private partnership that supports research, technological development and demonstration activities in fuel cell and $H_2$ energy technologies in Europe. Given this background, biological $H_2$ or biohydrogen production is a feasible alternative when compared to the commonly used technologies outlined above. The biological approach has significant advantages over chemical methods since it could be performed at relatively low temperatures (25–37 ℃), at atmospheric pressure and at relatively high rates (Trchounian and Trchounian, 2015). Efficient separation techniques from bioreactor headspaces have also been developed (Fan, L.S. et al., 2009). The biological production of $H_2$ can be classified into three major categories: (1) biophotolysis of water using algae and cyanobacteria, (2) photofermentation of organic compounds by photosynthetic bacteria and (3) dark fermentative production by heterotrophic microorganisms (Hallenbeck, 2005). The theoretical yields of $H_2$ from dark fermentations depend on the type of organism used in the fermentation (Mathews and Wang, 2009) but in general it is more efficient than photosynthetic processes (Horizonte 2020 Comisión Europea).

Of all the $H_2$-producing microorganisms described in the literature, the bacterium *Escherichia coli* is one of the most widely used for anaerobic fermentation due to its well-known metabolism and also because it is relatively easy to rewire its metabolic pathways through genetic engineering techniques (Atsumi and Liao, 2008; Blankschien et al., 2010; Chen et al., 2013; Choi et al., 2012; Clomburg and Gonzalez, 2010; Dharmadi et al., 2006; Ganesh et al., 2012; Joung et al., 2011; Maeda, Toshinari et al., 2008; Nakamura and Whited, 2003; Trinh and Srienc, 2009; Valle et al., 2012; Wang et al., 2011; Yu et al., 2010). The interest in this field is currently increasing, as evidenced by the exponential growth in publications since 2000. For instance, a search in SCOPUS using the terms 'hydrogen' (physics, chemistry, environmental engineering, biochemistry, technology, etc.), 'hydrogen and *Escherichia coli*' and 'hydrogen and *Escherichia coli* and engineering' indicates an exponential growth in the last 18 years (Fig. 1). It noteworthy that the growth rates of publications with the terms 'hydrogen' versus 'hydrogen and *Escherichia coli*' have been very similar in the last ten years, although the number of publications related to engineering and *E. coli* to improve $H_2$ production has increased significantly since 2000, which reflects the growing number of researchers interested in this topic and probably indicates a change in the paradigm of biofuel production in the near future.

In anaerobic fermentative hydrogen production (FHP), numerous carbon sources such as glycerol, glucose, formate, sucrose, lactose, galactose, arabinose, fructose, gluconate, maltose, mannitol, sorbitol, trehalose, and xylose can be used (Goyal et al., 2013; Rosales-Colunga and De León Rodríguez, 2015; Trchounian, 2015; Trchounian and Trchounian, 2015). Nevertheless, in order to make $H_2$ production by *E. coli* economically viable on a large-scale, organic substrates from agricultural, chemical, food or by-product wastes should be used (Trchounian, 2015). In this regard, glycerol

128 has become an abundant and inexpensive carbon source because it is generated as a by-

129 product in the biodiesel fuel industry (Sarma et al., 2012). This compound has been

130 widely used in the generation of $H_2$ by *E. coli* and other bacteria (Cofré et al., 2016;

131 Dharmadi et al., 2006; Hu and Wood, 2010; Kim et al., 2010; Sarma et al., 2012; Tran

132 et al., 2015; Trchounian and Trchounian, 2015; Valle et al., 2015a). Several advances

133 have been made in studies that address the possibility of improving yields and

134 productivities by using glycerol (Akhtar and Jones, 2008; Cofré et al., 2016; Henstra et

135 al., 2007; Hu and Wood, 2010; Trchounian and Trchounian, 2015; Valle et al., 2015a;

136 Valle et al., 2017), glucose (Maeda et al., 2007a; Seol et al., 2014; Seol et al., 2016;

137 Trchounian and Trchounian, 2014b; Yoshida et al., 2006; Zhu et al., 2011) or even both

138 together in the same culture medium (Trchounian et al., 2011; Trchounian et al., 2014;

139 Trchounian and Trchounian, 2013) as the main carbon sources. Glycerol is a highly

140 reduced carbon source compared to sugars and it therefore offers an effective

141 opportunity to obtain $H_2$ and other reduced products by bacteria (Ganesh et al., 2012;

142 Khanna et al., 2012). The energy content of pure glycerol is 19.0 MJ/kg, although it is

143 only 25.30 MJ/kg for crude glycerol, probably due to the presence of methanol and

144 traces of biodiesel (Strathclyde). Such a high energy content in crude glycerol indicates

145 its great potential to be an effective substrate for $H_2$ production.

146

147 The margin for increasing the efficiency of $H_2$ production is potentially high since, from

148 a microbial evolutionary perspective, the low biological $H_2$ yields in the natural strains

149 can be attributed to the fact that microorganisms capable of FHP have developed their

150 metabolic pathways preferentially for cell growth rather than for $H_2$ synthesis.

151 Therefore, the yields and productivities can be improved significantly by metabolic

152 engineering, which eventually will depend on the carbon source used in the

153  fermentation. For this reason, some efforts have been focused on obtaining $H_2$ from

154  glycerol (Cai et al., 2011; Davila-Vazquez et al., 2008; Rosales-Colunga and De León

155  Rodríguez, 2015). In this review, we describe the state of the art for the strategies used

156  to enhance $H_2$ production in *E. coli* by using glycerol as the main carbon source and the

157  results are compared with those obtained on using glucose.

158

159  **2.    Biosynthesis of $H_2$ in *E. coli***

160  *2.1 Fermentation of glycerol or glucose: from carbon source to formate*

161
162  $H_2$ is formed during the anaerobic mixed-acid fermentation in which pyruvate (PYR),

163  the final product of glycolysis, is converted into formate and acetyl-CoA, the precursors

164  of $CO_2 + H_2$ and ethanol, respectively (Dharmadi et al., 2006). Fermentation of glycerol

165  starts with its conversion to dihydroxyacetone phosphate (DHAP) in a process that is

166  mediated by a two-branch pathway: the oxidative branch by glycerol dehydrogenase

167  (GldA) and dihydroxyacetone kinase enzymes (DhaKLM), and the reductive branch by

168  glycerol kinase (GlpK) and glycerol 3-P dehydrogenase (GlpABC) enzymes. DHAP can

169  be metabolized in the glycolysis pathway to PYR, although a unique characteristic of

170  glycerol metabolism is that the conversion of phosphoenolpyruvate (PEP) into PYR is

171  coupled to DHA phosphorylation. In this process 1 $NAD^+$ is reduced, in the assimilation

172  of glycerol and in the synthesis of 1,3-bisphosphoglycerate (1,3-BPG) (Cintolesi et al.,

173  2012), to yield 1 mol PYR per mol glycerol (Fig. 2). In the case of glucose

174  fermentation, PYR is generated from glycolysis and 2 $NAD^+$ is also reduced in the same

175  step (conversion of glyceraldehyde 3-phosphate to 1,3-BPG), but the fermentation yield

176  is 2 mol PYR per mol glucose, i.e., double that obtained with glycerol (Fig. 2).

177  Independently of the substrate for FHP, the synthesis of formate from PYR is essential

178  for the production of $H_2$, which is formed concomitantly with Acetyl-CoA by pyruvate

179    formate lyase (PFL) in microaerobic and anaerobic conditions. Acetyl-CoA is then

180    reduced to ethanol to allow the NADH oxidation for glycolysis maintenance. PEP and

181    PYR are also the precursors for the synthesis of other fermentative end-products such as

182    succinate, acetate, acetolactate, lactate (Sawers and Clark, 2004), 1,2-propanediol

183    (PDO) (Murarka et al., 2008) and even butyric acid under certain conditions (Lugg et

184    al., 2008) (Fig. 2).

185    *2.2 From formate to $H_2$: FHL systems and hydrogenases*
186
187
188    One of the key elements in the strategy for improving $H_2$ production in *E. coli* is to

189    understand how the Formate Hydrogen Lyase (FHL) systems work. These multi-protein

190    complexes operate strictly under anaerobic conditions and they are not only able to

191    synthesize $H_2$ and $CO_2$ in a 1:1 molar ratio by decomposition of formate, but they are

192    also involved in $H_2$ recycling ($H_2 \rightarrow 2H^+ + e^-$). This is not the only way for the cell to

193    produce $H^+$ as it can also be formed in the oxidation of NADH (NADH $\rightarrow$ NAD$^+$ H$^+$),

194    which is produced during glycolysis (Das and Veziroğlu, 2001) and influences the way

195    in which FHL works.

196

197    $H_2$ synthesis is a process that is highly dependent on several factors such as pH$_{out}$,

198    carbon source, temperature, redox potential (E$_h$), presence of exogenous electron

199    acceptors, ratios of end products and $H_2$ concentration (Trchounian, 2015; Trchounian,

200    2012). These factors probably determine which FHL is more active. FHL consists of

201    two enzyme complexes – formate dehydrogenase H (Fdh-H) and [Ni-Fe] hydrogenase

202    enzymes (Hyd) – in *E. coli* (Bagramyan and Trchounian, 2003; Sawers, 2005)  and

203    other facultative anaerobe microorganisms (Sinha et al., 2015). The study of $H_2$

204    production has led to the discovery of multiple and reversible Hyd (Hyd-1, Hyd-2, Hyd-

205    3 and Hyd-4) enzymes. FHL-1 is composed of Hyd-3 complex encoded by the

*hycABCDEFGHI* operon, which is upregulated by an FhlA protein codified by the *fhl* gene (Self and Shanmugam, 2000). This complex is well characterized and is the main $H_2$-producing system during glycerol and glucose fermentation at acidic pH and it has been widely described and reviewed by Trchounian's group (Trchounian, 2015; Trchounian et al., 2012; Trchounian and Trchounian, 2015). The FHL-2 system is composed of Hyd-4 and Fdh-H and it is associated with $ATP_{ase}$. This is the least known of all the FHL and hydrogenase enzymes but it is also able to produce $H_2$ in both glucose- and glycerol-based media (Bagramyan et al., 2002; Mnatsakanyan et al., 2004; Trchounian and Trchounian, 2014b), although there is some controversy in this regard (Noguchi et al., 2010; Self et al., 2003; Skibinski et al., 2002) and its physiological role is not completely clear (Poladyan and Trchounian, 2009; Trchounian et al., 2012) (Fig. 2).

The other two hydrogenases, Hyd-1 and Hyd-2, play a minor role in $H_2$ synthesis and they are probably more related to the recycling of $H_2$, which again depends on pH, carbon source and $H_2$ concentration (Pinske et al., 2012), thus contributing to the reduced quinone pool (Trchounian et al., 2012). Hyd-1 is encoded by the *hya* operon and, despite the fact that it is not essential for anaerobic growth, it is needed to respond to the $[pH]_{out}$ shift from alkaline to acidic (Brøndsted and Atlung, 1994) and affects $H^+$ translocation across the membrane. Since the proton motive force ($\Delta p$) changes during a $[pH]_{out}$ shift, it has been suggested that Hyd-1 is required to maintain $\Delta p$ in an energy-conserving manner (Trchounian, 2015). Hyd-2 is encoded by the *hyb* operon, the expression is higher in alkaline media (Brøndsted and Atlung, 1994) and it is thought to operate as an oxidizing $H_2$ enzyme under glycerol and glucose fermentation at acidic pH. However, it is involved in $H_2$ production under glycerol fermentation at neutral and

231  slightly alkaline pH (Trchounian and Trchounian, 2009). Several pieces of evidence

232  suggest that either Hyd-1 or Hyd-2 operate preferentially under different conditions

233  (Trchounian, 2015).

234

235  The Hyd enzymes therefore appear to play important roles in increasing the fitness of

236  the bacteria to survive in different environments (Trchounian et al., 2012) (Fig. 2). The

237  physiological role of $H_2$ production mediated by Hyd enzymes seems to involve the

238  discharge of reducing power excess when other suitable electron acceptors, such as $O_2$,

239  are absent and for this reason $H_2$-producing Hyd enzymes are rapidly inhibited by $O_2$

240  (Laurinavichene et al., 2001; Lukey et al., 2010; Trchounian et al., 1998). It is

241  interesting to consider the relationship between $H_2$ and the $H^+$ cycle, since Hyd enzymes

242  have $H^+$ translocation activity and they interact with the $F_0F_1$-$ATP_{ase}$, which is also

243  involved in the $H^+$ cycle (Rossmann et al., 1991). FHL-2 function requires catalytically

244  competent $F_0F_1$-$ATP_{ase}$ and it is associated with $K^+$ uptake via the ATP-dependent TrkA

245  transporter. In this hydrogenase the uptake of $K^+$ is essential for $H_2$ synthesis, since the

246  mutation of the TrkA protein blocked the synthesis at $[pH]_{out}$ 7.5 on using glucose as the

247  carbon source (Trchounian, 2015). This is a key mechanism in the generation of $\Delta p$ and

248  the regulation of $[pH]_{in}$. In summary, Hyd-2 could be considered as a reversible enzyme

249  that can contribute to $H_2$ evolution more significantly in the absence of Hyd-3 and Hyd-

250  1. On the other hand, the oxidizing activity Hyd-1 and Hyd-2 depends on the presence

251  of an active $F_0F_1$-ATP synthase during growth at pH 7.5 and 5.5 with glucose or

252  glycerol fermentation. There is an inverse correlation between hydrogenase-1 and -2

253  activity at pH (5.5 and 7.5) during fermentative growth on glucose and a direct

254  correlation between hydrogenase activity at pH (5.5 and 7.5) during glycerol

255  fermentation. Since the $H^+$ gradient is essential to ATP and $H_2$ synthesis, it seems that

256   both Hyd-1 and -2 play an important role in energy conservation in fermenting *E. coli*

257   cells (Trchounian et al., 2011). Nonetheless, further studies are required to elucidate the

258   physiological relationship between the different hydrogenases and their interaction with

259   these factors (i.e., pH and carbon source) within the bacterial membrane. This research

260   might help to provide a new perspective in the bioenergetics of fermentation.

261

262   **3. Several environmental parameters that affect $H_2$ production in *E. coli***

263
264   It can be deduced from the above discussion of FHL complexes that the environment

265   has a marked impact on $H_2$ production. The most influential factors are outlined below.

266   *3.1 Formate transport and pH*
267
268   Formate or formic acid is a monovalent anionic substance at neutral and slightly

269   alkaline pH and this is one of the major mixed-acid fermentation products in many

270   obligate and facultative anaerobes. This molecule can be used for *de novo* nucleotide

271   biosynthesis in *E. coli* (Jensen et al., 2008). Formate is produced by the action of PFL

272   and can be exported from the cytoplasm or, in the presence of terminal electron

273   acceptors, can be eliminated by the periplasmic formate dehydrogenase oxygen-

274   dependent (FDH-O) or nitrate-dependent (FDH-N). However, formate is re-imported to

275   the cytoplasm and used as a substrate for $H_2$ production by the formate dehydrogenase

276   H-dependent (FDH-H) as a component of FHL-1 when the cells enter the late

277   exponential growth phase or when terminal electron acceptors are absent (Sawers,

278   2005). As a weak acid, formate may act as an uncoupling factor that dissipates Δp and

279   therefore leads to a decrease of $[pH]_{out}$ potentiates. Formate transport in *E. coli* is

280   carried out by the two formate channels named FocA (member of formate-nitrite

281   transporter family FNT), which has an important role in both the export and import of

282 formate (Beyer et al., 2013), and the putative formate transporter FocB, which is less

283 well characterized than FocA (Wang et al., 2009) (Fig. 2). These channels appear to

284 control formate translocation in a pH-dependent manner. However, it should be noted

285 that during glucose fermentation at a pH value higher than 7 the FHL-2 complex is

286 probably responsible for $H_2$ production, whereas when the pH is below 7 FHL-1 it is

287 more active (Trchounian and Trchounian, 2014a). Formate can be transported across the

288 membrane under different conditions. The FocB transporter preferentially imports the

289 formate to produce $H_2$ at different $[pH]_{out}$ with any carbon source (glucose or glycerol).

290 However, FocA exports formate during glucose fermentation at any pH and with

291 glycerol at pH 7.5, but it imports formate at pH values below 7 (Fan, Z. et al., 2009;

292 Trchounian and Trchounian, 2014a). From the results of these studies it can be deduced

293 that $H_2$ production during growth on different carbon sources depends on the *focA* and

294 *focB* genes. These channels seem to control formate translocation in a pH-dependent

295 manner and when both channels are absent it may lead to enhanced $H_2$ production

296 (Trchounian and Trchounian, 2014a). However, formate channels do not function in a

297 separate way and some interactions with other proteins have been identified. A study

298 published by Falke et al. (Falke et al., 2016) highlighted the glycyl-radical enzyme

299 (GRE) TdcE protein as a specific interaction partner of the formate-specific channel

300 FocA, which would play a similar role to that of PflB. Falke et al. (Falke et al., 2016)

301 ruled out the possibility that FocA-binding is a general property of GREs and only TdcE

302 and PflB interact with FocA.

303 *3.2 pH, hydrogenase activity and carbon source*
304
305 Trchounian's group has studied extensively the dependence of hydrogenase activities on

306 pH and their crosstalk functions (Trchounian, 2015; Trchounian et al., 2012;

307 Trchounian and Trchounian, 2015). $H_2$ production depends on $[pH]_{out}$ and carbon

source (glycerol or glucose) in the culture medium and FHL systems are involved in the regulation of $[pH]_{in}$ (Bock and Sawers, 2006); the results can be summarized as follows and in Table 1:

(1) At $[pH]_{out}$ 5.5 hydrogenases Hyd-1 and Hyd-2 seem to have similar functions in oxidizing hydrogen but Hyd-3 is involved in $H_2$ production, which has the highest rate when $H_2$ uptake by Hyd-1 and Hyd-2 showed low activity.

(2) At pH 6.5 Hyd-3 is the main $H_2$-producing enzyme during glycerol fermentation, although it might operate in a reverse direction and have significant $H_2$ uptake activity with glucose. Hyd-2 and Hyd-1 are involved in $H_2$ production in glycerol-containing media but operate in oxidizing mode in culture media that contain both glucose and formate.

(3) At pH 7.5 Hyd-3 with glycerol shows $H_2$ uptake activity but Hyd-2 and Hyd-1 are involved in $H_2$ production in glycerol-containing media, as described for pH 6.5.

*3.3 $H_2$ and $CO_2$ partial pressure*

$H_2$ is produced under strictly anaerobic conditions and studies carried out in *E. coli* have generally been performed in closed vials with headspace, although in several cases it has been reported that headspace pressure can have a negative effect on the synthesis of $H_2$, theoretically by the recycling operating mode of $H_2$ for Hyd-1 and/or Hyd-2 (Maeda, Toshinari et al., 2008). Therefore, low partial pressure fermentation is often applied to enhance $H_2$ production, as described in several studies (Dharmadi et al., 2006; Kim et al., 2006; Maeda, Toshinari et al., 2008; Mizuno et al., 2000). The concentration of $CO_2$ also has a negative effect on the $H_2$ production rate and yield because the synthesis of succinate consumes $CO_2$ and PYR via OAA synthesis. This

334  latter pathway competes with the synthesis of formate and therefore $H_2$ production

335  decreases – as a consequence, rapid gas removal and separation of $H_2$ are essential to

336  increase the production.

337

338  **4. Metabolic engineering strategies for improving the synthesis of $H_2$**

339  Metabolically engineered microbial strains can give higher yields of target products for

340  biotechnological applications. Different strategies for metabolic engineering have been

341  carried out in the last 10 years with several microorganisms, although *E. coli* is the most

342  commonly used for improving $H_2$ production. Metabolic pathways in *E. coli* have been

343  widely studied (Blattner et al., 1997), but there are still some gaps in our knowledge

344  about $H_2$ production.  For instance, very few studies have focused on the influence of

345  the carbon source in $H_2$ production. In this sense, several recent studies have shown

346  how the manipulation of certain genes can be beneficial for $H_2$ production using glucose

347  as the carbon source but they do not have the same effect on using glycerol and may

348  even have the opposite effect. For example, it has been reported that Hyd-1 and Hyd-2

349  are $H_2$-consuming enzymes during glucose metabolism, whereas they are $H_2$-producing

350  enzymes in the presence of glycerol at neutral and slightly alkaline pH (Trchounian et

351  al., 2011). In addition, anaerobic growth with glycerol is somewhat lower than with

352  glucose (Hu and Wood, 2010). Given the advances in the knowledge of metabolomics

353  and genetic manipulation techniques, the strategies for improving $H_2$ in *E. coli* have

354  evolved. In the past, genetic modifications were achieved through random processes

355  such as exposure to ultraviolet light, chemical mutagenesis etc., but nowadays genetic

356  engineering is widely used to obtain engineered strains with higher productivities

357  (Maeda et al., 2012). With the help of these new techniques, and based on the wide

358  availability of metabolic information on *E. coli*, several metabolic engineering strategies

359    to improve $H_2$ production can be designed *a priori* (Manish et al., 2007). It should be

360    noted, however, that genetic engineering is not always fruitful, as undesirable effects are

361    often encountered in the engineered organisms (Colletti et al., 2011). There is a need for

362    an extensive analysis and detailed understanding of metabolic fluxes and their

363    regulatory circuits that lead to $H_2$ formation. It is important to have a fundamental

364    knowledge of how the metabolic pathway flux responds to varying genetic and

365    environmental perturbations and whether the pathways can be deliberately redirected

366    towards $H_2$ synthesis rather than to the production of unwanted metabolites. The state of

367    the art of metabolic engineering in *E. coli* for $H_2$ production on using glycerol or

368    glucose as carbon sources is reviewed below and the most important strategies and

369    results reported to date are described and summarized in Table 2.

370

371    *4.1 Screening/High-throughput screening*
372

373    Screening and high-throughput screening methods can be useful to find new phenotypes

374    of $H_2$-producer knock out strains or potential genes to be overexpressed, as well as to

375    engineer further strains to improve yields and productivities. Mohd et al. (Mohd Yusoff

376    et al., 2013) reported four pseudogenes involved in $H_2$ metabolism with important roles

377    in cell physiology in a screening of 3985 *Escherichia coli* mutants from the Keio

378    Collection (Baba et al., 2006) by using $H_2$ chemochromic membranes. In another

379    screening of 150 single knock out mutants from the same collection, 12 novel strains

380    with enhanced yields for $H_2$ and/or ethanol production were detected in cells grown in a

381    glycerol-based medium by analyzing $H_2$ and ethanol production by gas chromatography

382    (Table 2B) (Baba et al., 2006; Valle et al., 2015a). In the same work, the knock out

383    genes that most significantly improved the parameters evaluated (including *gnd* and

384 *tdcE* genes) were combined and the resultant quintuple mutant significantly increased

385 the molar yields of $H_2$ production and glycerol consumption.

386

387 Barahona et al. (Barahona et al., 2016) suggested that there is a limitation in the

388 discovery or synthesis of better $H_2$-producing enzymes due to the absence of methods

389 for the high-throughput screening of $H_2$ production in biological systems. In an effort to

390 overcome this drawback, they engineered a natural $H_2$-sensing system of *Rhodobacter*

391 *capsulatus* to direct the emission of LacZ-dependent fluorescence in response to the

392 nitogrenase-produced $H_2$. This system was used in combination with fluorescence-

393 activated cell sorting flow cytometry to screen large libraries of nitrogenase Fe-protein

394 variants generated by random mutagenesis. This method was used to select an $H_2$-

395 overproducing Fe-protein variants lacking 40% of the wild type amino acid sequence,

396 and subsequently microbial $H_2$ production was improved by using directed evolution of

397 nitrogenases and hydrogenases in the selected protein variants (Barahona et al., 2016).

398 This strategy could be adapted to *E. coli* in order to improve $H_2$ production by

399 combining mutagenesis and high throughput screening.

400 *4.2 Mutagenesis of competitive pathways*
401
402 Several end-products synthesized in the mixed-acid fermentation, such as lactate,

403 acetate, ethanol and succinate, must be exported out from the cell in order to maintain

404 the $NADH/NAD^+$ balance and intracellular pH.

405

406 Since these metabolites are reduced molecules, their syntheses are presumably

407 competitive pathways of $H_2$ production. Therefore, the removal of the synthesis of one

408 or more of these end-products should theoretically result in an increased flux towards

409    H$_2$ synthesis. This strategy has been applied to several reported strains (Sawers and

410    Clark, 2004).

411    • Lactate. Deletion of the lactate dehydrogenase gene (*ldh*) helps to drain PYR

412        flux towards H$_2$ (Table 2A and B), although this occurs only when the medium

413        is acidic, since this condition stimulates the formation of lactate (Hallenbeck and

414        Ghosh, 2012).

415    • Succinate is another end-product whose synthesis can be reduced by knocking

416        out the fumarate reductase (FRD) operon (*frdABCD*) responsible for the

417        conversion of fumarate to succinate. This would increase intracellular PYR and

418        therefore H$_2$ production. Several publications have concerned the use of single

419        and double *frd* knock out genes that improve H$_2$ production in glucose- and

420        glycerol-based media (Table 2A). However, the improvement surprisingly varies

421        significantly between the single knock outs (Valle et al., 2015b) and the multiple

422        mutant (Valle et al., 2017), thus indicating that each FRD subunit may have

423        different roles, with H$_2$ production being more favorable when FrdB and FrdC

424        subunits are deleted.

425    • Ethanol. Blocking the synthesis of ethanol by inactivating alcohol

426        dehydrogenase (*adhE*) severely impaired H$_2$ formation and cell growth when

427        glycerol was employed as the carbon source (Murarka et al., 2008; Tran et al.,

428        2014). This finding indicates that the synthesis of ethanol is essential to maintain

429        the redox balance and also to allow the synthesis of formate and Acetyl-CoA.

430    • Acetate production can be avoided by removing acetate-producing enzymes

431        such as AckA-PtA (acetate kinase, phosphate acetyl-transferase) or PoxB

432        (pyruvate oxidase) (Murarka et al., 2008), although deletion of the *poxB* gene

433    did not increase $H_2$ synthesis because this mutation reduced cell viability (Tran

434    et al., 2014).

435  •  Butyrate is synthesized from acetyl-CoA via crotonyl-CoA. Despite the fact that

436    this metabolite has been detected in the culture under particular growth

437    conditions (Rosales-Colunga and De León Rodríguez, 2015), strategies for

438    removing butyrate have not been used to date, probably because its production is

439    relatively low.

440  •  1,2-Propanediol is synthesized by methylglyoxal synthase (MgsA) from DHAP

441    and it has been reported that the single knock out strain for the gene of this

442    enzyme increases slightly the production of $H_2$ in glycerol media (Tran et al.,

443    2014) (Table 2A).

444  •  The conversion of PYR into acetyl-CoA is catalyzed by the PYR dehydrogenase

445    complex (PDH) under aerobic conditions, but in anaerobic conditions PYR is

446    transformed into acetyl-CoA and formate by PYR formate lyase (PFL), which is

447    essential for $H_2$ synthesis. However, under microaerobic conditions the two

448    enzymes can be functional (Sawers and Clark, 2004). Despite PDH operon

449    genes (*aceEF*, *lpd*) having low expression levels in anaerobic conditions, the

450    activity of this enzyme could be a competitive pathway in formate synthesis and

451    therefore deletion of the *aceE* subunit resulted in an increased $H_2$ yield with a

452    glucose-based medium (Maeda et al., 2007a). However, significant

453    improvements were not observed on using glycerol (Tran et al., 2014).

454  •  Conversion of PEP into OAA by the anaplerotic enzyme PEP carboxylase (Ppc)

455    can be considered a competitive reaction since it diverts the C3 compound

456    toward succinate. For this reason, the knock out of this gene increases $H_2$

457    productivity significantly (Tran et al., 2014) (Table 2A).

- Hydrogenases (Hyd-1, -2, -3, -4) are the key controlling enzymes in the synthesis or oxidation of $H_2$ and their function depends on pH and carbon sources, as described previously. Thus, removal of the $H_2$-consuming activity of Hyd-1 and/or Hyd-2 by knocking out the large subunit of both enzymes ($\Delta hyaB\Delta hybC$) in an acidic culture medium is a strategy that has been successfully employed by several groups using both glycerol- (Maeda, T. et al., 2008) and glucose-based media (Ghosh and Hallenbeck, 2010; Maeda et al., 2007a; Maeda, Toshinari et al., 2008; Seol et al., 2014; Seol et al., 2016). In addition, the overexpression of Hyd-3, the most important enzyme that forms FHL-1, in FHP when glycerol is used at pH 7.5 increases $H_2$ production. Hence, the FHL expression may be manipulated by overexpression of *fhlA* (Horizonte 2020 Comisión Europea) or deletion of the *hycA* gene that encoded a repressor of FhlA expression (Penfold et al., 2003; Yoshida et al., 2005). Interestingly, a combination of *fhlA* overexpression and disruption of *hycA* did lead to a 2.8-fold increase in the $H_2$ formation rate (Yoshida et al., 2006) (Table 2A, B).

There are two additional formate dehydrogenases encoded by *fdnG* (a subunit of nitrogen-dependent formate dehydrogenase-N) and *fdoG* genes (a subunit of oxygen-dependent formate dehydrogenase-O) that consume formate but do not produce $H_2$. However, the deletion of *fdoG* did not improve the $H_2$ yield in a glycerol-based medium and the *fdnG* mutant showed only a moderate improvement in $H_2$ production (Maeda et al., 2007a; Rossmann et al., 1991).

Other enzymes or regulator proteins involved in formate consumption that may compete with the synthesis of $H_2$ are outlined below:

(1) Nitrate reductase A (whose α-subunit is encoded by the *narG* gene) consumes formate in the conversion of nitrate into nitrite by using electrons produced from formate by FDH-N. Deletion of the *narG* gene leads to a very modest $H_2$ productivity but in combination with other mutations the $H_2$ productivity increases significantly (Maeda et al., 2007a; Tran et al., 2014).

(2) FNR protein is a global DNA-binding transcriptional regulator that stimulates the transcription of several genes required for fermentation and anaerobic respiration and represses others like *hyf* operon, which encoded Hyd-4 (Salmon et al., 2003). As a consequence, deletion of the *fnr* gene leads to a three-fold increase in HyfR expression as the activator of *hyf* operon.

(3) The NarL protein is a global transcription repressor for the genes of FhlA, PFL, and nickel transporter *nik* operon. Inactivation of the *narL* gene led to a two-fold increase in the molar yield of $H_2$, with similar growth to that of the wild-type strain (Fan, Z. et al., 2009).

(4) Formate channels (FocA and FocB) appear to control formate translocation in a pH-dependent manner and the absence of both formate channels may lead to enhanced FHP (Trchounian and Trchounian, 2014a). Several strategies for metabolic engineering by manipulating these protein transporters with glycerol have been applied to the Δ*focA*Δ*focB* double mutant strain (Table 2A). However, the deletion of *focA* in multiple mutant strains did not increase $H_2$ formation and, indeed, it was even lower with respect to the wild type strain. These results indicate that the formate transporter is necessary for an increase in hydrogen production in these mutant strains (Tran et al., 2014) and this finding is consistent with those reported previously by Maeda et al. (Maeda et al., 2007a) for the conversion of glucose into hydrogen.

*4.3 Adaptive evolution*

Adaptive evolution is a set of environmentally induced mutations that confer growth advantages to the cell (Foster, 2007). In this strategy, an organism is subjected to serial or continuous cultivation for many generations under non-optimal conditions in order to select fitter genetic variants (Cooper et al., 2003). The specific growth rate in glycerol medium of the wild type *E. coli* strain – and even in derivate mutant strains – is quite low compared with that in glucose, which results in low $H_2$ productivity (Murarka et al., 2008). In an effort to overcome this limitation, Hu & Wood (Hu and Wood, 2010) obtained an efficient glycerol-utilizing strain by both adaptive evolution and chemical mutagenesis using a selection method based on increased growth on glycerol. The improved strain produced twenty times more $H_2$ in glycerol medium than the wild type strain. It was also found that the activity of several enzymes related to glycerol consumption were increased 4-fold with respect to the wild type strain, and a whole-transcriptome study revealed that several competitive pathways were repressed in that mutant.

*4.4 Homologous and heterologous expression*

Overexpression of *E. coli* native genes (homologous expression) or those from other species (heterologous expression) has been used to improve $H_2$ production in *E. coli*. The heterologous expression offers the advantages of obtaining more efficient enzyme activities based on kinetic parameters (Valle et al., 2017). The main strategies reported in the literature are described below:

534    (1) Homologous and heterologous hydrogenase-encoding genes. Several heterologous

535    hydrogenases and hydrogenase-related genes have been expressed in *E. coli*:

536    - The expression of HydA from *Clostridium butyricum* improved $H_2$ productivity

537      by around a factor of two (Subudhi and Lal, 2011).

538    - The expression of HydAEFG from *Clostridium* together with the expression of a

539      putative PYR flavodoxin/ferrodoxin oxidorectuase (YdbK) from *E. coli*

540      increased $H_2$ production by 1.5 times (Akhtar and Jones, 2009).

541    - The expression of the hydrogenase HoxEFUYH from the cyanobacteria

542      *Synechocystis* in a Hyd-1 and -2 *E. coli* mutant gave rise to a 41-fold higher

543      production (Maeda et al., 2007b).

544    - Expression of the NADPH-dependent hydrogenases from *Clostridium sp.* and

545      *Bacillus subtilis* together with the increment of the $NAD(P)H/NAD(P)^+$ ratio via

546      overexpression of *glp*X and *zwf* genes. This combination of heterologous

547      expression of NAPDH-dependent hydrogenases and autologous gluconeogenesis

548      and PPP enzymes led to an increase in the $H_2$ yield by 3.5-fold (Kim et al.,

549      2011).

550    (2) Anaplerotic enzymes involved in carbon redirection toward $H_2$ production. The

551    human mitochondrial GTP-dependent PEP carboxykinase (hPEPCK-M) that catalyzes

552    more efficiently the conversion of OAA to PEP has been used due to its kinetic

553    parameters, the use of an energy donor (GTP) and non-allosteric regulation. That

554    heterologous expression was found to enhance significantly the $H_2$ yield and glycerol

555    consumption (Valle et al., 2017).

556    (3) Overexpression of transcription factors. The FHL complex is composed of FDH-

557    H and Hyd-3. The biosynthesis of FHL and PFL are up-regulated by the action of

558    several transcriptional regulators, including the global transcriptional factors Fnr,

amongst other proteins. The transcription of the *fhl* regulon is controlled by the primary and secondary transcriptional activators FhlA and ModE. The biosynthesis of FDH-H also requires the expression of the *selC* gene, which encodes tRNA for the incorporation of selenocysteine to FHD-H. Therefore, the homologous overexpression of these encoded protein genes, *fhl*A, *pfn*r, *psel*C and *pmod*E, has led to an improved $H_2$ production in a glucose-based medium (Fan, Z. et al., 2009).

*4.5 Protein engineering*

Protein engineering to improve $H_2$ production by increasing catalytic reactions is a feasible approach. For instance, the *E. coli* Hyd-3 large subunit (HycE) has been modified and this enhanced the FHL complex formation. The method used by Maeda et al. (Maeda, T. et al., 2008) was based on mutagenesis of the HycE protein by an error-prone polymerase chain reaction (epPCR) and employed a host that lacked hydrogenase activity due to *hyaB hybC hycE* mutations. Seven enhanced HycE variants were obtained and the best contained eight mutations and showed a 17-fold higher $H_2$-producing activity than wild type HycE. Furthermore, shuffling of the DNA sequences of the three most active HycE variants provided a variant that increased $H_2$ production 23-fold. This was the first engineered hydrogenase obtained by random mutation and it seems to strengthen the interaction between the large and small subunits, thus enhancing the flow of electrons for formate dehydrogenase-H (Maeda, T. et al., 2008). The same method was also used to engineer the $\sigma^{54}$ transcriptional factor FhlA, which activates transcription of *fdhF*, and the *hyc*, *hyp* and *hydN-hypF* operons. The FhlA133 variant isolated by Sanchez-Torres et al. (Sanchez-Torres et al., 2009) gave a four-fold increase in $H_2$ production and the whole-transcriptome and promoter reporter analysis revealed that FhlA133 improved $H_2$ production by increasing transcription of all of the genes

585 activated by FhlA (FHL complex). Surprisingly, *fhlA133* also induced four oxidative-

586 stress proteins and 12-stress related genes during anaerobic fermentation. This finding

587 suggests that an increased FHL expression affects the cell physiology and that an

588 increased expression of anti-stress proteins could probably facilitate $H_2$ synthesis

589 (Sanchez-Torres et al., 2009).

590

591 *4.6 Optimizing environmental conditions by Design of Experiment (DOE)*
592
593 The environmental conditions may be optimized for a given engendered strain. $H_2$

594 production has been used as a response variable in order to reduce the number of

595 experiments required to optimize the conditions for maximum yields. For instance, a $3^K$

596 full factorial Box–Behnken design and response surface methodology (RSM) have been

597 employed for experimental design. Ghosh et al. (Ghosh and Hallenbeck, 2010) applied

598 this methodology and obtained a maximum molar $H_2$ yield on using glucose under the

599 optimal conditions at pH 6.5 in the *ΔhyaBΔhybCΔldhAΔfhlC* mutant strain (Rosales-

600 Colunga et al., 2010). Rosales-Colunga et al. (Rosales-Colunga et al., 2010) optimized

601 pH, temperature and substrate concentration of cheese, by using a strain deficient in a

602 protein repressor of Fhl activator factor (HycA) and lactose transporter (LacI). The

603 maximum yield obtained was comparable to the yield achieved in other $H_2$ production

604 processes with *Clostridium sp*. or mixed cultures (Table 2B). Another study in which

605 this method was used focused on optimizing culture media to improve the growth rate,

606 in order to obtain higher productivities of $H_2$ and ethanol and for scaling up the process

607 (Cofré et al., 2012).

608 *4.7 Metabolic Flux Analysis (MFA)*
609
610 The way in which anaerobic bacteria regulate flux distribution under different

611 environmental conditions remains to be elucidated. In this regard, the characterization

of metabolic fluxes is very important to design metabolic engineering strategies as the flux is a fundamental parameter of cell physiology (Cai et al., 2011). Flux balance analysis (FBA) was used to determine intracellular fluxes from substrate uptake to product formation rates by applying metabolite balancing, which is based on the stoichiometry of metabolic reactions (Varma and Palsson, 1994). The implementation of this approach with proteomic, metabolomics and transcriptomic data led to the concept of metabolic flux analysis (MFA). This approach has been applied to optimize the production of lysine, acetate, and ethanol. However, MFA for $H_2$ production has only attracted attention in recent years (Cai et al., 2011) and only Manish et al. (Manish et al., 2007) have reported the theoretical capability of the microorganism to produce $H_2$. They used existing experimental data to calculate fluxes in a batch culture and they commented on the limitations in the yield of $H_2$. MFA was also used to determine the feasible operating space for $H_2$ production against the varying yields of other metabolites. This kind of study has helped to show that ethanol and acetate – but not succinate or lactate – are necessary for $H_2$ production (Manish et al., 2007). It is interesting to note that this deduction was corroborated by experimental results (Murarka et al., 2008; Tran et al., 2014).

## 5. Effect of carbon source and redox balance in $H_2$ synthesis

Independently of the carbon source used, there are several genes that are involved in fermentative pathways, $H_2$ synthesis, TCA-reductive and fermentative pathways whose deletions have been widely reported and constitute the main targets for the metabolic redirection in engineered strains. For instance, the deletions of the genes of hydrogenases-1 and -2 (subunits Δ*hyaB* and Δ*hybC*, respectively), the repressor protein of FhlA (Δ*hycA*), the fumarate reductase (Δ*frdABC*), the lactate dehydrogenase (Δ*ldhA*),

637 the □-formate dehydrogenase-N ($\Delta fdnG$), and the phosphate acetyltransferase (Pta)

638 (underlined and bold in the gray square in Fig. 2), have been widely used to increase the

639 $H_2$ production rate using glucose or glycerol. However, not all of these strategies have

640 been tested using both carbon sources and there are some mutations that have only been

641 assayed with glycerol or glucose. In this sense, there are many mutation strategies, such

642 as deletion of acetate kinase ($\Delta ackA$), pyruvate oxidase ($\Delta pox$), pyruvate dehydrogenase

643 ($\Delta aceEF$), formate dehydrogenase-O ($\Delta fdoG$), phosphofructokinase ($\Delta pfkA$), that have

644 been tested in glucose-containing media but not in a glycerol C source that improves $H_2$

645 synthesis (marked as regular font in the gray square in Fig. 2)

646 On the other hand, some deletion strategies have worked in glycerol but not in glucose

647 media, for instance $\Delta rpiA$, $\Delta gldA$, $\Delta tdcB$, $\Delta tdcE$, $\Delta narG$, $\Delta mgsA$, $\Delta gatZ$. Nevertheless, it

648 has been reported than some mutations do not have the same final effect and even give

649 rise to an opposite result, as is the case of the gluconate dehydrogenase gene ($\Delta gnd$),

650 which has a positive effect when glycerol is used but its overexpression enhanced the

651 production of $H_2$ when glucose was used (marked in gray and also in the red square in

652 Fig. 2).

653 This differences in the engineering strategies on using glycerol or glucose are probably

654 a consequence of the numerous up- and down-regulation metabolic pathways caused by

655 the main regulator protein involved being a transcriptional dual regulator (CRP) (cAMP

656 receptor protein) that regulates the expression of over 180 genes. Many of these genes

657 are involved the central carbon pathways and are also regulated by the cra protein,

658 which carries out a glycolytic flux-dependent process. This latter process is determined

659 by the concentration of fructose-1,6-bisphosphate synthesized in glucose fermentation,

660 which is the molecular effector of the *cra* gene (Kochanowski et al., 2013). The absence

661 of a rapidly metabolizable carbon source, such as glucose, results in an elevated level of

cAMP and subsequently cAMP-CRP increases, which in turn causes catabolite repression. This protein also regulates the catabolism of secondary carbon sources (Soberón-Chávez et al., 2017) and could be related to the enhancement in $H_2$ synthesis reported for several mutant strains related to amino acid metabolism, antibiotic resistance (Table 2A) or pseudogenes (Table 2B).

The strategies outlined above do not always work when either glucose or glycerol are used as the C source. This could be due to the redox balance $NAD(P)H/NAD(P)^+$, which must be maintained at certain levels for an efficient synthesis of formate and thus $H_2$. For instance, the deletion of alcohol dehydrogenase ($\Delta adhE$), because it is a competitive pathway that consumes 2 NADH to produce ethanol, did not improve $H_2$ production. Furthermore, in the case of malate dehydrogenase (Mdh) and malic enzymes (MaeA, MaeB) that use NAD(P)H the synthesis of $H_2$ was even impaired (Valle et al., 2015b). The function of these enzymes and the regulation of the hydrogenase operon's gene expression are involved in maintaining the redox and energy balances and are connected to the proton motive force (PMF) in the intermembrane space. On the other hand, there are heterologous NAD(P)H-dependent hydrogenases that have been overexpressed in *E. coli* (Table 2) and the engineered strains required a higher $NAD(P)H/NADP^+$ ratio to improve $H_2$ production. This has been achieved by overexpressing enzyme genes involved in NADPH production. The carbon source and redox cofactors are two key factors to take into account in the bacterial physiology for improving $H_2$ synthesis.

## 6. Conclusions

In this review, the *E. coli* metabolic pathways most commonly modified with the aim of increasing carbon and electron flux towards $H_2$ biosynthesis are overviewed. The main

metabolic engineering strategies used for this purpose are also reviewed. This information may be useful to design new strategies for the scale-up of $H_2$ production with *E. coli*. The directed mutagenesis of Δ*hyaB*, Δ*hybC*, and Δ*hycA*, which involve hydrogenases-1, -2 and -3, together with Δ*fdnG*, Δ*ldhA*, Δ*frdBC*, and Δ*pta*, which are involved in competitive pathways of formate synthesis, are the most relevant deletions for metabolic engineering to improve $H_2$ regardless of the use of glycerol or glucose as carbon source. On the other hand, the overexpression of enzymes involved in glycerol assimilation (GldA), transcriptional factor Hyd-3 operon (FhlA), heterologous enzyme of human PEPCK, or hydrogenases have also been reported as potential strategies together with directed mutagenesis to enhance $H_2$. There are numerous other strategies (for instance, high-throughput screening, protein engineering, adaptive evolution or random mutagenesis) that have been applied in order to design engineered strains. It is also very useful to implement this information in modeling (MFA) or *in silico* studies in order to predict the optimized pathway to enhance the production of the target product. Although MFA is a suitable method for modeling, there is still a great deal of information that can be obtained from multiomic platforms, which are very valuable tools to design engineered strains that will be used as a microbial cell factory for $H_2$ production.

713

**Declaration of interest**

The authors declare that they have no conflict of interest.

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**Table and Figure caption**

Table 1. Activity (oxidizing or production mode) of hydrogenases 1, 2, 3 and 4 as a function of carbon source (glycerol or glucose) and $pH_{out}$. *In lacking Hyd-3 strains, in Minimal medium Hyd-2 activity is enhanced. **In MM and peptone medium. Hyd-3 activity is increased when Hyd-2 is deleted. [‡]Medium with glucose+ formate. [†]Low activity

1079

1080   Table 2. In an effort to facilitate the comprehension of the metabolic engineering

1081   strategies, the genotype of engineered mutant strains, the metabolic pathways redirected

1082   in every strain, strategies of metabolic engineering, environmental conditions such as

1083   pH, headspace, and parameters of hydrogen productivity, molar yields and the

1084   respective references, are described in for glycerol (A): *relativized molar yield respect

1085   to wild type strain values (mol $H_2$/ mol glycerol consumed). **Relativized productivity

1086   respect to wild type strain values (mol $H_2$/g DCW/h). ****Adding 10 mM formate to

1087   glycerol complex medium. [†]Complex-formate medium. [‡]Minimal medium with glycerol

1088   1%. [¥]Peptone glycerol medium 1%. For glucose fermentation (B): *Relativized molar

1089   yield respect to wild type strain values (mol $H_2$/ mol glucose consumed). **Relativized

1090   productivity respect to wild type strain values (mol $H_2$/g DCW/h //// mol $H_2$/mg protein

1091   /// mol $H_2$/ g DCW). *Cheese whey (20 g/L). **Lactose (5 g/L). ***Complex-formate

1092   medium. ****In vitro assay. *****Complex medium (contain fructose, galactose,

1093   maltose, lactose, glycerol citrate or succinate).

1094

1095   Figure 1. Curves representing the number of documents published from 1960 to 2017

1096   using different refined terms in the SCOPUS database: "hydrogen and *Escherichia coli*

1097   and engineering" (orange triangle); "hydrogen and *Escherichia coli*" (gray circles) and

1098   "hydrogen" (blue diamonds).

1099

1100   Figure 2. General metabolic pathways under anaerobic conditions, the different routes

1101   are represented by different colors. PPP (red), glycolysis (black), anaerobic respiration

1102   (TCA-cycle) (green); glycerol assimilation (brown); Entner–Doudoroff (orange),

1103   carbohydrates by using glycerol (*genes* in bold font) and for glucose fermentation

1104    (*genes* in regular font). The deleted genes are marked with a gray square and genes

1105    overexpressed are marked with a red square. Genes reported in both glycerol based

1106    medium and glucose based medium are underlined.
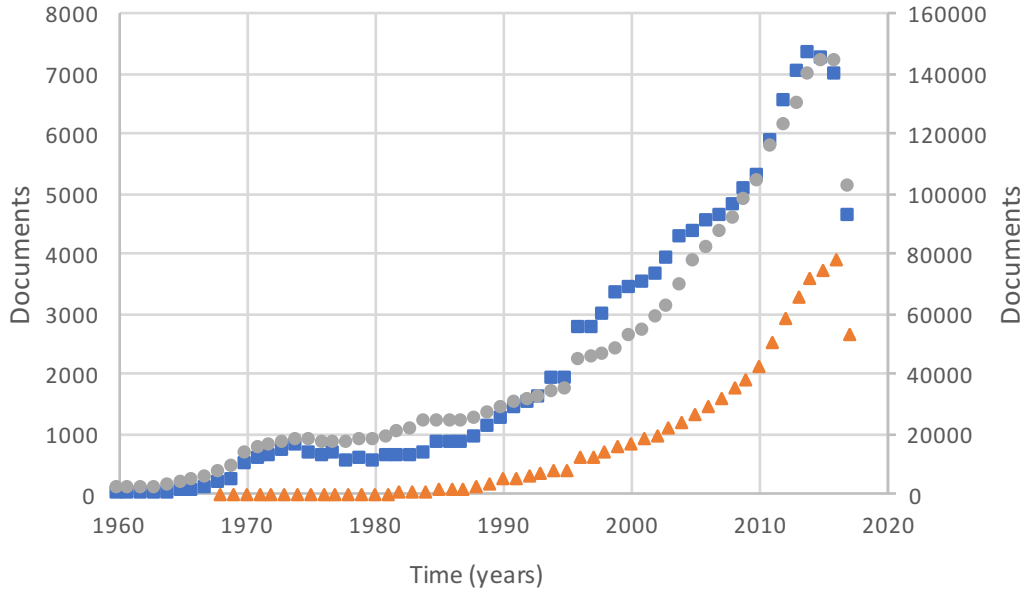
1107

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**Figure 1**



SCOPUS

**Figure 2**
**Click here to download high resolution image**

Figure 2

**Table 1**

| | pH$_{out}$ | | |
|---|---|---|---|
| | 5.5 | 6.5 | 7.5 |
| **Glycerol** | Oxidizing mode (H$_2$ $\rightarrow$ 2H$^+$ + 2e$^-$)) | | |
| | Hyd-1, Hyd-2$^\dagger$, Hyd-4 | Hyd-4 | Hyd-3, Hyd-4 |
| | Production mode (H$^+$+ 2 e$^-$ $\rightarrow$ H$_2$) | | |
| | Hyd-3** | Hyd-3**>Hyd-2, Hyd-1 | Hyd-2*> Hyd-1 |
| **Glucose** | Oxidizing mode (H$_2$ $\rightarrow$ 2H$^+$ + 2e$^-$) | | |
| | Hyd-1, Hyd-2, | Hyd-3 | Hyd-2 > Hyd-1 |
| | Production mode (H$^+$+ 2 e$^-$ $\rightarrow$ H$_2$) | | |
| | Hyd-3, Hyd-4 | Hyd-2, Hyd-1$^\ddagger$, Hyd-4 | Hyd-4, Hyd-3$^\ddagger$ |

**Table 2**

## Table 2A. Carbon source: Glycerol

| Strains, genotype (ID) | Phenotype | Metabolic pathways redirected | Metabolic Engineering strategies | pH$_{out}$ | Headspace (partial pressure) | Yield* | Produc-tivity** | References |
|---|---|---|---|---|---|---|---|---|
| BW25113 ΔhyaB ΔhybCΔhycE/pBS(Kan)HycE variant satHycE12T366 | Defective in Hyd-1, Hyd-2, Hyd-3 and overexpression of Hyd-3 catalytic subunit HycE with protein variation | Hydrogen synthesis by FHL-1 complex | Protein engineering | 7 | High | 30 | - | Maeda 2008[†] |
| BW25113 ΔhyaB ΔhybC ΔhycE/pBS(Kan)HycE variant shufHycE1-9 | Defective in Hyd-1, Hyd-2, Hyd-3 and overexpression of Hyd-3 catalytic subunit HycE with protein variation | Hydrogen synthesis by FHL-1 complex | Protein engineering | 7 | High | 23 | 9 | |
| MG1655 ΔfrdA::FRT Δpta::FRT-tet-FRT (SY03) | Defective of FRQ subunit FrdA and phosphotransacetylase (PtA) | TCA- reductive branch Fermentative pathway | Mutagenesis of competitive pathways | 6.3 | Low | 1.23 | 1.1 | Yazdani 2008[‡] |
| MG1655/pZSKLMgldA | Overexpression of dihydroxiacetone kynase (DhaKLM) and Glycerol dehydrogenase (GldA) | Glycerol assimilation | Homologous expression | 6.3 | Low | 1.10 | 1.5 | |
| BW25113 ΔfrdCΩkan$^R$/pCA24N-gldA (HW2) | Defective of FRQ subunit FrdC and overexpression of GldA | Glycerol assimilation and TCA | Mutagenesis of competitive pathways Adaptive evolution Chemical mutagenesis Homologous expression | 6.3 | High | - | 20 | Hu H. 2010[‡] |
| BW25113 ΔhyaBΩkan$^R$ | Defective of Hyd-1 large subunit (HyaB) | Reverse H$_2$ oxidizing mode | Mutagenesis competitive pathways | 6.5 | High | - | 1.76 | Trchounian 2011[¥] |
| BW25113 ΔhybCΩkan$^R$ | Defective of Hyd-2 large subunit (HybC) | Reverse H$_2$ oxidizing mode | Mutagenesis competitive pathways | 6.5 | High | - | 1.5 | |
| *E. coli* B SS1 | | - | High-throughput screening from isolated strains | 7 | | - | 1.67 | Suhaimi, 2012[¥] |
| BW25113 ΔhyfGΔfhlAΩkan$^R$ | Defective in Hyd-4 large subunit (HyfG) and protein regulator of *hyc*, *hyb* and *hya* operons expression (FhlA) | Reverse H$_2$ oxidizing mode | Mutagenesis competitive pathways | 6.5 | High | - | 1.7 | Trchounian 2012[¥] |
| BW25113 ΔhyaBΩkan$^R$ | Defective in Hyd 1 large subunit (HyaB) | Reverse H$_2$ oxidizing mode | Mutagenesis competitive pathways | 7.5 | High | - | 1.34 | Sanchesz Torres 2013[‡] |
| BW25113ΔfrdCΔldhAΔfdnGΔppcΔnarGΔmgsAΩkan$^R$ | Defective in FrdC, LdhA, FdnG, Ppc, NarG, methylglyoxal synthase (MgsA). | Fermentative pathway TCA reductive Branch Hydrogenase synthesis | Mutagenesis competitive pathways. Screening mutant strains Homologous expression | 6.5 | High | - | 4.3 | Tran 2014[‡] |
| BW25113ΔfrdCΔldhAΔfdnG ΔppcΔnarGΔmgsAΔhycAΩkan$^R$ | Defective in FrdC, LdhA, FdnG, Ppc, NarG, MgsA and repressor of the FHL complex (HycA). | | | | | 1.3 | 1.68 | |

| Strains, genotype (ID) | Phenotype | Metabolic pathways redirected | Metabolic Engineering strategies | pH$_{out}$ | Headspace (partial pressure) | Yield* | Produc-tivity** | References |
|---|---|---|---|---|---|---|---|---|
| BW25113Δ*frdC*Δ*ldhA*Δ*fdnG*Δ*ppc*Δ*narG*Δ*mgsA*Δ*hycA*Ωkan$^R$/pCA24N-FdhF | Defective in FrdC, LdhA, FdnG, Ppc, NarG, MgsA and HycA. Overexpression of formate dehydrogenase large subunit (FdhF) | Fermentative pathway TCA reductive Branch Hydrogenase synthesis | Mutagenesis competitive pathways. Screening mutant strains Homologous expression | 6.5 | High | - | 3.4 | Tran 2014[‡] |
| BW25113Δ*frdC*Δ*ldhA*Δ*fdnG*Δ*ppc*Δ*narG*Δ*mgsA*Δ*hycA*Ωkan$^R$ /pCA24N-FhlA | Defective in FrdC, LdhA, FdnG, Ppc, NarG, MgsA and HycA. Overexpression of FhlA | | | | | - | 3.1 | |
| BW25113Δ*frdC*Δ*ldhA*Δ*fdnG*Δ*ppc*Δ*narG*Δ*mgsA*Δ*hycA*Ωkan$^R$ | Defective in FrdC, LdhA, FdnG, Ppc, NarG, MgsA and HycA | | | | Low | 3.68 | 3.14 | |
| BW25113 Δ*focA*Δ*focB*Ωkan$^R$ | Defective of formate transporter FocA and FocB | Hydrogenase synthesis | Mutagenesis competitive pathways | 5.5 | High | - | 11 | Trchounian, 2014 |
| | | | | 6.5 | | | 3.2*** | |
| *BW25113* Δ*aroM*Ωkan$^R$ | Defective in shikimate kinase II | Aminoacids | Screening mutant strains Random mutagenesis | 7.5 | High | - | 1.7 | Tran 2015[‡] |
| BW25113 Δ*gatZ*Ωkan$^R$ | Defective in tagatose-1,6-bisphosphate aldolase 2 | Sugars | | | | | 1.4 | |
| BW25113 Δ*ycgR*Ωkan$^R$ | Defective in flagellar velocity braking protein, c-di-GMP-regulated | Motility | | | | | 2.4 | |
| BW25113 Δ*yfgI*Ωkan$^R$ | Defective in nalidixic acid resistance protein | Antibiotic resistance | | | | | 2.1 | |
| BW25113 Δ*ldhA*Δ*gnd*Δ*frdBC*Δ*tdcE*Ωkan$^R$ | Defective in LdhA, gluconate dehydrogenase (Gnd), FrdBC subunits and tdcE | Fermentative pathway TCA reductive Branch | Mutagenesis competitive pathways. Screening mutant strains | 7.5 | High | 1.33 | - | Valle 2015[¥] |
| BW25113 Δ*dcuD*Ωkan$^R$/pT-PEPCK | Defective in putative C4-dicarboxylate transporter (DcuD) and expression of human PEPCK mithocondrial | Putative C-4 transporter | Mutagenesis of competitive pathway Heterologous expression | 6.3 | Low | - | 2.46 | Valle 2017[¥] |
| *E. coli* SS1/hycE | Overexpression of Hydrogenase 3-large subunit hycE (*E. coli*) | Synthesis of hydrogen | Screening mutant strains Homologous expression | 5.8 | High | 1.41 | <1.0 | Soo 2017[¥] |
| *E. coli* SS1/hydA | Overexpression of Hydrogenase from *Clostridium* (HydA) | Synthesis of hydrogen | Screening mutant strains Heterologous expression | | | 1.26 | | |

### Table 2B. Carbon source: Glucose

| Strains, genotype (ID) | Phenotype | Metabolic pathways redirected | Metabolic Engineering strategies | pH$_{out}$ | Headspace (partial pressure) | Yield* | Produc-tivity** | References |
|---|---|---|---|---|---|---|---|---|
| BW25113 ΔhyaBΔhybCΔhycAΔfdoG kan$^R$/pCA24N-FhlA | Defective in Hyd-1 and Hyd-2, HycA and formate dehydrogenase-O (FdoG) | Hydrogenase synthesis | Mutagenesis of competitive pathway Homologous expression | 6.8 | High | 1.5 | 26.3*** | Maeda 2008 |
| | | | | | Low | | 141*** | |
| BW25113 ΔhyaBΔhybCΔhycAΔfrdCΔldhAΔaceEΔfdnG Ωkan$^R$ | Defective in Hyd-1 and Hyd-2, HycA, FrdC, LdhA, pyruvate dehydrogenase (AceE) and formate dehydrogenase-N (FdnG) | Hydrogenase synthesis fermentation, pyruvate metabolism and anaerobic respiration | Mutagenesis of competitive pathway Homologous expression | 6.8 | Low | 2 | 4.3 | Maeda 2007 |
| BW25113 ΔhyaBΔhybCΔhycAΔfdoGΔfrdCΔldhAΔpoxB Ωkan$^R$ | Defective in Hyd-1 and Hyd-2, HycA, FodG, FrdC, LdhA, and pyruvate oxidase (PoxB) | | | | | 2 | 4.6 | |
| BW25113 ΔhyaBΔhybCΔhycAΔfdoGΔfrdCΔldhAΩkan$^R$/pCA24N-FhlA | Defective in Hyd-1 and Hyd-2, HycA, FodG, FrdC, LdhA, and overexpression of FhlA | | | | | 2.5 | 4.4 | |
| W3110 ΔfocA::FRT/pfnr (ZF13) | Defective in Formate transporter A and overexpression of global transcriptional regulator (Fnr) of anaerobic metabolism; activator of fhlA and pflB genes, and of the hyp, nik (Ni$^{+2}$ ABC transporter) and moa (synthesis of Mb cofactor) operon genes, in pACYC177 vector with LacZ promoter and W3110 fnr gene. | Hydrogen synthesis, pyruvate metabolism, formate transporter | Mutagenesis of competitive pathway | 7.6 | High | - | 9.65 | Fan, 2009 |
| W3110 ΔnarL::FRT (ZF3) | Defective in global transcriptional regulator of anaerobic metabolism; represses the transcription of nik operon genes, fdhF and pflB genes; activates the transcription of formate dehydrogenase N, which couples formate oxidation to nitrate reduction. | | | | | 1.77 | - | |
| BW25113 ΔydfWΩkan$^R$ BW25113 ΔyclEΩkan$^R$ BW25113 ΔypdJΩkan$^R$ BW25113 ΔyqiGΩkan$^R$ | Defective in pseudogenes | Involved as leader peptides, ribosomal proteins and toxic proteins | High-through put screening | 7 | High | - | - | Mohd Yusoff 2013 |
| BW25113 ΔhyaBΔhybCΩkan$^R$ | Defective in Hyd-1 and Hyd-2 | Reverse H$_2$ oxidizing mode | Mutagenesis of competitive pathway | 5.5 | High | - | 1.43 | Trouchnian 2011 |
| | | | | 6.5 | | | 2 | |
| | | | | 7.5 | | | 1.4 | Trchounian |

| Strains, genotype (ID) | Phenotype | Metabolic pathways redirected | Metabolic Engineering strategies | pH$_{out}$ | Headspace (partial pressure) | Yield* | Produc-tivity** | 2013 References |
|---|---|---|---|---|---|---|---|---|
| DJT135 ΔhyaBΔhybCΩkan$^R$ ΔldhA, ΔfhlAΩCm$^R$ | Defective in Hyd-1 and Hyd-2, in LdhA and FhlA | Reverse H$_2$ oxidizing mode, fermentative pathways and synthesis of hydrogen. | Optimizing environmental conditions by Design of experiment (DOE) | 7.5 | Low | 1.69 | - | Ghosh, 2010 |
| W3110ΔhycAΔlacIΩkan$^R$ | Defective in HycA and gene lac operon repressor (LacI) | Hydrogenase synthesis and lactose fermentation | Optimizing environmental conditions by Design of experiment (DOE) | 7.5 | Low | 2.26** | 1.78* | Rosales-Colunga 2010 |
| BW25113 ΔhycA ΔhyaAB ΔhybBC ΔldhA ΔfrdAB Ωkan$^R$(SH5) | Defective in HycA, Hyd-1, Hyd-2, LdhA, FrdAB | Synthesis of hydrogen, fermentative pahtways and TCA-reductive branch | Mutagenesis of competitive pathway MFA | 7.0 | High | 1.42 | - | Seol, 2014 |
| BW25113 ΔhycA ΔhyaAB ΔhybBC ΔldhA ΔfrdABΔpfkAΔpta-ackAΩkan$^R$-adapted evolution-/pKD7-zwf-gnd (SH8) | Defective in HycA, Hyd-1, Hyd-2, LdhA, FrdAB, phosphofructokinase (PfkA), PtA, acetate kinase (AckA) and overexpression of NADP$^+$-dependent glucose-6-phosphate dehydrogenase (Zwf) and gluconate dehydrogenase (Gnd) | Pentose phosphpate pahtway (PPP) Embden-Meyerhof-Parnas (EMP) Synthesis of hydrogen, fermentative pahtways and TCA-reductive branch. | Mutagenesis of competitive pathway Adaptive evolution MFA | 7.0 | High | 1.32 | - | Seol, 2016 |
| | | | | 7.0 | High | 1.32 | - | |
| ΔldhA | Defective in LdhA | Lactose fermentation | Metabolic flux analysis | - | - | 1.35 | | Manish 2007 |
| W3110 ΔhycAΔldhA::Cm$^r$ΔfrdBC::Km$^r$ / FhlA (SR14) | Defective in LdhA and FrdB and FrdC subunits. | Lactose fermentation, anaerobic respiration and hydrogen synthesis | Mutagenesis of competitive pathway | 6.2 | High | 1.73 | - | Yoshida, 2006 |
| BL-21 (DE3)/pGEX-5X-hydA | pGEX-5X-3 vector carrying Clostridium butyricum hydA gene | Hydrogen synthesis | Heterologous expression | 7.1 | High | 1.68 | 1.9 | Subudhi, 2011 |
| BL21(DE3)ΔiscR/pAF-pYdbK HydA, HydF, HydG, HydE and CpFdx | Defective in the transcriptional regulator of the isc operon involved in the assembly of Fe-S clusters into proteins. | Hydrogen synthesis | Mutagenesis of competitive pathway Heterologous expression | 7.1 | High | 1.46 | - | Akhtar, 2009**** |
| BW25113 ΔhyaBΔhybC/pBS(Kan)Synhox | Defective in Hyd-1 and Hyd-2 large subunits and heterologous expression of hydrogenase (HoxEFUYH) from the cyanobacterium Synechocystis sp. PCC 6803 | Hydrogen synthesis | Mutagenesis of competitive pathway Heterologous expression | 6.8 | High | 41 | - | Maeda 2007***** |
| BL21 (DE3)/ pETDuet-HydA-HydE (HFdY) and pCDFDuet-HydF-HydG and pRSFDuet-1-FdxA-YumC and pACYCD-Zwf-GlpX (Strain HFdYzg) | Heterologous expression of hydrogenases (HydAEFG) from C. acetobutiricum FdxA from C. pasteurianum; YumC from Bacillus subtilis and Zwf from E. coli BL21 GlpX from E. coli BL21 | Hydrogen synthesis | Heterologous expression | 7.0 | High | 3.5 | - | Kim, 2011 |