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REVIEW ARTICLE

Mechanisms for hydrogen production by different bacteria during mixed-acid and photo-fermentation and perspectives of hydrogen production biotechnology

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Abstract

H_2 has a great potential as an ecologically-clean, renewable and capable fuel. It can be mainly produced via hydrogenases (Hyd) by different bacteria, especially *Escherichia coli* and *Rhodobacter sphaeroides*. The operation direction and activity of multiple Hyd enzymes in *E. coli* during mixed-acid fermentation might determine H_2 production; some metabolic cross-talk between Hyd enzymes is proposed. Manipulating the activity of different Hyd enzymes is an effective way to enhance H_2 production by *E. coli* in biotechnology. Moreover, a novel approach would be the use of glycerol as feedstock in fermentation processes leading to H_2 production. Mixed carbon (sugar and glycerol) utilization studies enlarge the kind of organic wastes used in biotechnology. During photo-fermentation under limited nitrogen conditions, H_2 production by *Rh. sphaeroides* is observed when carbon and nitrogen sources are supplemented. The relationship of H_2 production with H^+ transport across the membrane and membrane-associated ATPase activity is shown. On the other hand, combination of carbon sources (succinate, malate) with different nitrogen sources (yeast extract, glutamate, glycine) as well as different metal (Fe, Ni, Mg) ions might regulate H_2 production. All these can enhance H_2 production yield by *Rh. sphaeroides* in biotechnology Finally, two of these bacteria might be combined to develop and consequently to optimize two stages of H_2 production biotechnology with high efficiency transformation of different organic sources.

Keywords

Bacteria, hydrogenases, hydrogen production biotechnology, mixed-acid and photo-fermentation, mixed carbon sources

History

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Hydrogen as a potential fuel and its production by bacteria

H_2 has a great potential as an ecologically-clean, renewable and capable fuel due to the following: (1) it is generating no toxic by-products and produces only water; (2) H_2 has a higher (~3-fold) energy content (~−140 MJ/kg) than hydrocarbon fuels like oil (Hallenbeck, 2009; Momirlan & Veziroglu, 2005; Mudhoo et al., 2011). Most of the H_2 is now produced from water by the process of steam reforming or as a by-product from petroleum refining or chemicals production (Das & Veziroglu, 2001; Kapdan & Kargi, 2006). However, H_2 may be produced from biomass – agricultural products, industry, food, water and other organic wastes by bacteria during either photosynthetic or fermentative processes (Figure 1). Fermentation H_2 production is more efficient than a photosynthetic one: the rate of H_2 production is ~30 times higher and the cost is ~340 times lower (Hallenbeck, 2009; Han & Shin, 2004; Kapdan & Kargi, 2006; Levin et al., 2004; Mudhoo et al., 2011).

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Among H_2-producing bacteria during fermentation, *Escherichia coli* is the best-characterized bacterium, having metabolic pathways established clearly and modified by metabolic engineering (Ganesh et al., 2012; Kim et al., 2009; Maeda et al., 2007a,b, 2008a,b; Sanchez-Torres et al., 2009; Seol et al., 2012) and, importantly, there are many strains to manipulate genetically (Hu & Wood, 2010; Maeda et al., 2012).

During fermentation, the oxidation of a common sugar – glucose by this bacterium proceeds via a consequent biochemical pathway. At the stage of phosphoenolpyruvate some intermediates may be used for succinic acid formation, whereas all other end products, including formic acid (HCOOH), are formed from pyruvate (Bock & Sawers, 2006; Poladyan & Trchounian, 2009; Trchounian et al., 2012c) (Figure 2). The ratio of the products formed is variable and it depends on the concentration of glucose, external pH ($[pH]_{out}$), oxidation-reduction potential (E_h) and other factors (Riondet et al., 2000).

Further oxidation of formic acid to H_2 and carbon dioxide (CO_2) (see Figure 2)

$$HCOOH \rightarrow 2H + CO_2 \text{ and } 2H \rightarrow H_2 \quad (1)$$

is catalyzed by the formate hydrogen lyase (FHL) pathway consisting of two enzymes – formate dehydrogenase

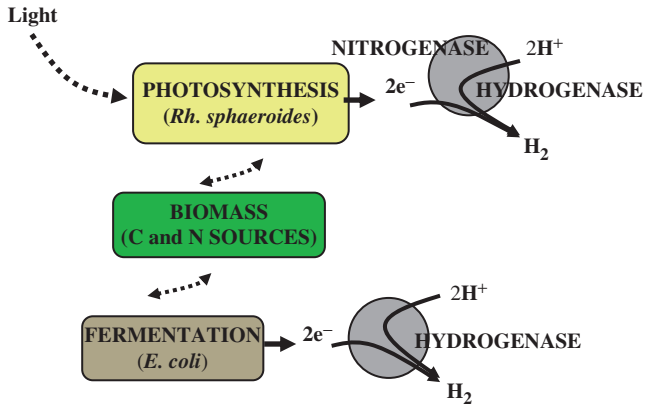

Figure 1. Two different ways of H_2 production by bacteria: photosynthesis (*Rh. sphaeroides*) and fermentation (*E. coli*). For both these ways hydrogenases could be the responsible enzymes, for photo-fermentation – under limited nitrogen conditions when nitrogenase is not active.

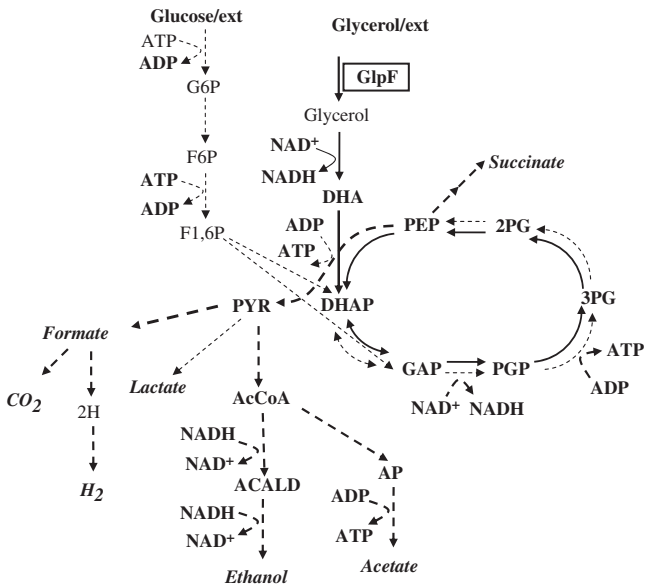

Figure 2. Combined putative pathways of glycerol and glucose fermentation in *E. coli*. The pathways are adapted from (Bagramyan & Trchounian, 2003; Booth, 2006; Poladyan & Trchounian, 2009; Trchounian et al., 2012c). Linear arrows indicate pathways only for glycerol fermentation, broken arrows indicate pathways only for glucose fermentation, and solid broken arrows indicate pathways for both glucose and glycerol fermentation. GlpF, glycerol facilitator protein; Metabolites: 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; AcCoA, acetyl-Coenzyme A; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; GAP glyceraldehyde-3-phosphate; NADH, dihydrodiphosphopyridine nucleotide; NAD^+, diphosphopyridine nucleotide; PGP, 1,3-diphosphate glycerate; PYR, pyruvate, G6P -glucose 6 phosphate, F6P-fructose-6 phosphate, F1,6P-fructose-1,6 diphosphate, AP-Acetyl-phosphate. See the text.

H (Fdh-H) and hydrogenase (Hyd) (Bagramyan & Trchounian, 2003; Bock & Sawers, 2006; Poladyan & Trchounian, 2009). Formate is a highly reducing (E_h for formate: H_2 couple is −420 mV) and potentially high energy compound (Andrews et al., 1997). Moreover, the change of standard free energy in this reaction is ∼3.5 kJ/mol but during *in vivo* conditions it is ∼−22 kJ/mol as calculated (Andrews et al., 1997) so as the FHL reaction can be coupled to energy conservation. However, the energetics of this reaction has not been well studied.

Interestingly, under fermentation conditions, CO_2 (see reaction 1) might be dissolved in H_2O: $CO_2 + H_2O \rightarrow HCO_3^- + H^+$, and then it can be involved in the regulation of cytoplasmic pH ($[pH]_{in}$) and used for metabolism by bacteria (Futatsugi et al., 1997). It should, therefore, be noted that the production of H_2 reduces organic acid formation but provides H^+ and represents an important factor in the regulation of $[pH]_{in}$ in bacteria (Slonczewski et al., 2009).

H_2 production and the FHL pathway have intrigued microbial physiologists, biotechnologists and specialists in hydrogen energy for many decades. The study has led to the discovery of multiple and reversible [Ni–Fe] Hyd enzymes in *E. coli* (Bock & Sawers, 2006; Poladyan & Trchounian, 2009; Trchounian et al., 2012c), of which Hyd-3 has been shown as a main H_2-producing enzyme during glucose fermentation (Sauter et al., 1992). In addition, under glucose fermentation, the other one – Hyd-4 proposed (Andrews et al., 1997; Skibinski et al., 2002) might also function as a H_2-producing enzyme but under different conditions, especially at neutral and slightly alkaline $[pH]_{out}$ (Bagramyan et al., 2002; Mnatsakanyan et al., 2004; Trchounian & Trchounian, 2009).

Moreover, under anaerobic conditions, it has been discovered that *E. coli* can also ferment glycerol producing H_2; the latter has been observed at acidic (Dharmadi et al., 2006; Murarka et al., 2008; Trchounian et al., 2011b) and alkaline $[pH]_{out}$ (Trchounian & Trchounian, 2009; Trchounian et al., 2011b). This is the novel intriguing finding contradicting an idea about the inability of these bacteria to grow on glycerol under anaerobic conditions in the absence of fumarate as an electron acceptor (Booth, 2006; Ganesh et al., 2012; Varga & Weiner, 1995). Glycerol metabolism represents a relatively simple cluster of biochemical reactions leading to the entry points in the lower section of glycolysis (Poladyan & Trchounian, 2009; Trchounian et al., 2012c) (see Figure 2). Glycerol is suggested to be transported into the cell by GlpF, glycerol facilitator protein. Then, as reported by Gonzalez's group (Murarka et al., 2008) under fermentative conditions, glycerol is converted to dihydroxyacetone phosphate (DHAP), which is a glycolytic intermediate (see Figure 2). The conversion of DHAP into phosphoenolpyruvate (PEP) takes place in several steps through the action of common glycolytic enzymes (see Figure 2, solid broken arrows). While PEP is converted into pyruvate by the glycolytic pathway during the metabolism of glucose, a unique characteristic of glycerol metabolism is that the conversion of PEP into pyruvate is coupled to DHA phosphorylation (Cintolesi et al., 2011; Ganesh et al., 2012). This coupled reaction is a critical component of glycerol fermentation in *E. coli* and generates a cycle in the metabolic pathway (see Figure 2). However, glycerol metabolism is being intensively studied (Cintolesi et al., 2011; Ganesh et al., 2012) and succinic, acetic and formic acids and ethanol are shown to be produced under acidic conditions ($[pH]_{out}$ 6.3); no lactic acid was detected (Murarka et al., 2008). This is linked to the availability of CO_2, which is produced by formate oxidation (see reaction 1) through FHL, but H_2 has a negative impact on fermentation end product formation (Dharmadi et al., 2006; Gonzalez et al., 2008). Note, glycerol is a low cost, abundant

and highly reduced carbon source compared to sugars and offers an effective opportunity to obtain H_2 and other reduced products by bacteria (Ganesh et al., 2012; Khanna et al., 2012). Nowadays, glycerol is produced in large volumes as a by-product during biodiesel production from raw materials (Sarma et al., 2012).

The ability to produce H_2 in the presence of light (H_2 photo-production), the natural way (see Figure 1), is found largely in purple bacteria, than in the dark (Levin et al., 2004). H_2 production rate by purple bacteria under the light, whether it is in growing cultures or suspensions of non-growing cells, is much more than in the dark (Kondratieva & Gogotov, 1981; Tsygankov et al., 1998). Moreover, H_2 production, as a rule, is essentially increased in the presence of different organic substrates – organic acids, sugars, spirits and other organic compounds (Gabrielyan & Trchounian, 2009a; Kapdan & Kargi, 2006).

In mineral spring waters of mountains in Armenia mostly non-sulfur purple bacteria exist, whereas sulfur and green photo-bacteria exist in small numbers (Gabrielyan & Trchounian, 2009a; Paronyan, 2002). Mineral spring water has a rather rich specific spectrum of non-sulfur photosynthetic bacteria. Some of these bacteria belong to *Rhodobacter sphaeroides*, which are able to produce H_2 depending on carbon and nitrogen sources and the other factors (Gabrielyan et al., 2010; Gabrielyan & Trchounian, 2012; Paronyan, 2002). Probably, Hyd enzyme is responsible for H_2 production under limited nitrogen conditions (Kondratieva & Gogotov, 1981; Levin et al., 2004). Moreover, a combination of *Rh. sphaeroides* with *E. coli* can be used for H_2 production biotechnology (Manish et al.,& Banerjee, 2008; Mudhoo et al., 2011; Redwood et al., 2008). That is why H_2 production mechanisms by *Rh. sphaeroides* are reviewed.

Hydrogenases and pathways for H_2 production by *E. coli* during mixed acid fermentation

E. coli possesses four Hyd enzymes (Figure 3); three of these – Hyd-1, Hyd-2 and Hyd-3 are characterized well and the fourth one – Hyd-4 is only postulated and its function should be clarified (Poladyan & Trchounian, 2009; Trchounian et al., 2012c). All these Hyd enzymes might function in bacteria together forming membrane-associated H_2 cycling, which is a novel phenomenon.

Hydrogenase 1 and 2

Hyd-1 is encoded by the *hya* operon; three *hyaA, hyaB* and *hyaC* genes, which encode large and small subunits (Menon et al., 1991) (see Figure 3). The other genes could encode different polypeptides, however, their functions are unknown. They probably have a role in the modification of Hyd-1 structural subunits on activity level. The expression of *hya* is induced during fermentation under anaerobic conditions in an acidic medium (King & Przybyla, 1999) and by the presence of formate but not nitrate (NO_3^-) during growth on glucose (Brondsted & Atlung, 1994; Richard et al., 1999). However, the increase in the amount of Hyd-1 during the fermentation is not due to regulation at the transcriptional level (Pinske et al., 2012b). Moreover, Hyd-1 is not required for anaerobic growth but this is required to respond to a $[pH]_{out}$ shift from alkaline

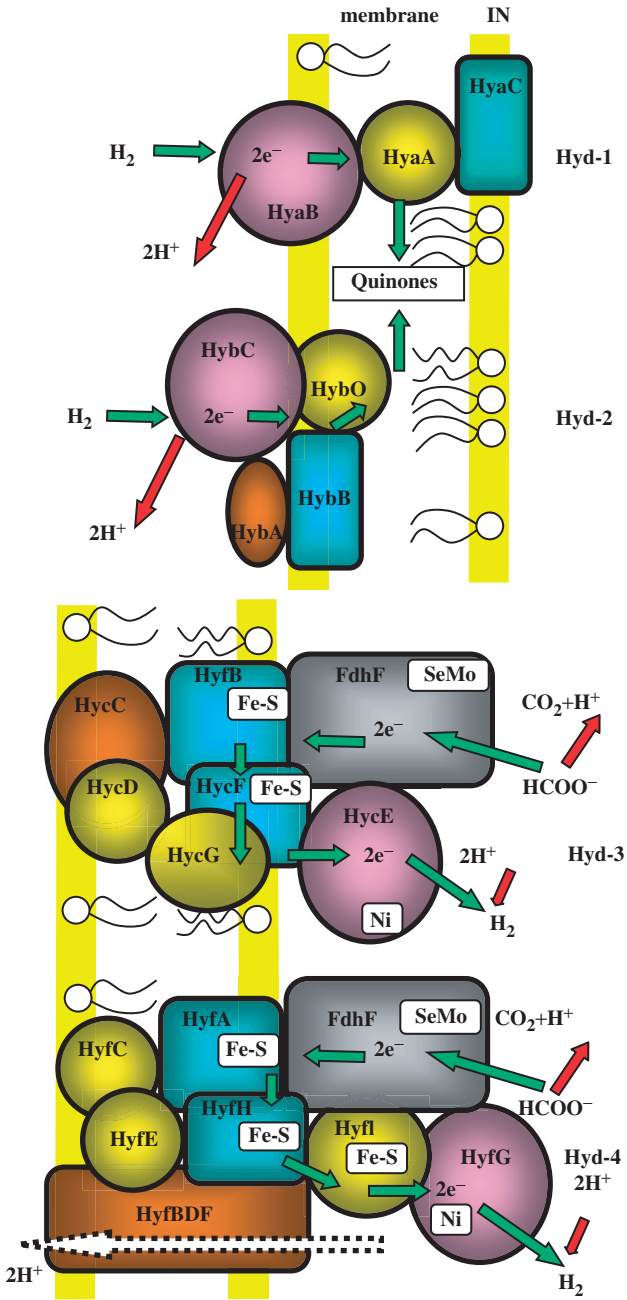

Figure 3. Structural composition and organization of different hydrogenases in *E. coli* membrane: Hyd-1 (*hya*), Hyd-2 (*hyb*), Hyd-3 (*hyc*) and Hyd-4 (*hyf*). Hyd-3 and Hyd-4 are shown to form different FHL pathways during glucose fermentation. For Hyd-1, Hyd-2 and Hyd-3, see Forzi & Sawers, 2007; for the others, see the text.

to acidic conditions (Brondsted & Atlung, 1994). Since the proton motive force (Δp) is changed during a $[pH]_{out}$ shift (Slonczewski et al., 2009; Trchounian et al., 1998;), Hyd-1 is suggested to function to maintain Δp in an energy-conserving manner.

Hyd-2 is encoded by the *hyb* operon (Menon et al., 1994; Richard et al., 1999). Hyd-2 contains four different, large and small subunits (Dubini et al., 2002) (see Figure 3). The large subunit, HybC, has similarity with a large subunit of Hyd-1 (Ballantine & Boxer, 1986). It is oxidizing H_2 to H^+ and is probably involved in their translocation by the other subunit, being an integral protein (Laurinavichene et al., 2001; Laurinavichene & Tsygankov 2001). HybO small subunit with HybC, forming the core enzyme, is associated with two

other Hyb proteins to complete the Hyd-2 complex (Dibini et al., 2002). Its maximal expression is attained in alkaline medium (Brondsted & Atlung, 1994).

Some *hyb* gene products are suggested to be involved in maturation and regulation of Hyd-2 and Hyd-1 large subunits (Blokesch et al., 2001; Hube et al., 2002; Menon et al., 1994). Therefore, metabolic cross-talk between Hyd-1 and Hyd-2 at the level of gene expression and enzyme activity is proposed. This cross-talk is also suggested when Hyd-2 but not Hyd-1 activity is enhanced when Hyd-3 activity is lost or when Hyd-1, but not Hyd-2 activity, is reduced when Hyd-3 activity is abolished (Menon et al., 1994). Moreover, Hyd-3 activity is increased when *hyb* but not *hya* is deleted (Redwood et al., 2008). Altogether it is understood when interaction between Hyd enzymes is toward the recycling of H_2, however, further detailed study is required.

Certain evidence has been obtained that Hyd-1 and Hyd-2 both operate preferentially under different conditions (Laurinavichene & Tsygankov 2001; Laurinavichene et al., 2002; Pinske et al., 2012a). Unlike Hyd-1, Hyd-2 is inhibited by formate (Ballantine & Boxer, 1986; Richard et al., 1999). Hyd-2 is also sensitive to oxygen (Laurinavichene et al., 2002) and a higher concentration of this protein is observed under anaerobic conditions (Laurinavichene & Tsygankov 2001; Laurinavichene et al., 2001; Richard et al., 1999). The study of Hyd-1 and Hyd-2 enzymes' oxidation–reduction properties has revealed maximal Hyd-1 activity in oxidizing environments (E_h of +30 mV to +100 mV), whereas Hyd-2 maximal activity occurs in more reducing conditions (E_h below −80 mV) (Laurinavichene et al., 2001, 2002; Redwood et al., 2008). The latter is confirmed by the absence of Hyd-2 activity under aerobic conditions (Lukey et al., 2010) and by the redox dye (E_h of −80 mV) reduction with Hyd-1but not Hyd-2 (Pinske et al., 2012a). Moreover, activity of Hyd-1 and Hyd-2 has been shown to be different depending on the H_2 concentration (Pinske et al., 2012a).

Hydrogenase 3

Hyd-3 is encoded by the *hyc* operon (Bock & Sawers, 2006; Sauter et al., 1992). The first *hycA* gene is a regulatory one for the whole operon and its protein product, HycA, antagonizes expression of several other genes, particularly *fdhF* (Bock & Sawers, 2006). The mechanism of regulation by *hycA* is unknown (Sauter et al., 1992; Seol et al., 2012; Skibinski et al., 2002). The effect of HycA is possibly realized via binding to a formate sensitive protein, FhlA (Rossmann et al., 1991; Schlensog & Bock, 1990). The large subunit of Hyd-3 is encoded by the *hycE* gene (Bohm et al., 1990), whereas the *hycB*, *hycF* and *hycG* genes encode the small subunits of Hyd-3 (Sauter et al., 1992). HycE is Ni-containing [Fe–S]-protein (Ballantine & Boxer, 1985; Rossmann et al., 1994). On the other hand, *hycB* might cause pleiotropic effects as it is involved in the functioning of the other Hyd enzymes (Bagramyan et al., 2002; Trchounian et al., 2012c). Furthermore, HycI forms a protease required for the Hyd-3 large subunit maturation (Bock & Sawers, 2006; Forzi & Sawers, 2007; Rossmann et al., 1994).

The *hyc* operon expression is dependent on formate and $[pH]_{out}$. It can be activated by FhlA (Rossmann et al., 1991) such regulation has also been studied recently (Poladyan & Trchounian, 2009; Trchounian et al., 2012c). Moreover, oxygen and NO_3^- suppress both the *hycB* and *fdhF* gene expression (Sauter et al., 1992; Wu & Mandrand-Berthelot, 1987).

Based on the data regarding the structure and membrane localization of the enzyme, expression of the genes encoding its subunits, maturation and interaction, a model for Hyd-3 in *E. coli* has been proposed (Bagramyan & Trchounian, 2003; Poladyan & Trchounian, 2009; Sauter et al., 1992). According to this model, two catalytic components, Fdh-H and a large subunit of Hyd-3, associated with the cytoplasmic side of the membrane, interact with other Hyd-3 subunits (see Figure 3). With the complex electrons a transfer route is proposed. The model requires experimental validation because of the little information about small subunits and precise pathways of oxidation–reduction equivalents transfer within the enzyme.

Hydrogenase 4

Hyd-4 is proposed by Andrews et al. (1997) to be encoded by the *hyf* operon. Nine of the genes encode proteins that are homologous to seven Hyd-3 subunits, however, they have not been isolated and studied yet. The *hyfG* and *hyfI* genes encode large and small subunits, respectively, and the *hyfR* gene encodes a formate-sensitive regulatory protein. Protein products of three other genes, *hyfD, hyfE* and *hyfF*, probably represent integral membrane proteins that lack analogs in Hyd-3. These proteins are assumed to give H^+-translocation activity to Hyd-4; however, such activity has not been shown yet. These proteins might underline characteristic functions of Hyd-4 if they actually exist. This would be important for FHL energetics. It is possible that, as Hyd-3, Hyd-4 is also a Ni-containing protein including [Fe–S] clusters.

In spite of limited information about Hyd-4 subunits, the other model (see Figure 3) also suggests location of Fdh-H and Hyd-4 large subunit in membrane cytoplasmic side. These two proteins bind to the membrane via other subunits of Hyd-4 (Andrews et al., 1997). Moreover, the *hycB* gene product has been shown to be required for the activity of Hyd-4 at neutral and slightly alkaline $[pH]_{out}$ upon glucose fermentation (Bagramyan et al., 2002; Trchounian et al., 2012c) and would be, therefore, considered as a part in FHL functional complex. This could explain why *E. coli* mutants lacking Hyd-1, Hyd-2 and Hyd-3 do not produce H_2 (Sauter et al., 1992; Self et al., 2004).

FHL and its different forms

Hyd-3 and Hyd-4 are suggested to be H_2 producing enzymes and together with Fdh-H they form two different FHL pathways (Andrews et al., 1997; Bagramyan & Trchounian, 2003). Since Hyd-3 and Hyd-4 are encoded by distinguished operons and are characterized by different subunit composition and organization in the membrane (see Figure 3) it is reasonable to assume that different FHL forms are functionally active under different conditions and therefore they play distinct roles in bacteria. Factors, determining functional activity of multiple membrane systems in bacteria, include temperature, $[pH]_{out}$, E_h, concentration of substrates and/or products of fermentation, the presence of some exogenous

electron acceptors and ratios of end products (Poladyan & Trchounian, 2009; Seol et al., 2012; Trchounian et al., 2012c). These conditions probably determine different FHL forms.

Actually, our study with *E. coli* grown during glucose fermentation at neutral and slightly alkaline $[pH]_{out}$ has shown that H_2 production is not observed in *fdhF* and *hyf* mutants (Bagramyan et al., 2002; Mnatsakanyan et al., 2004). Interestingly, H_2 production is detected in various *hyc* mutants lacking large and small subunits of Hyd-3, but it is not formed in the *hycB* mutant (Bagramyan et al., 2002). These results point out that the production of H_2 by *E. coli* in neutral and slightly alkaline medium involves the second FHL. Some contradiction has been reported by different authors (Noguchi et al., 2010; Skibinski et al., 2002; Self et al., 2004) but it could be due to the role of *hycB* and different experimental set ups. The second FHL may be required for generation of CO_2 for use in the formation of oxaloacetate, from phopshoenol-pyruvate during fermentation, but this is not clear at all.

Role of pH in induction of the Hyd enzymes

Acidification of the medium has been shown to promote induction of Hyd-3 (Rossmann et al., 1991; Sauter et al., 1992). Such induction was observed at slightly alkaline $[pH]_{out}$ (Mnatsakanyan et al., 2002, 2004). The mechanism of such induction might be a result of the increased concentration of fermentation end products – organic acids, causing a decrease in $[pH]_{out}$ (Slonczewski et al., 2009). This may also be attributed to formate transport from an external medium into the cell and its further oxidation (see reaction 1) by Hyd 3 or Hyd-4.

Although information on regulation of FHL components, synthesis and maturation by $[pH]_{out}$ becomes increasingly available, Slonczewski and co-workers (Hayes et al., 2006; Noguchi et al., 2010) have reported that *hycB* expression under aerobic conditions is greater (~6-fold) at $[pH]_{out}$ 8.7 than pH 5.0. They seem to indicate that *hycB* has a role in strengthening bacterial adaptation to an alkaline medium. On the other hand, this may specify again an important and unusual role of *hycB* in the second FHL under fermentation at neutral and high $[pH]_{out}$ (Bagramyan et al., 2002; Mnatsakanyan et al., 2004) and also pH regulation.

Regulation of cytoplasmic pH by hydrogenases

The other interesting idea that has been proposed by Bock & Sawers (2006) is that FHL in *E. coli* is involved in regulation of $[pH]_{in}$. Such a hypothesis is based on the following observations. First of all, FHL catalyzes decomposition of formic acid to H_2 and CO_2 (see reaction 1) so that the enzyme neutralizes the acidic products of fermentation. This process depends on $[pH]_{out}$ (Rossmann et al., 1991). Then, formic acid, the end product of fermentation, is a weak acid that may act as an uncoupling factor dissipating Δp (Slonczewski et al., 2009; Trchounian, 2004). Decrease in $[pH]_{out}$ potentiates such effects of acids formed during fermentation.

It seems unlikely that FHL catalyzing terminal reaction of mixed-acid fermentation in *E. coil* operates independently from other fermentation enzymes. This viewpoint can be supported by the following arguments. First, FHL functioning requires catalytically competent F_0F_1-ATPase interacted with the K^+ uptake system TrkA (Bagramyan et al., 2003; Trchounian et al., 1997, 2009, 2012b,c; Trchounian, 2004). If so, mutations in TrkA have been shown to be accompanied by changes in H_2 production at slightly alkaline $[pH]_{out}$ (Trchounian et al., 1997, 1998). Thus, FHL activity and H_2 production depend on K^+ concentration and therefore in K^+-depleted cells H_2 production is blocked. The inactivation of fermentation enzymes by K^+ depletion is also suggested.

Interestingly, Wood's group (Maeda et al., 2007c) has recently obtained that Hyd-3 in *E. coli* might operate in a reverse direction having significant H_2 uptake activity like Hyd-1 and Hyd-2 during sugar fermentation. This has been confirmed by Trchounian et al. (2011b). Moreover, Hyd-1 and Hyd-2 can also function in a reversible mode upon glycerol fermentation (Trchounian & Trchounian, 2009; Trchounian et al., 2011b). Each Hyd enzyme is, therefore, likely to function in one direction, depending on the fermentation substrate, $[pH]_{out}$ and other conditions; this is to recycle H_2.

Reversibility of hydrogenases and recycling of H_2

Our study with H_2 production during glycerol fermentation by *E. coli* has suggested the operation of different Hyd enzymes in reversible mode. Three novel principal findings have been already reported: (1) H_2 production is $[pH]_{out}$ dependent (Murarka et al., 2008; Trchounain et al., 2011a,b); the highest rate is determined at $[pH]_{out}$ 5.5 (Trchounian et al., 2011a,b). (2) At neutral and slightly alkaline $[pH]_{out}$ Hyd-2 and Hyd-1 are involved in H_2 production, no relation with FHL activity is observed (Trchounian & Trchounian, 2009) whereas at acidic $[pH]_{out}$ conditions FHL complex consisting of rather Hyd-3 than Hyd-4 is required for H_2 production (Gonzalez et al., 2008; Trchounian et al., 2011a,b). Moreover, at acidic $[pH]_{out}$ the requirement of Hyd-3 and Hyd-4 is different (Trchounian et al., 2011b). Then, increased H_2 production at a low $[pH]_{out}$ is probable when H_2 uptake activity is absent, so as Hyd-1 and Hyd-2 worked in a reversed H_2-oxidizing mode have low activity (Trchounian et al., 2013b). Hyd function seems to be similar with each other during glycerol and glucose fermentation at low $[pH]_{out}$ (Bagramyan et al., 2003; Trchounian et al., 2012c). (3) A relationship between Hyd activity and the F_0F_1-ATPase is determined (Blbulyan et al., 2011; Trchounian et al., 2009, 2013a). However, it is necessary to note that detailed mechanisms for H_2 formation by Hyd enzymes during glucose and glycerol fermentation are unclear currently.

The recycling of produced H_2 is, therefore, required for H_2 metabolism during glucose and glycerol fermentation. The H_2 recycling was suggested for glycerol fermentation conditions that could change H_2 production rate by double *hyaB hybC* mutant (compared to the wild type) (Trchounain et al., 2011b). The proposal about H_2 recycling is likely to the results of Redwood et al. (2008) on compensatory uptake function during H_2 production but under different conditions. Moreover, H_2 production in a double *hyaB hybC* mutant was increased (Trchounain et al., 2011b) that could be explained with the loss of *hyb* but not *hya* genes (Redwood et al., 2008). This seems to be in favor with the conclusion of Murarka et al. (2008) that H_2 evolved by *E. coli* FHL complex is being

recycling during glycerol fermentation and to the suggestion by Zbell & Maier (2009) about a role of different levels of *Salmonella enterica* Hyd-1 to recycle H_2 production.

Thus, *E. coli* and some other bacteria are known to have a set of [Ni–Fe]-containing Hyd enzymes: H_2-uptake Hyd are of equal or perhaps greater concern in some systems (Bock & Sawers, 2006; Forzi & Sawers, 2007; Poladyan & Trchounian, 2009), because they frequently co-exist with H_2-producing Hyd and can recycle H_2 within the bacteria, greatly diminishing overall H_2 yield (Trchounian et al., 2012c). Some metabolic cross-talk between different Hyd enzymes is suggested by different groups of researchers (Sauter et al., 1992; Trchounian et al., 2012b,c) and might have a role in H_2 recycling as well. The physiological role of Hyd enzymes mediated H_2 production appears to be the discharge of excess reducing power, necessary when other suitable electron acceptors such as O_2 are absent (Laurinavichene et al., 2001; Lukey et al., 2010; Trchounian et al., 1998). It is not surprising, therefore, that H_2-producing Hyd enzymes are rapidly inhibited by O_2. For fermentative H_2 production, this is no obstacle, as the entire H_2 metabolism takes place in anaerobic conditions.

It would also be interesting to consider H_2 cycling interaction with H^+ cycle since Hyd enzymes have H^+ translocation activity and by interaction with the F_0F_1-ATPase can be, therefore, involved in H^+ cycle. This is a key mechanism in generating Δp and regulating of $[pH]_{in}$. An interaction between these two key cycles within the bacterial membrane might be a novel look at the bioenergetics of fermentation.

H_2 production by photo-fermentation

H_2 production by different bacteria during photo-fermentation (see Figure 1) is known to involve a coordinated action of two enzymes: [Mo–Fe]-containing nitrogenase and [Ni–Fe]-containing Hyd (Gabrielyan & Trchounian, 2009a; Kim et al., 2008; Kondratieva & Gogotov, 1981). Nitrogenase is suggested, with the help of the energy from ATP synthesized by the F_0F_1-ATPase, can reduce H^+ to H_2. The latter, therefore, is produced as a by-product of the nitrogenase. This enzyme activity is inhibited in the presence of oxygen, nitrogen and carbon sources ratio and the other factors (Colbeau et al., 1980; Sasikala et al., 1991).

Hyd enzyme in non-sulfur purple bacteria such as *Rh. sphaeroides* is an H_2-uptake, one that utilizes H_2 and therefore is antagonistic to nitrogenase activity (Colbeau et al., 1980; Sasikala et al., 1991). Probably the H_2 uptake does not depend on light, but as against a photo-production of H_2, usually is inhibited by CO_2. It could not be ruled out another important ability of the Hyd enzyme to produce H_2 that follows from a relationship between Hyd and nitrogenase as suggested by Kondratieva & Gogotov (1981) and Kars et al. (2008). To produce H_2 under N_2 limitation conditions, Hyd can be using H^+ from ATPase reaction and electrons from ferredoxin (Figure 4). *Rh. sphaeroides* Hyd enzyme might be reversible depending on the conditions; the reversibility of Hyd enzyme is similar to the situation with Hyd enzymes in *E. coli* (Maeda et al., 2007c; Trchounain et al., 2012c). Importantly, the yield of H_2 in Hyd-catalyzed reaction is much higher than that of the nitrogenase-catalyzed reaction (Das et al., 2006; Hallenbeck, 2009). The Hyd activity in *Rh. sphaeroides* and its relationship with nitrogenase and the F_0F_1-ATPase might be determined by carbon and nitrogen sources and other different factors.

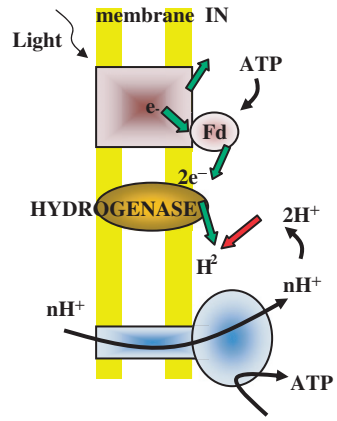

Figure 4. H_2 production way through hydrogenase in *Rh. sphaeroides* and probable interplay of hydrogenase with the H^+ translocation ATPase. Fd is ferredoxin; see the text.

H_2 production by *Rh. sphaeroides* at the light

Our recent study with H_2 production during photo-fermentation under anaerobic conditions by *Rh. sphaeroides* strains isolated from mineral springs of Armenia and its relationship with ATPase activity has shown the following. First, the strains were capable of growing and producing H_2 in anaerobic conditions at the illumination of 1500 lx, $[pH]_{out}$ 7.0–7.2 (28 °C –30 °C) using succinate or malate and lysine as the carbon and nitrogen sources, respectively (Gabrielyan & Trchounian, 2009b). This process was suppressed at the presence of the F_0F_1-ATPase inhibitors – *N,N′*-dicyclohexylcarbodiimide (DCCD) and NaN_3. In addition, H_2 production disappeared with a protonophore. Interestingly, increase in $[pH]_{out}$ from 7.0 to alkaline value was determined. Moreover, membrane vesicles demonstrated marked ATPase activity inhibited significantly by DCCD and NaN_3. In view of that, under limited nitrogen conditions the activity and direction of Hyd enzyme might be changed (Kim et al., 2009), used inhibitors suppress H^+-translocating systems of bacterial membrane, and in anaerobic conditions the activity of the F_0F_1-ATPase at *E. coli* and the other bacteria (Gabrielyan & Trchounian, 2009b; Hayes et al., 2006). It is possible to admit the activity of Hyd and the role of this ATPase in H_2 production by *Rh. sphaeroides* (Figure 4). To confirm a role of Hyd in H_2 production, specific inhibitor for Hyd enzyme in *Rhodobacter* sp. – diphenylene iodonium (Magnani et al., 2009) was applied. The complete inhibition of H_2 production was observed recently (Hakobyan et al., 2012a).

To characterize the role of nitrogen sources in order to stimulate growth and to enhance H_2 production by *Rh. sphaeroides* in N_2 limited conditions, different amino acids and yeast extracts were further tested. Though at a higher concentration, amino acids may inhibit the growth and activity of different bacteria, a low concentration of amino acids is required for bacterial growth and H_2 production (Klemme, 1989; Merugu et al., 2010). Several studies also

Table 1. Effects of various carbon and nitrogen substrates on H_2 yield of *Rh. sphaeroides* MDC 6521 during growth up to 72 h in phototrophic anaerobic conditions.

	Nitrogen sources			
Carbon sources	Amino acid and it's concentration ($mol\,m^{-3}$)	Yeast extract ($2\,g\,L^{-1}$)	H_2 yield ($mol\,m^{-3}$)	Reference
Malate	glutamate, 7	+	0.73	(Gabrielyan
Succinate	–		5.16	et al., 2010)
	glutamate, 7	–	0.74	(Hakobyan
		+	6.44	et al., 2012b)
	glycine, 5		3.62	(Gabrielyan
	glycine, 7		0.75	et al., 2010)
	lysine, 7		0.86	(Gabrielyan & Trchounian, 2012)

investigated the toxicity of some amino acids for growth of various microorganisms (Gabrielyan & Trchounian, 2012; Klemme, 1989; Merugu et al., 2010). *Rh. sphaeroides* strains studied were shown to be able to grow and to produce H_2 using various amino acids (Gabrielyan et al., 2010). However, succinate rather than malate as carbon source and glutamate rather than lysine as nitrogen source (Table 1) were effective to enhance H_2 production (Gabrielyan et al., 2010; Paronyan, 2002). Besides, the concentration-dependent effect of the other amino acid – glycine on *Rh. sphaeroides* strains growth and H_2 production was also established when relatively high concentration of glycine had inhibitory effect (Gabrielyan & Trchounian, 2012). This is similar to the results that glycine at a high concentration affected the photosynthetic growth and H_2 photo-production by different bacteria (Igeno et al., 1995; Merugu et al., 2010; Xu et al., 1998). Probably, the inhibitory effect of glycine at a high concentration is mediated with NH_4^+, which could be accumulated during glycine metabolism (Andreesen, 1994; Xu et al., 1998). On the other hand, glycine at a low concentration can stimulate photosynthetic electron transfer chain components synthesis (Neidle & Kaplan, 1993). Thus, the effects of glycine on *Rh. sphaeroides* are clear but the mechanism of the effect has yet to be explained.

The suitability and limitation of the other nitrogen source – yeast extract to support cell growth and to enhance H_2 photo-production by *Rh. sphaeroides* strains was further investigated during the anaerobic growth at the light. The increased (2–3.5 fold, for certain conditions) growth rate and the enhanced (~7-fold) H_2 yield was obtained for bacterial cells with yeast extract ($2\,g\,L^{-1}$) compared with glutamate (Booth, 2006). All effects (Table 1) obtained are suggested to be mediated by the photosynthetic pigments synthesis stimulation shown by the changes of typical peaks in absorption spectra (Gabrielyan & Trchounian, 2012; Hakobyan et al., 2012b). Such explanation is likely to that in a different study with *Rh. sphaeroides* showing the requirement of light harvesting pigment–protein complexes for H_2 production (Kim et al., 2006; Zhu et al., 2007).

Thus, the optimal culture conditions with carbon and nitrogen sources for *Rh. sphaeroides* bacterial growth stimulation and enhanced H_2 production are likely to be well established.

If Hyd enzyme is responsible for H_2 photo-production by *Rh. sphaeroides* (Figure 4), and like to *E. coli* (see Figure 3) (Bagramyan & Trchounian, 2003; Ballantine & Boxer, 1985; Forzi & Sawers, 2007; Rossmann et al., 1994; Trchounain et al., 2012c), *Rh. sphaeroides* Hyd is suggested to be a [Ni–Fe]-containing enzyme and to depend on various metal ions too (Zhu et al., 2007), the effects of Ni and other metal ions were also under study. The Ni (II) and Mg (II) ions effects on bacterial growth and H_2 production by *Rh. sphaeroides* were established (Hakobyan et al., 2012c). The highest growth rate was obtained with $(4–6) \times 10^{-6}\,mol\,m^{-3}$ Ni^{2+} and $5\,mol\,m^{-3}$ Mg^{2+}. pH of the growth medium changed from 7.0 to 9.2–9.4 during the bacterial growth up to 72 h in spite of Ni^{2+} added but $[pH]_{out}$ increased in different manner with Mg^{2+}. In the presence of $(2–4) \times 10^{6} molm^{3}$ Ni^{2+} the marked (~2.7-fold) enhanced H_2 yield was established. Moreover, the H_2 yield determined in a medium with the other metal ion $-80 \times 10^{-6} molm^{-3}$ Fe^{2+} was also higher than that with $40 \times 10^{-6}\,mol\,m^{-3}$ Fe^{2+}. These effects of metal ions might be due to incorporation into or stimulating the responsible enzymes and/or related pathways as suggested with *E. coli* (Bock et al., 2006; Forzi & Sawers, 2007; Trchounain et al., 2012c); further study is required. However, the results obtained point out that new regulatory ways to improve H_2 production by *Rh. sphaeroides* are important to develop the technology.

Perspectives of H_2 production biotechnology

H_2 is stated to be produced by different bacteria during mixed-acid and photo-fermentation due to Hyd enzymes (see Figure 1).

The first example of H_2 production by bacteria is with mixed-acid fermentation. In this respect, *E. coli* is the best characterized and most widely used bacteria. This bacterium possesses four Hyd enzymes encoded by different operons and forms two different FHL pathways composed of different Hyd enzymes (see Figure 3). The effects of different mutations in these operon's genes on H_2 production are important being dependent upon the environmental conditions. It is clear that multiple Hyd enzyme expression and their reversible functioning depend on fermentation substrate and $[pH]_{out}$ (Trchounian et al., 2012c). These properties of Hyd enzymes appear to play an important role in increasing the fitness of the bacterium in order to survive a variety of growth environments.

It can be concluded that our knowledge on multiple Hyd enzymes and different FHL functioning in H_2 production by bacteria is not exhaustive although biochemical and molecular biological mechanisms of terminal stages of fermentation are rather clear (Trchounian et al., 2012c). Further study would clarify the factors regulating H_2 production and the role of Hyd enzymes and FHL pathways in energy transformation in the bacterial cell membrane, regulation of $[pH]_{in}$ and adaptation of bacteria to different environments. It is of significance to reveal FHL complex formation and its association or binding to other transport systems and enzymes, formation of functional assemblies and their effectiveness in catalysis of fermentation reactions as well. The most important is to understand a metabolic cross-talk

between the Hyd enzymes suggested (Blokesch et al., 2001; Colbeau et al., 1980; Trchounian et al., 2012c). Moreover, the requirement of the F_0F_1-ATPase for Hyd enzymes and FHL activity is established (Bagramyan et al., 2002, 2003; Trchounian et al., 2011a), but mechanisms of this requirement should also be studied.

Recently, Wood's group (Hu & Wood, 2010; Maeda et al., 2007a,b, 2008a,b, 2012; Sanchez-Torres et al., 2009) have used the *E. coli* K-12 library containing all non-lethal deletion mutations to rapidly construct multiple, precise deletions in the *E. coli* genome to re-direct the metabolic flux toward H_2 production. Ceasing H_2 uptake by inactivating Hyd-1 and Hyd-2 by deleting *hyaB* and *hybC,* respectively, is among simple approaches for metabolically engineering *E. coli* to enhanced H_2 production during glucose fermentation (Maeda et al., 2008a,b). Manipulating with *hyaB, hybC, hycA* and many other genes and overexpressing of *fhlA* coding transcriptional regulator for FHL especially Hyd-3 or Hyd-4 (Rossmann et al., 1991; Schlensog & Bock, 1990). To re-direct formate metabolism, a single fermentative *E. coli* strain has been engineered that produces ~141-fold more H_2 from formate and ~3-fold more H_2 from glucose than the wild type strain (Maeda et al., 2008b). Different *E. coli* strains were also developed to enhance H_2 production by the other groups (Kim et al., 2009; Seol et al., 2012). Moreover, a new *E. coli* strain which lacks the fumarate reductase gene [(fumarate reductase catalyzes the pathway from PEP to succinate (see Figure 2)] has been engineered to produce 20-fold more H_2 from glycerol (Ganesh et al., 2012). Use of recombinant DNA technology in strains, construction and other new and more effective approaches have recently been reviewed for potential strategies with whole-cell and cell-free and the two systems compared (Meda et al., 2012).

However, sugar fermentation is now well understood in detail, but this substrate seems to be expensive and with different organic wastes it spreads together with other substrates – organic acids, other sugars and glycerol (mixed carbon sources) and, therefore, glycerol fermentation might be a useful pathway to produce H_2 by bacteria (Ganesh et al., 2012; Khanna et al., 2012). In this case, the effects of cultivation conditions and $[pH]_{out}$ on the fermentation of glycerol and the production of end products by *E. coli* (Murarka et al., 2008) and the establishment of Hyd enzymes responsible for H_2 production (Trchounian & Trchounian, 2008; Trchounian et al., 2011b) may also be usefully implicated for enhanced production of fuels and reduced chemicals.

The above become significant for H_2 production technology from organic wastes. A new strategy to regulate Hyd enzymes activity should be developed when a mixed carbon source (glucose, formate and glycerol at least) is present. Interestingly, co-utilization of glucose and glycerol by *E. coli* has been studied for enhanced production of aromatic compounds (Martinez et al., 2009) and H_2 (Trchounian & Trchounian, 2013). However, there are rather many questions to be answered.

The other situation on H_2 production is with photo-fermentation when the highest rate of photo-biological H_2 production has been achieved with strains belonging to the purple non-sulfur bacteria *Rhodospirillaceae*, such as *Rh. sphaeroides*, which have been investigated to some extent (Gabrielyan & Trchounian, 2009a; Hallenbeck, 2009; Kondratieva & Gogotov, 1981; Levin et al., 2004; Mudhoo et al., 2011; Ooshima et al., 1998).

Phototrophic microorganisms are a potential for H_2 production which is connected with the absorption of light energy and, hence, can increase the efficiency of the solar radiation (Levin et al., 2004; Rupprecht et al., 2006). Hyd enzyme might also be responsible for H_2 production by these bacteria (Gabrielyan & Trchounian, 2009a; Kars et al., 2008). It is important to create anaerobic conditions, select temperature, $[pH]_{out}$, carbon and nitrogen sources ratio, light intensity and etc. (Gabrileyan et al., 2010; Gabrielyan & Trchounian, 2012; Kapdan & Kargi, 2006; Kim et al., 2006; Ooshima et al., 1998; Sasikala et al., 1991. 1995; Uyar et al., 2007). The optimum growth temperature for the photosynthetic bacteria is in the range of 30 °C –35 °C and $[pH]_{out}$ is 7.0.

The substrates providing significant H_2 photo-production by a number of purple bacteria are malate, pyruvate, succinate and lactate (Gabrielyan & Trchounian, 2009a,b; 2012; Gabrielyan et al., 2010; Gadhamshett et al., 2008; Guillaume & Patrick 2009; Levin et al., 2004;). Other compounds (for example acetate, propionate) can also be used depending on the properties of different species and strains (Kapdan & Kargi, 2006). Among substrates lactate is especially interesting, as a significant amount of lactate is a part of waste products of some manufactures. The H_2 photo-production by non-sulfur purple bacteria depends on the content of different metal ions in the growth medium, especially Ni or Mg ions, as shown recently (Hakobyan et al., 2012c). Moreover, a high yield of H_2 production from pure and crude glycerol is also possible by photo-fermentation (Guillaume & Patrick et al., 2009). Such a yield, when crude glycerol is used, is close to a theoretical maximum (Sarma et al., 2012).

Strategies using molecular genetics have had some notable successes improving the H_2 production efficiency of purple bacteria. The light harvesting antenna size was reduced to increase light capture efficiency (~50%); uptake Hyd enzymes and alternative electron link pathways that compete with H_2 production were ''knocked out'' (~30%–35%) and culture parameters were optimized (Franchi et al., 2004; Kondo et al., 2002; Vasilyeva et al., 1999). To date, the most economic H_2 generation method using purple bacteria involves using carbohydrate-rich wastewater from industrial food processing for bacterial H_2 fermentation (Kapdan & Kargi, 2006; Mudhoo et al., 2011).

Different ways to enhance H_2 production by photosynthetic non-sulfur purple bacteria are considered including not only choosing effective substrates (Gabrielyan et al., 2010; Gabrielyan & Trchounian, 2012), but also exploring H_2 metabolism pathways (Kars et al., 2008; Kondratieva & Gogotov, 1981; Wang & Wan, 2009). These are regulating electron transfer routes within bacterial cell membranes and activity of Hyd enzymes. Together with our results on the effects of different nitrogen sources and metal ions on H_2 photo-production by *Rh. sphaeroides* (Gabrielyan et al., 2010; Gabrielyan & Trchounian, 2012; Hakobyan et al., 2012bc) all these might delight novel pathways in controlling H_2 production.

Finally, although H_2 fermentative production is more efficient than photosynthetic methods (Hallenbeck, 2009; Mudhoo et al., 2011), H_2 production biotechnology can be further developed using a combination of *E. coli* and *Rh. sphaeroides* in different steps during the continuous process: this study has been already begun at the laboratory scale (Manish & Banerjee, 2008; Mudhoo et al., 2011; Redwood et al., 2008).

In general, effective sources and optimal conditions for H_2 production by different bacteria are currently under intensive study. Almost two times more H_2 per mol glycerol can be produced from crude glycerol compared with pure glycerol (Sarma et al., 2012). However, it is interesting that production could be more if glucose is replaced with the other sugars and glycerol (mixed carbon) in a complex medium (Maeda et al., 2007b; Sanchez-Torres et al., 2013; Trchounian & Trchounian, 2013). Using mixed bacterial cultures or a continuous mode instead of batch cultures for H_2 production is an approach undergoing as well (Sarma et al., 2012; Selembo et al., 2009). However, the situation seems to be complicated and intensive study is required. The other important novel study would relate to interplay between biofilm formation and H_2 production by bacteria (Domka et al., 2007; Maeda et al., 2007b). Different gene expression for four Hyd enzymes in *E. coli* has been found during biofilm formation, and that may change H_2 production activity.

There are many other topics and perspective approaches to develop H_2 production biotechnology but they will be considered separately.

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Declaration of interest

The author has no conflict of interest.

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