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Fermentative hydrogen production in recombinant *Escherichia coli* harboring a [FeFe]-hydrogenase gene isolated from *Clostridium butyricum*

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ARTICLE INFO

Article history:
Received 24 January 2011
Received in revised form
2 April 2011
Accepted 5 April 2011
Available online 12 May 2011

Keywords:
Clostridium butyricum
[FeFe]-hydrogenase
Heterologous expression
Fermentative hydrogen production

ABSTRACT

The [FeFe]-hydrogenase (*hydA*) from *Clostridium butyricum* TERI BH05-2 strain was isolated to elucidate its molecular characterization. A 1953 bp DNA fragment encompassing the ORF and the putative promoter region of *hydA* gene was PCR amplified and subcloned into pGEM®-T-Easy cloning vector (pGEM®-T-*hydA*). The *hydA* DNA sequence revealed the presence of a 1725 bp length ORF (including the stop codon) encoding 574 amino acids with a predicted isoelectric point and molecular mass of 6.8 and 63097.67 Da, respectively. The *hydA* ORF was PCR amplified from pGEM®-T-*hydA* and inserted into a prokaryotic expression vector to create a recombinant plasmid (pGEX-5X-*hydA*) and transformed into *Escherichia coli* BL-21. The recombinant *E. coli* BL-21 was investigated for fermentative hydrogen production under anaerobic condition from glucose. Heterologous expression of the *Clostridium butyricum hydA* resulted in 1.9 fold increase in hydrogen productivity as compared to that from the wild type strain, *C. butyricum* TERI BH05-2. The hydrogen yield of the recombinant strain was 3.2 mol H_2/mol glucose, 1.68 fold higher than the wild type parent strain.

1. Introduction

Hydrogen (H_2) is renewable, efficient, and environmentally benign and thus considered as a promising energy source for the future [1]. Anaerobic microorganisms have evolved unique mechanisms for hydrogen production. The facultative and strict anaerobic bacteria such as *Escherichia coli*, *Enterobacter* species [2,3] and Clostridia species [4,5], respectively, have been reported for fermentative conversion of organic substrates to hydrogen.

In Clostridia species fermentative hydrogen production proceeds from the anaerobic metabolism of pyruvate by the following electron transfer chains: pyruvate: ferredoxin oxidoreductase (PFOR), NADH: ferredoxin oxidoreductase (NFOR), and hydrogenase [6]. Pyruvate: ferredoxin oxidoreductase (PFOR) from several anaerobic or microaerobic species of microorganisms reduces ferredoxin as it breaks down pyruvate to acetyl-CoA. Recently, Agapakis et al. have reported for creation and characterization of a synthetic electron transfer circuit that couples hydrogen evolution with the breakdown of glucose in *E. coli* via heterologous expression of PFOR, Ferredoxin, and [FeFe]-hydrogenase [7].

Hydrogenase enzyme is principally the prime candidate in these fermentative microbes which catalyzes the reduction of

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0360-3199/$ – see front matter
doi:10.1016/j.ijhydene.2011.04.027

protons into hydrogen. Based on the sequence of constituent polypeptides and the structure of the metal center active site, hydrogenases are categorized into three groups: [NiFe]-hydrogenase, [FeFe]-hydrogenase and [Fe]-hydrogenase [8,9]. In *E. coli* the native [NiFe]-hydrogenases are coupled to NADH with a reducing potential of −320 mV, while [FeFe]-hydrogenases are partnered with the electron carrying protein ferredoxin which can have a significantly stronger reducing potential, close to that of the H_2/H^+ (−420 mV) [10]. Hence, [FeFe]-hydrogenases thermodynamically favor hydrogen production. Compared to [NiFe]-hydrogenase, [FeFe]-hydrogenase is 10 times more active as a hydrogen producer [11]. Clostridial hydrogenases belong to the group of [FeFe]-hydrogenase most of which are cytoplasmic, soluble and monomeric [12]. [FeFe]-hydrogenases from *Clostridium* species contain several 'ferredoxin-like' domains which enhances hydrogenase interaction with other ferredoxins and provides an electron transport channel toward the hydrogenase active site [13]. Among few hydrogenases those are characterized the [FeFe]-hydrogenase I of *Clostridium pasteurianum* is the most extensively studied enzyme [12,13]. Furthermore, [FeFe]-hydrogenases have been characterized and recombinant strains have been constructed for several species of *Clostridium*; *C. acetobutylicum*, *C. pasteurianum*, *C. tyrobutyricum*, *C. perfringens* and *C. paraputrificum* [14–19].

[FeFe]-hydrogenases serve as excellent enzymatic modules for recombinant expression in a synthetic system because of their high hydrogen production activity, conserved structure including simple maturation pathway [7]. Heterologously expressed [FeFe]-hydrogenases have been characterized from several species in vitro and in vivo [20–22]. In vivo heterologous expression of [FeFe]-hydrogenase alone is sufficient for small, measurable hydrogen production from *E. coli* and this hydrogen production can be enhanced with the co-expression of ferredoxins from several organisms [21]. Mishra et al. isolated a novel [Fe]-hydrogenase gene from *Enterobacter cloacae* IIT-BT 08 [23]. Overexpression of this [Fe]-hydrogenase in *E. coli* BL-21 resulted in 3.12 mol of hydrogen yield from 1 mol of glucose [24].

In the present study, we report the isolation, molecular characterization and heterologous expression of [FeFe]-hydrogenase gene from *C. butyricum* TERI BH05-2, in *E. coli* BL-21.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Clostridium butyricum TERI BH05-2, a dark fermentative hydrogen producing bacterium, was isolated from sediment samples collected from Yamuna river (contaminated with organic pollutants), flowing below the Delhi–Noida-Direct flyway, New Delhi, India (28_3704000N 77_1502100E) [25]. pGEM®- T-Easy (Promega, USA) plasmid was used for cloning and sequencing of *C. butyricum* TERI BH05-2 *hyd*A gene. Plasmid pGEX-5X-3 (Pharmacia, USA) was used to over express the *hyd*A gene. Selection for the presence of plasmids was carried out in the presence of 100 μg/ml ampicillin. For DNA extraction, batch cultures of *C. butyricum* TERI BH05-2 strain were grown anaerobically at 37 °C in modified DMI medium. The modified DMI medium was composed of (per liter): 5.24 g of NH_4NO_3, 6.72 g of $NaHCO_3$, 0.087 g of K_2HPO_4, 0.5 g of $MgCl_2$, 0.0075 g of $MnSO_4$, 0.025 g of Na_2S, 0.02 g of $FeSO_4$, 4 g of malt extract, 4 g of yeast extract and 10 g of glucose [25]. Modified DMI medium is a modified form of DMI medium [26]. The DMI medium was composed of (g/l): NH_4HCO_3, 5.24; $NaHCO_3$, 6.72; K_2HPO_4, 0.125; $MgCl_2$, 0.1; $MnSO_4$, 0.015; Na_2S, 0.5; $FeSO_4$, 0.01; resazurin, 0.01; and carbon source as a substrate, 17.8. *E. coli* BL-21 (Promega, USA) cells were grown anaerobically at 37 °C in Luria Bertani medium (5 g yeast extract, 10 g peptone and 10 g NaCl per liter). Recombinant *E. coli* BL-21 was cultivated in Luria Bertani medium supplemented with ampicillin (100 μg/ml).

2.2. DNA isolation and manipulation

RNA free genomic DNA was extracted from *C. butyricum* TERI BH05-2 by employing the method as described previously [27]. The plasmid DNA was prepared from *E. coli* cells with an alkaline lysis technique using GET™ plasmid miniprep kit (G-Biosciences, USA). The PCR amplified *hyd*A ORF was gel purified and digested with *Bam*HI restriction enzyme by incubating at 37 °C for 2 h. The digested *hyd*A gene fragment was ligated into *Bam*HI digested pGEX-5X-3 prokaryotic expression vector.

2.3. Nucleotide sequence determination of *C. butyricum* TERI BH05-2 strain hydA gene

A pair of oligonucleotide PCR primers: UpSt-FW/RV-1 (Table 1) were designed, based on the published nucleotide sequences of [FeFe]-hydrogenase genes (*hyd*A coding sequence with its upstream putative promoter sequence) from other *C. butyricum* bacterial strains, as available in the NCBI database. A 1953 bp DNA fragment was amplified from genomic DNA of *C. butyricum* TERI BH05-2 strain by PCR using the above mentioned designed PCR primer pair. The PCR conditions consisted of an initial denaturation step for 2 min at 95 °C, 30 cycles of repeated denaturation step at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension of 10 min at 72 °C. The PCR amplified DNA fragment was gel purified and cloned into pGEM®-T-Easy plasmid vector. The ligated plasmid construct was transformed into the *E. coli* JM109 strain as per the manufacturer's instructions (Promega, USA). Presence of insert was confirmed by restriction enzyme digestion and nucleotide sequencing. The recombinant plasmid construct was designated as pGEM®-T-*hyd*A (Fig. 1). ORF finder tool (http://www.ncbi.nlm.nih.gov) was employed for determination of *hyd*A ORF sequence. The instructions were followed as described in the above mentioned web site. BLASTN was used to search for nucleotide identity of *hyd*A coding region. BLASTP was used to search for deduced amino acid identity of *hyd*A coding region. The theoretical isoelectric point (pI) and molecular weight (MW) of *hyd*A gene encoding hydrogenase enzyme were determined by employing web-based software (http://br.expasy.org/cgi-bin/pi_tool). ScanProsite tool (http://expasy.org/tools/scnpsit3.html) was employed for functional domain analysis of *hyd*A amino acid sequence.