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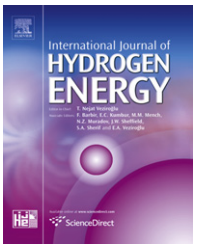

Uncharacterized *Escherichia coli* proteins YdjA and YhjY are related to biohydrogen production

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ABSTRACT

Biohydrogen has gained importance as an alternative energy source, and advances in molecular biology and biotechnology have raised the quality and efficiency of biohydrogen production from various microorganisms and substrates. Here, *Escherichia coli* proteins YdjA and YhjY have been identified as essential in biohydrogen production from glucose. The mutations *ydjA* and *yhjY* reduced biohydrogen productivity compared to the parent strain from 40 to 4 and 29 μmol/mg protein, respectively. Through transcription analysis, it was determined that YdjA and YhjY are positive effectors of the FHL complex since their inactivation repressed *fhlA*. In addition, the FHL expression of the repressor gene, *hycA*, increased for the *ydjA* mutant, so YdjA reduces transcription of the HycA repressor. Hence, two new proteins have been identified that are important for biohydrogen production.

1. Introduction

The merits of biohydrogen as a fuel source remain undisputed due to its higher energy content compared to hydrocarbon fuels [1,2]. The necessity of replacing fossil fuels also has been discussed extensively since petroleum prices have increased dramatically and there is continued anxiety about the level of green house gases (GHG) in the atmosphere [3]. Critically, Kim et al. reported that only water vapor is produced once biohydrogen is combusted, and no pollutants evolve which may contribute to the GHG phenomena [4].

Many methods and sources are available for biohydrogen production through physical, chemical or biological approaches [1]. Haijun has reported chemical and physical approaches such as partial oxidation of fossil fuels and steam reforming of natural gas are producing large amounts of hydrogen, however these processes have created environment pollution and increase the cost of hydrogen production [5]. On the other hand, hydrogen also has been produced through the water–gas shift reaction and as a by-product of petroleum refining, gasification of coal, and electrolysis of water which are grouped as chemical or electrochemical methods. Indeed, methods that have been mentioned have high costs since some of them require high temperature >850 °C (physical properties) [1,6]. Another studies have reported that the addition of a specific chemical to the fermentation process by

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obligate anaerobic microorganisms influenced bacterial activity to finally increase biohydrogen production [7,8]. Among them, the biological path seems relevant for large production of biohydrogen due to substrate availability for microorganisms. Biohydrogen from biological methods, especially dark fermentation [9,10], offers utilization of renewable sources such as agricultural feedstocks, starch, biomass, organic wastes as well as sludge from industries [11–14].

The formation of hydrogen is catalyzed and controlled by hydrogenase (H_2ase). H_2ase genes have been described and characterized from many researchers in the last few decades [15]. H_2ase has been classified into three groups, [NiFe]-, [FeFe]- and [Fe]-hydrogenases based on the metal content of their active sites [16]; these enzymes catalyze the conversion of hydrogen to the protons and electrons, through a redox reaction ($H_2 \leftrightarrow 2H^+ + 2e^-$) [3]. Biosynthesis of H_2ases requires accessory proteins for maturation. Each H_2ase needs different maturation proteins but also shares similar proteins such as guanine nucleotide-binding proteins (GTPases). There are many proteins that are involved in the maturation steps, including HypABCDEF (metallochaperones for NiFe insertion), HycI, encoding the endopeptidase and SlyD responsible for nickel insertion. Similarly, [FeFe]-hydrogenases require HydE, HydF and HydG proteins to complete the biosynthesis [17,18].

Escherichia coli is esteemed as a robust bacterium for research development due to its well characterized proteins and the accessibility of its complete genome [19,20]. Additionally, genetic and protein engineering have been used to enhance biohydrogen production in *E. coli* [3,19,21,22]. *E. coli* has four membrane-bound hydrogenases which are responsible for hydrogen synthesis and hydrogen uptake referred to as hydrogenase 1, hydrogenase 2, hydrogenase 3 [3,19], and hydrogenase 4 [15,23]. Among them, hydrogen 3 has a dramatic effect in hydrogen synthesis. Hydrogenase 3 is encoded by the *hyc* operon and is comprised of the large subunit *hycE* and the small subunit is encoded by *hycG* [24].

Glucose is converted to pyruvate and NADPH through glycolysis pathways. Afterward, pyruvate formate lyase (PFL) will catalyze the conversion of pyruvate to formate along with acetyl coenzyme A, then the formate hydrogen lyase (FHL) complex system will be activated to synthesize two moles of hydrogen from two moles of pyruvate in mixed-acid fermentations [3]. In addition, succinate is one of the intermediates in the tricarboxylic acid cycle (TCA) either in facultative or strict anaerobes [25]. Succinate is synthesized from phosphoenolpyruvate through the fumarate reductase reaction (FrdABCD). Accumulation of succinate might reduce hydrogen yield due to consumption of phosphoenolpyruvate to oxaloacetate and finally succinate through fumarate instead of sythesized pyruvate to hydrogen through formate [26,27]. Therefore, deletion of the *frdC* gene results in increased the biohydrogen yield by about two fold [24]. Meanwhile data encountered through disruption of the *pflB* and *ldhA* genes has testified that genes are responsible in succinic acid production during mix-acid fermentation in *E. coli* [25].

The FHL complex is one of the essential components for biohydrogen in *E. coli*. The FHL complex consists of formate dehydrogenase (FDH-H) encoded by *fdhF* [23], hydrogenase 3 (from the *hyc* operon), while essential activator of FHL (*fhlA*) and repressor (*hycA*) are regulators for the system. Sanchez-Torres et al., used random mutagenesis over the whole *fhlA* gene to increase biohydrogen production nine fold, by protein engineering [22].

Organic acids and ethanol have been reported as excess by-products derived from the anaerobic process of biohydrogen production [28,29]. Overabundance of unnecessary or accessory products can be decreased by metabolic engineering techniques based on the anaerobic pathway [30]. Numerous studies have shown that metabolic engineering as mentioned in the previous paragraph can enhance production of biohydrogen. It is essential to understand the particular by-products produced in each reaction during anaerobic pathways in order to clarify appropriate modification and alteration using metabolic engineering.

Therefore, many researchers believe that the metabolic pathway of biohydrogen production by *E. coli* is completely understood; however, to date, an exhaustive search of genes related to hydrogen production has not been conducted. To address this, here we elucidated uncharacterized genes related to biohydrogen production by screening the entire Keio mutant library (3985 isogenic mutants) with chemochromic membranes (GVD Corp., Cambridge, MA). These membranes are formed by a thin film of WO_3 covered with a catalytic layer of palladium and are used to detect biohydrogen gas produced by colonies via a colorimetric response [22,23]. Using this exhaustive screen, we found that the uncharacterized proteins YdjA and YhjY are important for biohydrogen production in *E. coli*.

2. Materials and methods

2.1. Bacteria strains, selection and growth

The strains used are described in Table 1. Parent strain *E. coli* BW25113, the Keio mutants, and the ASKA plasmid strains

Table 1 – *E. coli* strains and plasmids used in this study.

Strains and plasmids	Genotype/relevant characteristics	Reference
BW25113	F^- *Δ(araD – araB)567 ΔlacZ4787(::rrnB-3) λ⁻ rph-1 Δ(rhaD – rhaB)568 hsdR514*; parental strain for the Keio collection.	Yale Coli Genetic Stock Center
BW25113 *ydjA*	BW25113 *ΔydjA::kan* Km^R	[31]
BW25113 *yhjY*	BW25113 *ΔyhjY::kan* Km^R	[31]
BW25113 *ldhA*	BW25113 *ΔldhA::kan* Km^R	[31]
BW25113 *fhlA*	BW25113 *ΔfhlA::kan* Km^R	[31]
BW25113 *fdhF*	BW25113 *ΔfdhF::kan* Km^R	[31]
BW25113 *hypC*	BW25113 *ΔhypC::kan* Km^R	[31]
ASKA JW2701	Harboring *fhlA* Cm^R	National Institute of Genetics
ASKA JW2968	Harboring *hypC* Cm^R	
ASKA JW0886	Harboring *pflB* Cm^R	
ASKA JW4040	Harboring *fdhF* Cm^R	

Km^R is kanamycin resistance; Cm^R is chloramphenicol resistance.

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(*fdhF*, *hypC*, *pflB* and *fhlA*) were obtained from the National Institute of Genetics, Mishima, Shizuoka, Japan. The mutants referred to as BW25113 *ydjA*::*kan* (*ydjA*) and BW25113 *yhjY*::*kan* (*yhjY*), were obtained from the Genome Analysis Project in Japan through their Keio collection [31]. Both strains were mutated at the *ydjA* and *yhjY* alleles, respectively. Strains were routinely streaked on Luria-Bertani (LB) plates containing 100 μg/ml kanamycin or 30 μg/ml chloramphenicol where appropriate and grown at 37 °C for 15–18 h. Single colonies of streaked strains were grown aerobically in a shaking incubator at 120 rpm at 37 °C in LB medium prior inoculation for fermentation. The cell turbidity was measured at 600 nm with a UV/VIS spectrophotometer (JASCO V-530), and the total cell protein values were quantified based on optical density (OD) and total proteins in *E. coli* (Protein assay kit, Sigma Diagnostics, St. Louis, MO, USA), 0.22 mg/OD/ml [24].

2.2. Fermentation and biohydrogen assay

Cells suspensions from the overnight culture (1 ml) and fresh complex medium containing glucose or formate (9 ml) [32] were mixed in 34 ml crimp-top serum vials inside an anaerobic chamber. In order to provide anaerobic conditions through the process, the inoculum and fresh medium were sparged with nitrogen for 5 min, respectively, prior inoculation process [23]. The biohydrogen assay was conducted for 24 h with two independent cultures. The amount of biohydrogen generated in the headspace was measured using gas chromatography (GC) with a thermal conductivity detector. Specifically, 50 μl of sample was measured using GC as described by Maeda and his colleagues [17]. Also, glucose minimal medium without tryptone and yeast extract was used for organic acids determination in each culture. Samples for organic acid analysis were centrifuged to remove suspended solid and supernatant was then filtered with 0.45 μm pore size syringe filter before being analyzed by High Performance Liquid Chromatography (HPLC) (Shimadzu LC-10AD with conductivity detector CDD-6A equipped with Shim-packed SCR-102H (8.0 mm LD × 300 mm L) × 2 column) as reported previously [33].

2.3. Mutants verification

The two mutants used in this study were verified by polymerase chain reaction (PCR). Two pairs of specific primers for the *ydjA* and *yhjY* genes were used (Table 2). PCR amplification was done by using KOD plus reagent (TOYOBO CO., Ltd), the mixture consists of 2 μl 10× Buffer for KOD plus, 2 μl dNTPs (2 mM), 1.6 μl $MgSO_4$ (25 mM), 0.3 μl each primer (20 mM), 1.0 μl template (0.2–4 ng/μl) and 0.5 μl KOD plus enzyme (1.0 U/μl). Total amplification reaction was 20 μl and amplified for 30 cycles which each cycle consisting denaturation at 95 °C run for 1 min followed by annealing 25 °C for 1 min and elongation at 68 °C for 5 min, the cycle was pre-denatured at 95 °C for 5 min.

2.4. Total RNA extraction and quantitative RT-PCR

RNA was obtained from cell pellets during the exponential phase of the fermentation process. Exponential phase was defined as a log phase of hydrogen production rate along with higher glucose consumption rate. Whereas, pattern of

Table 2 – Primers designed according to the target genes in expression analysis and for strain verification.

Name/targeted gene/function	Sequence	Relevant characteristics
fhlA Transcriptional activator	f – 5′-GTGTAT TGCAGGAACAG GAGTTTG-3′ r – 5′-GAATACGT TCAGGCGG TAATAGAG-3′	Activator of FHL complex [31]
fdhF formate dehydrogenase-H	f – 5′-GGATT TCTACGGT GCGACTTAC-3′ r – 5′-GGTACT CGTCGGT GAGTTTGTC-3′	Enzymes catalyze the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ [1]
hypB GTPase	f – 5′-CACTG GACGATAA CGGTATTCTG-3′ r – 5′-AAGTT GAGATAC GGCAA CAGGT-3′	Nickel liganding into hydrogenase large subunit [1]
hypF Carbamoyl phosphate phosphatase	f – 5′-ATC TTCACGGC GATGTCTGT-3′ r – 5′-CTGT GCTCTGGC GTATAGTGAA-3′	Required during assembling of Ni–Fe metallocenter [23]
hycE Large subunit	f – 5′-ATCAG CTGACTG TCACCGTAAAG-3′ r – 5′-GTAAT CCAACACT TAGTGC CCTTC-3′	Large subunit of hydrogenase 3 (Hyd-3) [22]
ldhA lactate dehydrogenase	f – 5′-GCTAAC TTCTCTC TGGAAGGTCTG-3′ r – 5′-GACATA CTCCACA CCGAGTTCC-3′	Converting pyruvate to lactate [23]
rrsG Housekeeping gene	f – 5′-TATTG CACAATG GGCGCAAG-3′ r – 5′-ACTTAAC AAACC GCCTGCGT-3′	Housekeeping gene for expression data [56]
hycA Repressor gene	f – 5′-CTACTG CAATTC GCTGGTTCAG-3′ r – 5′-CGACGT AATACT CGATGGTGTG-3′	FHL complex repressor gene [4]
pflB	f – 5′-CGGTAT CGACTACCTG ATGAAAG-3′ r – 5′-CAGAG ATGTCGT \AGCCGT ATTTC-3′	Coded for pyruvate formate lyase (PFL) [25]

(*continued on next page*)

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Table 2 – (continued)

Name/targeted gene/function	Sequence	Relevant characteristics
ydjA-confirm	f – 5′-GTAAT GACCCAA CCGGTACT-3′ r – 5′-GTAAC CCTCATT CCGTGAAG-3′	Confirmation absence of *ydjA* gene (this study)
yhjY-confirm-f	f – 5′-CCATA CTTTCCCT CGCTATGTACCC-3′ r – 5′-AGTTAGC ACCCACTATC GCCACAAC-3′	Confirmation absence of *ydjA* gene (this study)
k1	5′-CAGTCATA GCCGAATA GCCT-3′	Confirmation presence of kanamycine [30]
k2	5′-CGGTGC CCTGAAT GAACTGC-3′	Confirmation presence of kanamycine [30]

biohydrogen production and glucose consumption rate were observed before the appropriate exponential time was chosen for mRNA extraction sample. One ml fermentation sample was mixed in RNAlater solution at 1:1 ratio (Formerly Ambion, Cat#AM7024, 250 ml, from Applied Biosystems) in 2 ml screw cap tubes before centrifuging at 130 000 rpm, 2 min. The cell pellet was immersed in 100 ml of dissolved dry ice in ethanol for 10 s and stored at −70 °C prior to RNA extraction. Total RNA was isolated using a RNeasy kit (Qiagen, Inc., Valencia, CA) and bead beater Wakenyaku Co. Ltd, Kyoto Japan, model 3011b as described by Ren et al. [34]. In addition, a StepOne Real-Time PCR system and Power SYBR green RNA-to C_T 1-Step kit (Applied Biosystems, Foster City, CA) were used to affirm transcription analysis. Quantitative real-time reverse transcription PCR (qRT-PCR) was used for the transcription analysis using targeted genes (Table 2). The *rrsG* primer (16S rRNA) was used as a housekeeping gene to normalize the values obtained from the analysis. Additionally, at least three technical replicate samples were performed. In this study, seven primers were designed to analyze the target genes namely *hycE*, *fhlA*, *fdhF*, *hypF*, *hypB*, *hycA*, *pflB* and *ldhA*. The length of each primer was about 25–30 bp (Table 2). Fifty ng/μl of RNA samples were used as a template from each strain, *ydjA* and *yhjY*, respectively. Meanwhile, RNA from parent strain was applied as reference template in all analysis. The expression level was quantified through relative quantification for qRT-PCR ($2^{-\Delta\Delta CT}$) [35].

2.5. *Complement analysis test*

Four ASKA plasmids (ASKA) were tried, ASKA2701, ASKA2968, ASKA0886 and ASKA4040 harboring *fhlA*, *hypC*, *pflB* and *fdhF*, respectively, under an isopropyl-D-1-thiogalactopyranoside (IPTG) inducible promoter (Table 1). Plasmids were purified using a plasmid extraction kit, QIAprep Spin Miniprep kit (Qiagen, Inc., Valencia, CA), and competent cells were prepared according to Datsenko and Wanner (2000). Fifty microliter of competent cells were mixed with 5 μl of purified plasmid prior to electroporating using the Bio-Rad Gene Pulser II unit. The transformed cells were incubated in 1 ml of LB for at least 1 h at 37 °C before spreading on LB agar plates with specific antibiotic selection. Overnight transformed cells were used for the inoculum for the biohydrogen assay as described previously, after a single isolation of the transformant through streaking on the same agar plate. In addition, 0.1 mM and 1.0 mM of IPTG were assimilated into medium at initial stage of fermentation process; the initial OD was measured around 0.4–0.6 vary in each strain. IPTG was added purposely for induction to the ASKA complement strains to allow over expression of target genes [24].

3. Results and discussion

There are many species of bacteria favorable for biohydrogen production through fermentation such as *Clostridium* sp., *Enterobacter* sp., *Escherichia* sp, *Klebsiella* sp., [12,30,36]. For example, Chong and co-workers have shown that *Clostridium butyricum* is an efficient strain for biohydrogen production in dark fermentations using palm oil mill effluent as carbon and nitrogen sources [12]. *E. coli* is as renown for molecular genetics research [1]; thus, in this study, *E. coli* BW25113 was chosen to explore means to increase biohydrogen production.

3.1. *Involvement of* E. coli*'s genes,* ydjA *and* yhjY *in biohydrogen production*

By screening the complete KEIO collection, *ydjA* and *yhjY* were identified as key genes for biohydrogen production in *E. coli* since these mutations dramatically decreased biohydrogen production as indicated by membrane screening (available to see as a supplementary file). These mutants showed no blue color on the membrane, which indicated the mutants did not produce hydrogen gas and that YdjA and YhjY are important for hydrogen metabolism. Importantly, there are no reports of the function of YhjY, and the structure of YdjA indicates that it is a nitroreductase [37] although there have not been any studies to confirm its activity; both proteins have not been identified as important for hydrogen production previously. In order to prove the above qualitative discovery, biohydrogen produced from the glucose by *ydjA* and *yhjY* mutants was thoroughly measured.

Fig. 1 shows the amount of biohydrogen and final biomass difference during fermentation. The wild-type strain was able to produce biohydrogen as soon as after 1-h fermentation and kept producing it up to more than 200 μmol. In contrast, the *ydjA* and *yhjY* mutants did not produce significant hydrogen; after overnight fermentation, the *ydjA* strain produces about 16 μmol and two folds less hydrogen was produced by the *yhjY* strain.

3.2. *Literature survey on* ydjA *and* yhjY *genes*

Based on screening the Keio collection, BW25113 *ydjA*::*kan* and BW25113 *yhjY*::*kan* were identified as candidates to be explored for their specific gene response to biohydrogen metabolism. Reported, *ydjA* in *Bacillus subtilis* is grouped in the

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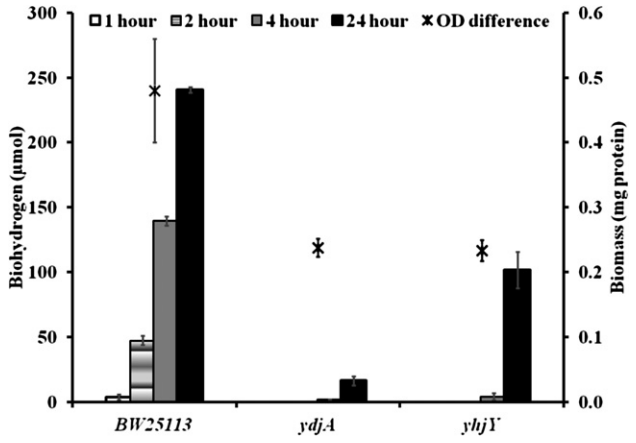

Fig. 1 – Time course of biohydrogen amount (▯ column) and optical density (OD) difference based on protein content (✱-scatter) after a 24 h fermentatiom with 100 mM of glucose in BW25113 (parent strain), *ydjA* and *yhjY* strains.

ydiR, *ydiS* and *ydjA* operon. It resides in the prophage 3 region in *Bacillus subtilis* and it functions to support activity of a DNA restriction enzyme. *ydjA* also exhibited some homology with a DNA restriction enzyme of *Lactococcus* plasmid PTR2030 (LlaI-3) and this enzyme is known as a methyltransferase [1]. *ydjA* is holding an accession number of EG11134 in EcoGene database and ECK1763 obtained from K-12 gene database. The gene is located at 1 846 149–1 846 700 bp and coding for about 183 amino acids [37,38]. *ydjA* has been described as one of the genes that coded for nitroreductases (NTRs). NTR is commonly used in bioremediation technology especially when dealing with hazardous nitroaromatic compounds. NTRs polypeptides were encoded by more than 210 amino acids, however *ydjA* gene consists only 190 amino acids in total, thus it has been classified as one of the smallest NTRs found to date [1,39]. Interestingly, through literature survey, no report has mentioned *ydjA* and *yhjY* associated to biohydrogen metabolism or influence in hydrogen metabolism. Recent closer report has claimed that *yhjY* protein is known as lipase and embedded in outer membrane cells. Translated *yhjY* protein was 25.1 kDa in weight, and its sequence is located between *yhjX* and *tag* gene in *E. coli* genome accordingly. In the study, an expression level of outer proteins has been identified, predicted, cloned and analyzed using molecular tools and techniques. Unfortunately, doubtful result was obtained whereas the present of *yhjY* protein could not be determined due to difficulty in expression experiment. However it concludes that most of the protein is found in inclusion bodies and does not related to glycolysis pathway at any stage [15,16]. In addition, *yhjY* is coding for about 232 amino acids and the gene is located at 3 710 259–3 710 957 bp in *E. coli* gene bank. *YhjY* is holding a EcoGene accession number EG12269 and ECK3535 in K-12 Gene accession number, respectively [30].

3.3. *Role of* ydjA *and* yhjY *mutants in biohydrogen production from glucose fermentation*

The data obtained from fermentation of mutants have shown almost no biohydrogen produced due to the mutation in the *ydjA* and *yhjY* alleles. In general, mutation has bounded the ability of mutant for biohydrogen generation through glucose degradation. According to Table 3, productivity was measured based on biohydrogen amount over the amount of cells (mg of protein) which contributes to biohydrogen production, generally. The wild-type strain had the highest productivity followed by *yhjY* and *ydjA* with 40, 29 and 4 μmol/mg protein, respectively (Table 3). Low productivity was measured both in glucose and formate fermentation from mutants compared to parent strain. Ten folds lower has observed from *ydjA* and about two folds given by *yhjY* in glucose fermentation. Moreover, similar results were found from formate degradation with 168, 3 and 67 μmol/mg protein obtained from parent strain, *ydjA* and *yhjY*, respectively. It is obvioius these strains are deficient in biohydrogen production through glucose and formate degradation. However, the strains have shown satisfactory growth rate throughout the experiment. The growth rate (μ) was determined under anaerobic conditions using complex glucose whereas similar rate has shown from *yhjY* and parent strain at 1.14 ± 0.06 and 1.31 ± 0.14 (1/h), respectively. Intriguingly, lower growth rate is slightly observed in *ydjA* strain, with 0.82 ± 0.07 (1/h), almost half rate different. Simple conclusions from ydjA strain, the mutation on ydjA allele has affected the growth and extinguish the production of biohydrogen. Another essential parameter is the hydrogen production rate (HPR). HPR has given parallel

Table 3 – Hydrogen production rate, biohydrogen productivity, and final pH of each strain from the fermentation of glucose and formate after 24 h at 37 °C.

Strain	Biohydrogen production rate (μmol/mg protein/h)	Biohydrogen productivity (μmol/mg protein)	Final pH	Biohydrogen production rate (μmol/mg protein/h)	Biohydrogen productivity (μmol/mg protein))	Final pH
	Glucose[a]			Formate[b]		
BW25113	1.7 ± 0.1	40 ± 1	4.8	7.0 ± 0.4	168 ± 8	7.1
ydjA	0.19 ± 0.03	5 ± 1	4.8	0.13 ± 0.03	3 ± 1	7.3
yhjY	1.2 ± 0.2	29 ± 4	4.8	2.79 ± 0.01	67 ± 0	6.5

a Glucose as substrate during fermentation.
b Formate as substrate during fermentation.

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response to the growth rate of all strains, the production rate of *ydjA* is just only 0.2 μmol/mg protein/h compared to *yhjY* and wild-type with 1.2 and 1.7 μmol/mg protein/h. As reported, it is important to technically keep initial cell viability at compatible value, in order to achieve the comparable productivity at the end of fermentation [1]. Therefore; mutation in *yhjY* has not given complement effect to the cell growth even the strain showed substantially diminish biohydrogen productivity. In addition, *ydjA* strain showed uttermost complement to the cell growth and probably, one of the reasons to the reduction in biohydrogen productivity from glucose.

The difference of glucose concentration was examined from broth in each set of fermentation to clarify consistent understanding of the metabolic degradation flows of hydrogen metabolism. Wild-type presented typical attributes of glucose utilization with 40–50% had consumed throughout the fermentation and was producing biohydrogen as by-product. Meanwhile, mutant strains (*ydjA* and *yhjY*) slightly reflect to the glucose decrement, with lower consumption had shown 20–30% reduction, respectively. Minute reduction due to acidic condition in the broth that causes inhibition in the middle of the fermentation process since slower growth was observed. Van Ginkel and Logan have proven that pH value is an immense factor affecting biohydrogen evolution due to undissociated forms of acetic or butyric acid during fermentation. The pH value between 4.5 and 5.5 has revealed tremendous result of inhibition on the bacteria and suggested higher pH would be optimum atmosphere to raise biohydrogen yield [40]. Summarized by Fang and Liu, pH control is one of crucial parameters especially affecting hydrogenase activity in hydrogen production and reported, optimum pH varied from pH 9.0 to pH 4.0 using batch mode fermentation [41]. In another report by Morimoto and her colleagues, pH was not controlled during hydrogen production fermentation and the fermentation was started at neutral pH. The optimum pH was found at pH 5.0–6.0, while the final pH is dropped to acidic conditions pH 3.0–4.0 due to formation of acids throughout the fermentation process [42].

In this study, all fermentation experiments were started at neutral pH around 7.0–7.2 with un-control pH condition. Similar condition has applied between parent strain and mutants strain. Equal condition is important to see the different between both strains in term of biohydrogen production and not exactly on the pH changes. However, final pH was determined at the end of each batch. Indeed, the pH has fallen to acidic level around pH 4–5 in all strains. Initial pH was not controlled accurately; it depends on medium preparation itself. The descent effect of pH during fermentation is agreed with mixed acid fermentation; whereas, organic acids are one of the by-products being presented during hydrogen metabolism [42–44]. On the other hand, final pH in glucose and formate has shown a big difference due to their native metabolic pathways, whereas in glucose degradation pathway, it tends to produce organic acids as by-products and finally reduce the pH value in the fermentation process [42]. Meanwhile, from formate as initial substrate, almost no possible organic acids able to synthesize as by-products, hence the final pH has not changed very much from the initial pH.

In addition, acetic acid is reported as substantive by-products in dark fermentation, which delivers maximum yield at 4 moles of hydrogen from each mole of acetate, stoichiometrically [45]. Nevertheless; other products such as butyric, lactic and alcohols also contribute in the process despite demonstrate modest effects [4]. According to the data obtained, it is important to clarify the organic acids accumulation, since each mole of organic acids will give different moles of hydrogen based on balanced equation. Perhaps, mutation has driven accumulation of undesired organic acids that did not contribute to biohydrogen yield beside inhibit fermentation process such as lactic acid accumulation [46].

3.4. *Organic acids analysis from* ydjA *and* yhjY *using minimal medium*

Final samples from fermentation were used to analyze organic acids accumulation. Data presented was computed from standard graph form each acid prepared beforehand. Five main acids were detected with acetic acid and lactic acid dominated in all strains (Fig. 2). Elaborated previously, acetic acid plays essential role during biohydrogen production with higher mole hydrogen produced from a mole of acetic [47,48]. The data obtained clearly support the fact which highest acetic acid obtained in parent strain, 7.9 mM. Meanwhile, higher concentration of lactic acid has shown in *yhjY* strain with 13.1 mM. Reported by Mohd Yasin et al., lower pH due to accumulation of lactic acids might interrupt bacteria internal pH and will suppress bacterial cells growth as observed from *yhjY* sample [46]. Higher lactic acid will quash pH value and affecting biohydrogen evolution [49].

On the other hand, formic acid has revealed extra information especially in formate degradation behavior. Formic acid was not detected from parent strain, indicating that formic acid has been utilized for biohydrogen production during the process at similar phenomenon that was exhibited by *yhjY*. Indeed; absence of formic acid in *yhjY* was not only directed to biohydrogen production since the amount of biohydrogen just half amount compared to parent strain. As understand, formic acid consumed has gone to another by-products such as CO_2 by means of formate dehydrogenase (FDH_O, FDH_N) [16]. Meanwhile; about 5.6 mM of formic acid is

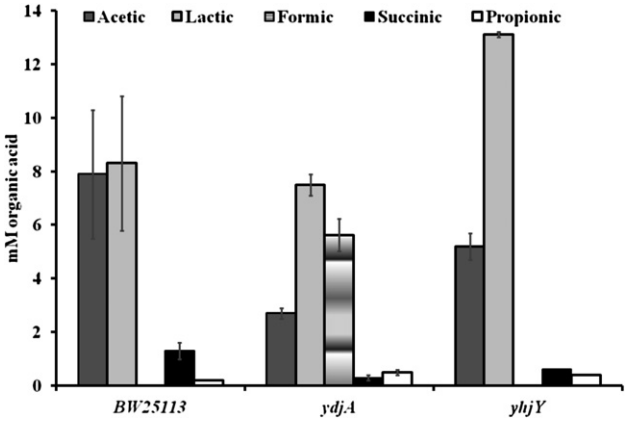

Fig. 2 – Organic acid composition detected using HPLC after a 24 h fermentation with minimal glucose for the BW25113 (parent strain), *ydjA* and *yhjY* strains.

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observed in *ydjA* strain, it demonstrated that *ydjA* mutant cannot consume formate for hydrogen production.

Reported hydrogenase 3 is responsible for hydrogen production from formate [1]. *ydjA* is failed to consume formic acid most probably due to inefficient gene responsible for formate conversion. Since in *ydjA* fermentation process, there is remaining formic acid in the broth after 24 h fermentation, so it is believed that it is one of the indicators which showed the inefficient responsible genes for formate conversion to hydrogen in *ydjA* strain. Meanwhile; through *yhjY* strain, minute hydrogen has shown in the end of fermentation although no formic acid detected, probably another genes were accidentally activated and has consumed formic acid to another by-product instead of hydrogen.

On the other hand, lactic acid is one of fatty acids produced during fermentative hydrogen production processes [10]. Amount of lactic acid in parent strain and *ydjA* has shown similar pattern however *yhjY* strain has shown about two folds higher than both strains. According to metabolic pathway of glucose degradation toward hydrogen production, lactate is one of intermediates produced through pyruvate synthesis [50]. Based on data obtained, most of pyruvate seems has been consumed for lactate production pathway instead of consumed for formate production and finally for hydrogen production [51]. As a wrap up, only lactic acid and formic acid have triggered sensible effect on hydrogen metabolism in both mutants equated with parent strain.

3.5. *Biohydrogen production by* ydjA *and* yhjY *mutants from formate*

The background of this experiment was to find out if the defective biohydrogen production by *ydjA* or *yhjY* mutation due to the inability of FHL activity that was responsible for biohydrogen production in *E. coli*. Formate was used instead of glucose as carbon source. The idea of this experiment was to interpret the mutants' capability in formate degradation through glycolysis pathway. As described beforehand, through glycolysis pathways formate is one of the essential intermediates for biohydrogen production. According to the data prevailed from formate fermentation, no biohydrogen was given during fermentation thus, it complement effect acquisitioned from glucose fermentation (Table 3b). The data obtained has given fundamental basis that *ydjA* mutant might be related to formate uptake deficiency instead of only glucose degradation problem. On the other hand, small amount of biohydrogen has appeared from *yhjY* but still lower then parent strain at similar pattern during glucose fermentation. Based on the data it enhances our knowledge and gave some indicators for further analysis especially for transcription analysis. Meanwhile, due to non-disassociated compound as by-products during formate degradation, not much pH changes have shown in the broth at the end formate fermentation [40].

In another aspect, similar pattern has shown in HPR from both glucose and formate fermentation in all strains. However, no pH change was observed at the end of fermentation since theoretical pathways had shown no acids accumulation under formate fermentation [52]. Mutation in *ydjA* has interrupted the functional gene of either formate or glucose conversion to biohydrogen. Meanwhile, the functional gene in *yhjY* showed lower conversion compared to parent strain and assumed that some of the related genes responsible on the formate degradation or formate synthesis have been interrupted by mutation.

3.6. *RNA isolation and transcription analysis*

In hydrogen metabolism, there are a few essential genes that have been extensively studied to illuminate the function and efficiency of their regulation system in *E. coli* such as maturation genes, coded by hypA-F [23], FHL complex regulator components *fhlA* and *hycA* [22], hydrogenase 3 with their big subunit coded by *hycE* [23]. Also another components in FHL complex, *fdhF* [18]. Thus; in this study, the study has focused in these particular genes to seek out their behavior and response due to mutation in specific targeted alleles.

Described by Vardar-Schara et al., *fdhF* gene is one of the important genes functioning in conversion of formate to biohydrogen [16]. This is important analysis to verify role of *fdhF* in mutant during biohydrogen production. On the other hand, *fhlA* is responsible to the activation of FHL complex in biohydrogen metabolism, therefore; excision of this gene abolished transcriptional activation of FHL complex consequently inhibits biohydrogen evolution as by-product [15,22]. Inactivation of *fhlA* will cause depletion on biohydrogen productivity and disrupt transcription of *fdhF* and the *hyc* operon [1,22]. In addition, FHL complex requires accessory proteins for maturation process such as *HycI* protease, *hycABCDEF* for assembling of Ni–Fe metallocenter [23], and putative electron carrier *HydN*. Meanwhile, *fdhF* plays crucial function of formate conversion to $2H^+$,$2e^-$ and CO_2. Over expression of *fhlA* has induced expression of *fdhF* consequently caused 6.5 folds increase of biohydrogen yield and enhances large subunit expression level (*hycE*) [16]. Indeed; malfunction of these genes (*fhlA*, *hypC* and *fdhF*) absolutely abolishes hydrogen metabolism, nevertheless; only one mutant showed positive correlation through complement experiment with plasmid harboring specific alleles (ASKA).

RNA was successfully isolated from pellets of each strain. Seven primers as target genes were used to elucidate expression difference between wild-type and mutants during fermentation. Most of targeted genes were related to FHL complex in *E. coli*. Frequently reported, hydrogen was produced through formate degradation by FHL complex pathway [53]. Meanwhile, *rrsG* gene was used as housekeeping gene while parent strain played as reference gene to normalize the expression data in C_T value [54].

ydjA showed repression in all target genes related to FHL complex except from *hycA* and *hycE* genes. Highest down regulation had shown in *hypB* gene, with 3-folds followed by *fhlA*, *hypF* and *fdhF* at almost 2-folds repression, respectively (Fig. 3). In contrast, *hycA* showed high expression at 4-folds over than parent strain. The expression of *hycA* has given higher repression force to the FHL complex system since *hycA* functioning as repressor activator to the FHL regulations [4]. Meanwhile, complementary effect observed from *fhlA* expression level, whereas, two folds repression has delivered against parent strain. The repression in *fhlA* is conceived due to higher expression level observed from *hycA* gene. The role

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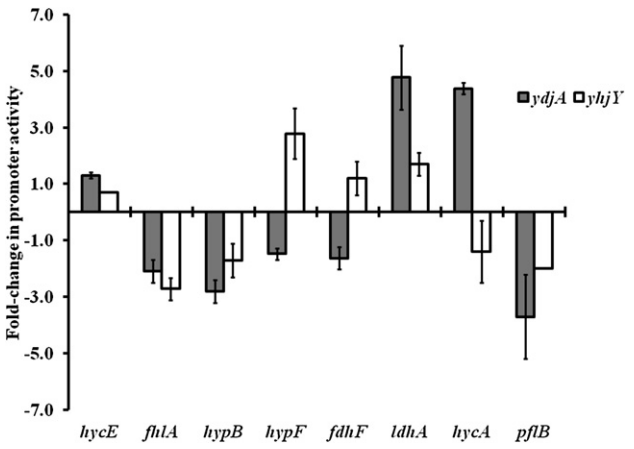

Fig. 3 – Fold change in promoter activity during transcriptional analysis obtained from targeted genes (*hycE*, *fhlA*, *hypB*, *fdhF*, *ldhA*, *hycA* and *pflB*) using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) against *ydjA* and *yhjY* RNA as a template.

of HycA regulator seems very important since Sanchez-Torres has reported that over expression of *hycA* has delivered 1–4 folds reduction in biohydrogen production [22]. Consequently, impetus expression level from *hycA* and repression in *fhlA* have been believed due to knockout of *ydjA* gene. The mutant has regulated repressor activity of the FHL operon in hydrogen metabolism subsequently this complex is not efficiently functional. Moreover, *ydjA* mutants also have shown suppression effect in *fhlA* gene which is responsible as activator gene for FHL complex [55]. Prevailing knockout of activator regulator of FHL complex definitely threatens the efficiency of the regulation system [22].

According to organic acids analysis, lactic acid accumulation in *ydjA* strain was nearly equal to parent strain; however, no relevant reason has been obtained from gene analysis from *ldhA* as targeted gene. Through transcription gene analysis, it prevails that the mutation has influenced a few of genes in FHL complex such as *fhlA*, *hypB* and *hycA* genes, and finally jeopardize FHL complex activities. On the other hand, different trend has been demonstrated from *yhjY* strain. Repression has been observed from *hycE*, *fhlA* and *hypB* genes, 1.5, 1.7 and 2.7 folds, respectively. *hypB* is one of essential components of *hycE* maturation protein; thus, optimum expression of these genes would be necessary for the efficient regulation in hydrogen metabolism. Therefore, expression analysis showed that *yhjY* mutant has restricted the function of FHL regulation system. Meanwhile, other gene analyses that were *ldhA* and *fdhF* had shown an expression compared to parent strain as baseline. The expression of *fdhF* is important evidence due to organic acids analysis that showed no formic acid accumulation; hence, the expression of *fdhF* revealed reliable factor support to this data. In addition; repression of *fdhF* gene in *ydjA* mutant also had shown complementary effect of formic acid in fermentation. *fdhF* gene in *ydjA* mutant has lost its ability in the formate conversion; consequently, higher formic acids observed.

Through the result obtained, *yhjY* has barred some of FHL components like *fhlA* and *hypB* however another components still active such as *hycE* and *hypF*. Thus, it believes, the degradation of substrates has not occurred efficiently; moreover, lower biohydrogen amount was observed. On other hand, low expression in *hycA* indicated minor effect of repression activity in the cells. As a result, some essential genes that related to biohydrogen metabolism were functioning in native mode since maturation gene like *hypF* has shown higher expression [24].

3.7. *Complement test analysis through fermentation*

Four ASKA plasmids expressing the *fhlA*, *fdhF*, *pflB* and *hypC* genes were electroporated into each mutant to try to complement the biohydrogen metabolism deficiencies created by the *ydjA* and *yhjY* mutations. Most of the plasmids did not overcome the *ydjA* and *yhjY* mutations, however, the *ydjA* strain harboring the *fdhF* ASKA plasmid inducted with 0.1 mM IPTG increased hydrogen. Harboring ASKA *fdhF* in *ydjA* has delivered 76 μmol of biohydrogen amount compared 218 μmol from wild-type. Presence of *fdhF* ASKA has partly cured *ydjA* deficiency and produces biohydrogen during fermentation. Even though the production was not as high as parent strain but it has shown some increment than before. Unfortunately, *yhjY* did not come out with any positive indicator from ASKA complement experiment.

Data expressed from transcriptional and complement analysis had given solid consideration on the genes that associate with biohydrogen metabolism through *fdhF* on the transcription level. *ydjA* mutant has found committed to *fdhF* function with the recovery rate about 30% compared to parent strain.

4. Conclusions

Mutations in *ydjA* and *yhjy* reduce biohydrogen production during glucose and formate fermentation. Since the *ydjA* mutation caused slower growth compared to the parent strain and the *yhjY* mutation, this may be one of the factors contributing to reduced biohydrogen for the *ydjA* strain. Gene expression from both strains showed that more than one essential genes have been shut down due to mutation in these alleles. No difference in response occurred when glucose and formate were used as substrates for both strains. According to the fermentation data, transcription and complement analysis, YdjA is associated with FdhF function during hydrogen synthesis. Hence, two important proteins were identified that are related to biohydrogen production.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijhydene.2012.08.115.

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