INTERNATIONAL JOURNAL OF HYDROGEN ENERGY XXX (2017) 1–7

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli*

Satenik Mirzoyan [a], *Pablo Maria Romero-Pareja* [a,b], *Maria Dolores Coello* [b], *Armen Trchounian* [a], *Karen Trchounian* [a,*]

[a] *Department of Biochemistry, Microbiology and Biotechnology, Faculty of Biology, Yerevan State University, 0025, Yerevan, Armenia*

[b] *Department of Environmental Technology, Universidad de Cádiz, Poligono Rio San Pedro S/n, 11510, Puerto Real, Cadiz, Spain*

ARTICLE INFO

Article history:
Received 18 June 2017
Received in revised form
15 July 2017
Accepted 17 July 2017
Available online xxx

Keywords:
Escherichia coli
Hydrogenases
Glycerol, glucose and formate utilization
Bioenergy and biohydrogen
pH

ABSTRACT

Biohydrogen production by *Escherichia coli* during fermentation of the mixture of glycerol, glucose and formate at different pH values was studied. Employing mutants lacking large subunits of different hydrogenases (Hyd), it was reported that, at pH 7.5, H_2 production was produced except in a *hyaB hybC hycE* triple mutant, thus suggesting compensatory H_2-producing functions of the Hyd enzymes. Activity of Hyd-4 was revealed in glucose assays at pH 7.5 in the triple mutant whereby 62% of the wild type level of H_2 production was derived from Hyd-4. In formate assays, it was shown, that, first, the *hyaB hybC* double mutant had a H_2 production ~3 fold higher than wild type, indicating that Hyd-1 and Hyd-2 oxidize H_2, and second, that at pH 5.5, Hyd-4 and Hyd-3 were responsible for H_2 production. These findings are significant when applying various carbon sources such as sugars, alcohol and organic acids for biohydrogen production.

Introduction

The current climate of shrinking fossil fuel resources and continually increasing energy demand requires the identification of efficient, renewable and ecologically friendly new sources of energy. Molecular hydrogen, or dihydrogen (H_2), fits these criteria, with its high energy density – 142 kJ/g, and having water as only product of combustion [1,2]. H_2 is produced during dark fermentation by bacteria through biological conversion of organic substrates such as alcohols (glycerol), organic carboxylic acids (e.g. acetic, formic, succinic acids), different sugars (e.g. glucose, lactose, xylose), which are present in organic wastes originating from agricultural and industrial processes [3,4]. Glycerol is massively produced as a by-product during biodiesel generation (1 kg of glycerol is produced for every 10 kg of biodiesel synthesized) [5]. Different sugars and organic acids (e.g. acetic, formic, succinic) are present in agricultural and industrial wastes, making these potentially valuable energy sources [6–9].

Gonzalez et al. [10] reported a decade ago that polyols, such as glycerol, can be metabolized under anaerobic fermentative

* *Corresponding author*. Department of Biochemistry, Microbiology and Biotechnology, Yerevan State University, 1 A. Manoogian Str., 0025, Yerevan, Armenia.
E-mail address: k.trchounian@ysu.am (K. Trchounian).

http://dx.doi.org/10.1016/j.ijhydene.2017.07.126

Please cite this article in press as: Mirzoyan S, et al., Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli*, International Journal of Hydrogen Energy (2017), http://dx.doi.org/10.1016/j.ijhydene.2017.07.126

conditions by *Escherichia coli* at pH 6.3 and further, at pH 7.5 [11]. Products of the process depend on external pH but biohydrogen was always detected. As a consequence of such a significant finding, different mixtures of inexpensive and readily available carbon sources such as sugars (lactose, xylose etc.) and glycerol have been tested to improve H_2 production by *Escherichia coli* [12–15]. Moreover, different mixtures of carbonaceous substrates have been tested by various research groups in co-cultivation experiments with the aim of transformation these organic substrates into valuable chemicals [16–18].

During mixed acid fermentation *E. coli* produces H_2 at different pHs. H_2 is produced via membrane-bound [Ni–Fe] hydrogenase (Hyd) enzymes. Their activation depends on various external factors such as the type of fermentation substrate, external pH etc. [19–22]. The mechanisms underlying the expression of genes encoding the Hyd enzymes and, furthermore, their posttranslational maturation, assembly and activity, as well as reciprocal interaction with other membrane proteins are an intense focus of study. An important feature of Hyd enzymes in *E coli* is their reversibility with regard to H_2 production and oxidation [9,19]. The main H_2 evolving formate hydrogen lyase (FHL-1) complex consists of Hyd-3 and formate dehydrogenase (FDH-H), is active at low pH [19]. The other formate hydrogen lyase complex – FHL-2 formed by Hyd-4 and FDH-H is proposed to be active at high pH [23]. Although, it is well documented [24–26] that synthesis of both FHL complexes is under transcriptional control, their differential synthesis is not clearly defined.

External pH is important for the activity of Hyd enzymes [27,28], which makes a key contribution to the enhancement of H_2 production. Moreover, in the case of Hyd-4 activity, the fermentation substrate is important, as well as its concentration. Thus, Hyd-4 is active within a glucose concentration range from low to moderately high (0.2%) [22]. Several other considerations are highly relevant for enhancing biohydrogen generation, including understanding the physiology of the Hyd enzymes, the involvement of heavy metals and stimulating and inhibiting metabolites [15,29–31]. Moreover, the choice of strains, and whether the genes encoding some of the Hyd enzymes and their regulatory proteins are absent, has a major influence on H_2 production [32,33].

Currently, a key issue in the field of biohydrogen production is the fermentation or utilization of various mixed carbon sources released from wastes of industry or agriculture. Other major goals are the assessment and the improvement of the process of H_2 production and a major aim is to understand what controls the activity of Hyd enzymes.

Fermentation of a combination of three carbon sources (glucose, glycerol and formic acid) by *E. coli*, which constitutes itself a novel approach, with different external pHs, is conducted in the current work, targeting conditions for enhancement of H_2 production and on providing further insights into the Hyd enzymes involved in H_2 generation.

Materials and Methods

Bacterial strains and cultivation

The characteristics of *E. coli* strains used in the study, BW25113 or MC4100 (wild type parents) and mutant strains with defects in the genes coding Hyd enzymes are described in Table 1.

Bacterial cells culture was grown overnight (O/N) under anaerobic fermentative conditions and transferred into buffered growth medium containing peptone (20 g L^{-1}) at pH of 7.5, 6.5 and 5.5, with salt compositions as follow: 15 g L^{-1} K_2HPO_4, 1.08 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 7.5); 7.4 g L^{-1} K_2HPO_4, 8.6 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 6.5), and 1.08 g L^{-1} K_2HPO_4, 15 g L^{-1} KH_2PO_4 and 5 g L^{-1} NaCl (pH 5.5). The medium was simultaneously supplemented with the following carbon sources: glucose (2 g L^{-1}), glycerol (10 g L^{-1}) and sodium formate (0.68 g L^{-1}). Kanamycin (25 μl mL^{-1}) was also added when appropriate (see Table 1).

Bacterial cultures were grown in sealed flasks with closed lids under fermentative conditions for 18–24 h at 37 °C; anaerobic conditions in the medium were achieved by displacing O_2 during autoclaving [11,21,22]. The medium pH was determined using a pH-meter with selective pH-electrode (HJ1131B, Hanna Instruments, Portugal) and adjusted to required values (see above) with 0.1 M NaOH or 0.1 N HCl. Bacterial growth was monitored by means of measuring bacterial culture absorbance by spectrophotometric method (600 nm) (Spectro UV–Vis Auto, Labomed, USA).

Table 1 – Characteristics of *E. coli* wild type parents and mutant strains used.

Strains	Genotype	Absent hydrogenase subunit or related protein	References
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rha* BAD$_{LD78}$	Wild type parent	[11]
MC4100	F-*araD139* Δ (*argF-lac*)*U169* λ-*rpsL150 relA1 deoC1 flhD5301* Δ(*fruK-yeiR*)*725*(*fruA25*) *rbsR22* Δ(*fimB-fimE*) *632* (::IS1)	Wild type parent	[21,34]
JW0955 Km^{Ra}	BW 25113 Δ*hyaB*	Large subunit of Hyd-1	[11,28]
JW2962 Km^{Ra}	BW 25113 Δ*hybC*	Large subunit of Hyd-2	[11,28]
JW 2691Km^{Ra}	BW 25113 Δ*hycE*	Large subunit of Hyd-3	[35]
JW2472 Km^{Ra}	BW25113 Δ*hyfG*	Large subunit of Hyd-4	[9,37]
MW 1000	BW25113 Δ*hyaB* Δ*hybC;*	Large subunits of Hyd-1 and Hyd-2	[9,37]
FTD147	MC4100 Δ*hyaB* Δ*hybC* Δ*hycE*	Large subunits of Hyd-1, Hyd-2 and Hyd-3	[26]
FTD150	MC4100 Δ*hyaB* Δ*hybC* Δ*hycE* Δ*hyfG*	Large subunits of Hyd-1, Hyd-2, Hyd-3 and Hyd-4	[26]

[a] Resistant to kanamycin.

Please cite this article in press as: Mirzoyan S, et al., Evidence for hydrogenase-4 catalyzed biohydrogen production in *Escherichia coli*, International Journal of Hydrogen Energy (2017), http://dx.doi.org/10.1016/j.ijhydene.2017.07.126