Enhanced Bioenergy Production through Integration of Molecular Biotechnology and Bioprocess Technology Approaches

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Enhanced Bioenergy Production through Integration of Molecular Biotechnology and Bioprocess Technology Approaches

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently, submitted for any other degree at Kyushu Institute of Technology or at any other institutions.

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Chapter 1

General overview and big picture

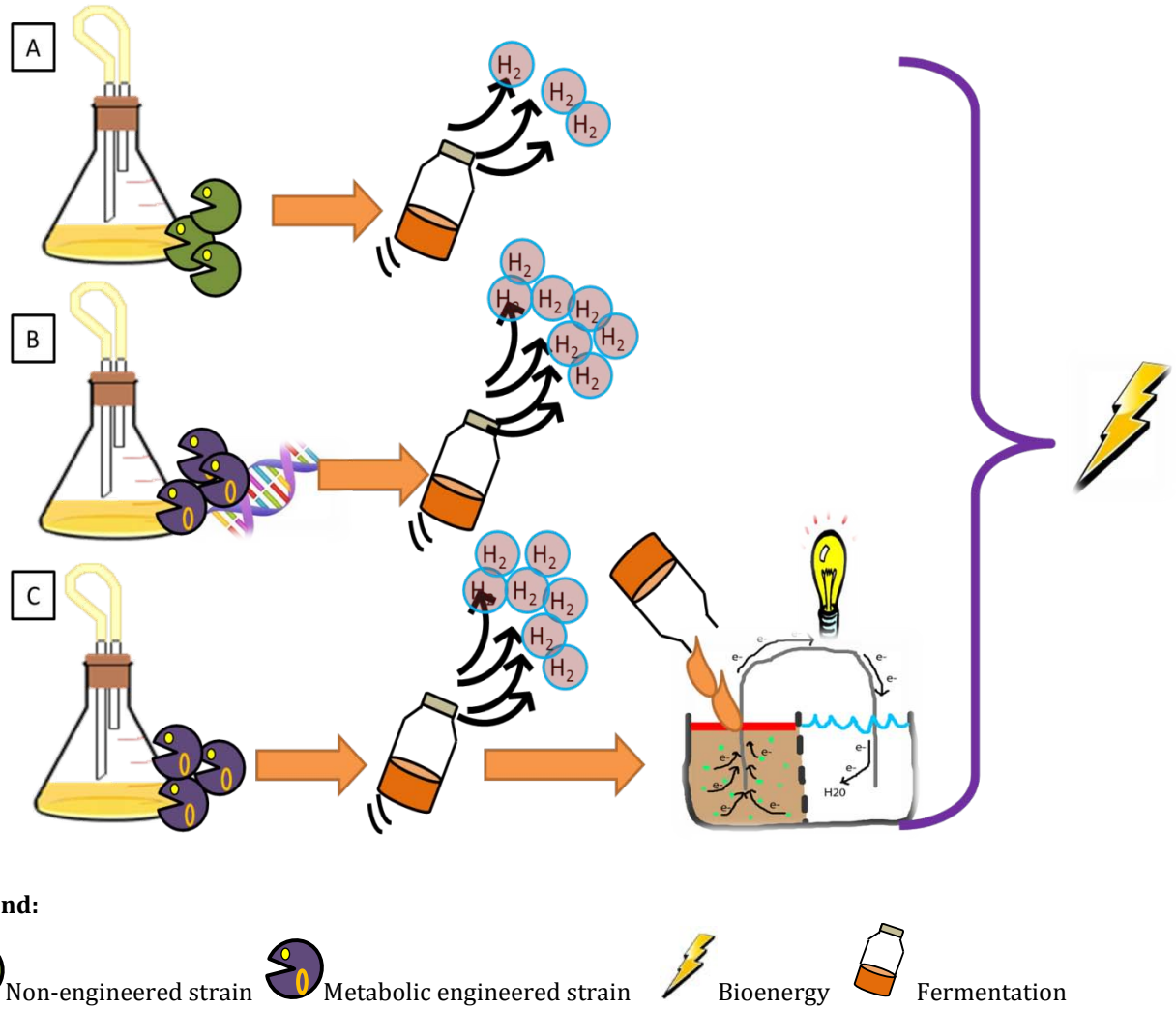
1. Integration of biohydrogen production and microbial fuel cell

Hydrogen is one of the alternative renewable energy sources that could be produced from numerous renewable substrates (Mohd Yasin et al., 2011; Ntaikou et al., 2008; Yusoff et al., 2009).. Typically, the hydrogen production is accompanied by secretion of other substances such as VFAs, lipids, proteins, and other smaller monomers.

Development of bioenergy generation (hydrogen and electricity) was aimed through bioprocess biotechnology and molecular biotechnology disciplines for the future energy-supplying technology that could contribute to a low carbon society. Hence, in this study, hydrogen production was produced from glucose by *Escherichia coli.* Specifically, *E. coli`s* uncharacterized genes related to bacterial hydrogen production was investigated to analyze the gene functions how they are responsible for hydrogen evolution. Henceforth, molecular biotechnology approaches alike metabolic engineering were performed to the specific targeted genes in order to elevate hydrogen production in terms of yield and productivity. The information of the elucidated genes was used for the consecutive experiment in order to enhance hydrogen production in *E. coli.* In this study, octuple engineered strain was constructed and delivered higher hydrogen yield compared from previously reported septuple engineered strain. The yield obtained close to the theoretical yield of hydrogen using *E.coli* from glucose as substrate. On the other hand, since the fermentation effluent from hydrogen generally rich with fermentable nutrients that might be useful as a precursor for another fermentation process. Thus, here we are suggesting an application of microbial fuel cell (MFC) for the production of bioelectricity using one initial substrate. In this study, production of electricity from MFC was

performed using sludge as an inoculum and substrate. The electricity generation was improved by additional pre-treatment to the sludge as substrate. The MFC application is effective for the utilization of excess nutrient rich substrate from glucose fermentation especially from hydrogen production process. At the same time, the utilization of MFC application will helps on the reduction of pollutant in the effluent such as chemical oxygen demand and biological oxygen demand. As a brief summary, dual bioenergy production (hydrogen and electricity) is potential valuable by implementation of hydrogen production in integration of MFC application from a single initial substrate (Figure 1.1)

Figure 1.1a shows hydrogen produced from non-engineered strain. The fermentation was carried out using glucose or formate as a substrate. Then, through investigation of gene functions for an uncharacterized gene related to bacterial hydrogen production, we can obtain the new knowledge, which can be useful for the metabolic engineering of *E. coli* to produce higher hydrogen production as a product (figure 1.1b). Finally, the effluent wasted from hydrogen production will be utilized in the MFC for bioelectricity generation (figure 1.1c). Currently we have proved the bioelectricity generation using sludge as a substrate and inoculum through MFC application. Thus, the utilization of hydrogen effluent might be an ideal strategy for the dual bioenergy generation (hydrogen and electricity) from a single substrate.



Legend:

Non-engineered strain Metabolic engineered strain Bioenergy Fermentation

Figure 1.1: Big picture of bioenergy generation in application of anaerobic degradation process molecular biotechnology approaches and biotechnology application Hydrogen produced from uncharacterized gene of *E. coli* obtained through hydrogen membrane screening (a). Hydrogen production using *E. coli* metabolic engineered strain (b). The effluent wasted from hydrogen production will be utilized in the MFC for electricity generation (c).

2. Energy crisis, current and future trend of renewable energy

World energy crises and fossil fuels depletion had contributed a rapid research study to overcome the energy demand and supply. Oil shock eruption one of the major contributors that lead to the instability crude oil price and shoot up to the unexpected price per barrel of oil. Since then, many research efforts have been carried out to minimize the crude oil dependent and optimize energy consumption for more value added products. The initiatives were started from the oil shock in 1970s (Timilsina and Shrestha, 2011). An accompanied research activities on the energy and renewable energy have been overwhelmed approximately early of twentieth century. For the academic records, it easily discovered that, specific journals related to these topics were recognized in the international cited peer reviewed level. Consequently, the impact factor had increased by the first quarter of year 2000. As example, Renewable Sustainable Energy journal existed since 1997 and ten years later, it gained first impact factor at 3.7 and kept increasing to 6.0 after five years. Same pattern applied to the International Journal of Hydrogen Energy and Bioresource Technology whereas their impact factor has increased about 0.5/year over time from a year 2007. That growing pattern has proven that these related topics gradually gained an interest by the society and become prominent topic within researchers internationally. In general, these journal are broadly published the issues of energy reconstitution, renewable energy, biomass consumption, management and environmental concern related. All the issues are directly or indirectly associated to the biofuels and energy generation. For instance, Journal Biofuels, Bioproducts and Biorefining was introduced in 2007 and right after two years it gained their promotion with 2.3 impact factor and keep increasing year by year.

All these trends demonstrated the biofuels and bioenergy have promised a bright future for the research and development growth. The topics were steadily gained an interest over the

time due to world concern topic of energy crisis, environmental pollution and oil price. The practicality of the biofuels produced would be in co-operated with environmental concerns and industrial applications. The biofuels is reckoned as biohydrogen, biomethane, bioelectricity, biomass consumption/utilization and microalgae cultivation for fuels. In complement to the industry demand, the application of molecular biotechnology proficiencies might be useful to enhance the outcomes of specific biofuels as major product or to enhance biofuels pathways. The integration of bioprocess and molecular biotechnology knowledge is important to elevate the nature yield of the biofuels production instead of modification of medium composition or substrate to product ratio. On the other hand, the thriving of this knowledge may overcome the metabolic barrier in the wild-type for the industrial application and it will evolve multidisciplinary parameters in the metabolic and bioprocess system. Furthermore, this study may assign a lot of new finding that will contribute to another spectrum of research for the future suggestion.

3. Anaerobic degradation process and its application for bioenergy

Fermentation technology is one of the evergreen technologies that promised a great knowledge for the anaerobic degradation and by-products formation especially for the commercialize industry such as the utilization of lactic acid bacteria (LAB) for the production of healthcare supplements (health and nutrition), or yeast (*Saccharomyces cerevisiae)* in beer industry. Also rise of fermented food products (cheese, cider, kimchi), beverage (alcoholic drink), and other supplement products (probiotic drink, enzyme production) (Furet et al., 2004; Paludan-Müller et al., 1999; Romano et al., 2003). Anaerobic process involves in a few steps, hydrolysis, acidogenesis, acetogenesis and methonagesis. Methane is a dead end product in anaerobic fermentation process. On the other hand, hydrogen is one of the intermediate by products

produced during the anaerobic degradation process. In the process, acidogenic bacteria excrete enzymes for hydrolysis, convert soluble organics to VFAs and alcohols, then accompanied with acetogenic bacteria that convert the remaining products into acetic acid, H_2 and CO_2. Harvesting the hydrogen during anaerobic fermentation proses was an awesome idea especially in the industry since the hydrogen not a greenhouse gases and it can be used as an energy precursor.

MFC is a technology in combination between anaerobic degradation process and electrochemistry field. MFC is a fantastic tool that may produce electricity from various types of substrate. Reported, acetate is a highly influence substrate in MFC process (Pant et al., 2010). Thus, the utilization of biohydrogen effluent considers a great idea since the broth is rich in organic acids (acetic acid, propionic acid, formic acid and so on) after the fermentation process figure 1.2. The consumption of effluent from biohydrogen production not only produce energy but also helped in degradation process to give lower COD amount at the end of MFC process.

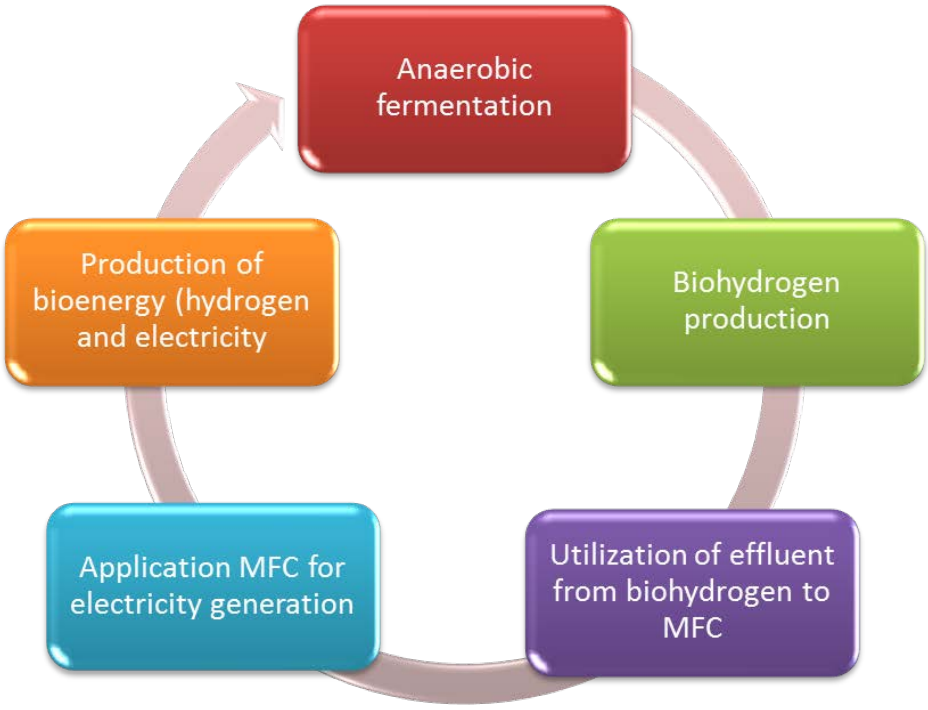


Figure 1.2: The schematic diagram of the anaerobic degradation application for the bioenergy generation

4. Biohydrogen metabolism

Hydrogen reported as cleanest energy as a fuel. It offers a great potential as renewable energy since it can easily produce from glucose as initial substrate (Morimoto et al., 2004). Another advantage of hydrogen it does not contributed to greenhouse gasses either through combustion or electricity production through fuel cells (Atif et al., 2005). There are several methods are available to produce hydrogen gas as fuel such as through physical, chemical or biological approaches (Maeda et al., 2008a). Yang has reported chemical and physical approaches such as partial oxidation of fossil fuels and stem reforming of natural gas were producing large amounts of hydrogen, however this processes have created environment pollution and increased the cost of hydrogen production (Haijun et al., 2006). On the other hand,

hydrogen also can be produced through water-gas shift reaction. Hydrogen from by-product of petroleum refining, gasification of coal, and electrolysis of water were grouped as chemical or electrochemical methods. Indeed, mentioned methods had not satisfied to the cost and application since some of the method required high temperature >850◦C (physical properties) (Jo et al., 2007; Maeda et al., 2008a). The production of hydrogen through biological approach (biohydrogen) using microorganisms has become a new alternative technology development that gives a variety of hydrogen production methods (Mohd Yusoff et al., 2012). Fermentation technology or anaerobic degradation is well practiced technology especially for industrial application especially due to less energy intensive, low cost of maintenance and large amount of idle biomass available as initial substrate (Chong et al., 2009; Gómez et al., 2009; Ntaikou et al., 2008; Wang et al., 2007). It also require moderate condition such ambient temperature and pH around 5.5 to 6.5 (Wang and Wan, 2009). Figure 1.3 shows general anaerobic degradation of glucose as substrate. Through degradation process, many by-products secreted such as organic acids, ethanol and CO_2 beside hydrogen itself (Ren et al., 2006). Organics acids and ethanol have been reported as excess by-products derived from the anaerobic process of biohydrogen production (Arooj et al., 2008; Li et al., 2007). Overabundance of unnecessary or accessory products can be increased and decreased by metabolic engineering techniques based on the anaerobic pathway (Datsenko and Wanner, 2000). For enhanced biohydrogen production, 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. Thus, these by products are important to determine biohydrogen production yield in anaerobic fermentation process.

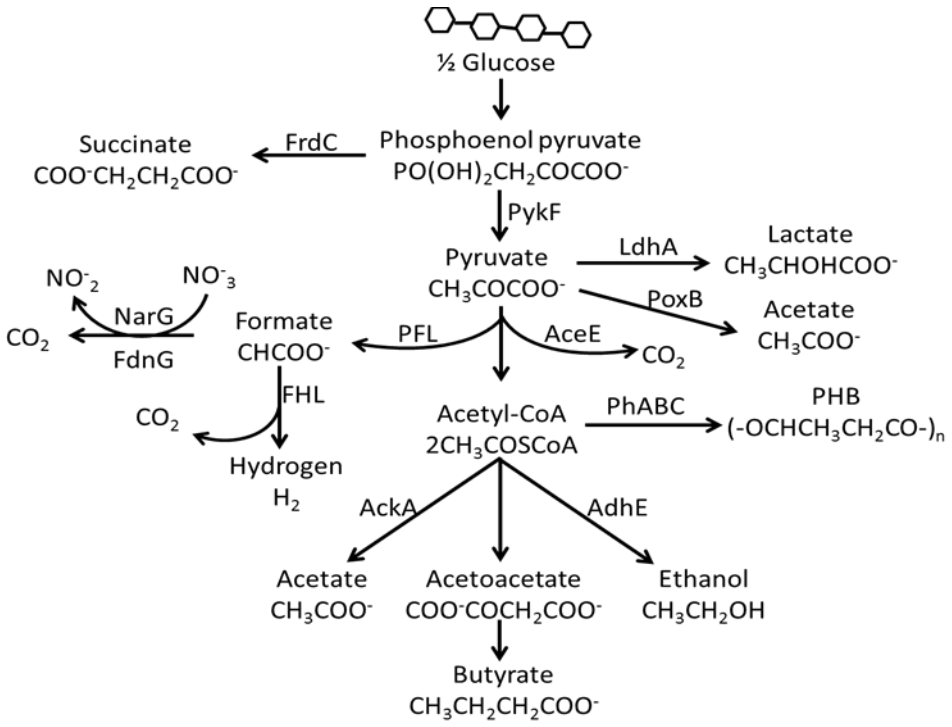


Figure 1.3: Possible metabolic pathway of fermentative hydrogen evolution and other by-products during biohydrogen production.

Two decade ago, Bailey (1991) had coined the knowledge of metabolic engineering, The metabolic engineering become upmost study in many fields included hydrogen production related. The flexibility of metabolic engineering may enhance hydrogen production in many ways such as re-direct the metabolic degradation pathways, eliminate unnecessary by-products, knock out regulator repressor, or overproduce essential genes. The metabolic engineering also reported may solve the hydrogen production barrier by increase carbon flow towards hydrogen production and increase substrate consumption (Mathews and Wang, 2009). Since then, tremendous progress has been overwhelmed in application of metabolic engineering of specific strain. *E. coli* one of the well-used bacteria in metabolic engineering and gene modification so do in hydrogen production studies. Maeda and his colleague have reported the application of metabolic engineering to enhance bacterial hydrogen production (Maeda et al., 2008a). A lot of

efforts have been done in modification and knock out of the relative genes in order to produce higher hydrogen yield (Maeda et al., 2007b). No doubt the metabolic engineering is one of ideal approach in bacterial hydrogen study in order to abolish bioprocess and metabolic barriers (Oh et al., 2011).

5. Microbial fuel cells (MFC)

MFC technology has rapidly grown as a potential renewable power supply system that can utilize organic materials as a fuel. Rises of MFCs would promise a sustainable wastewater treatment and management toward efficient and sustainable energy sources. Thus, MFCs defined as economically and environmentally friendly technologies (Sun et al., 2011). MFCs are an outstanding device that expeditiously converts energy stored in chemical bonds in organic compounds into electrical energy through the catalytic reactions by microorganisms, during the biodegradation (Pant et al., 2010). MFCs utilize bacteria to catalyst fermentable organic and simultaneously generate power. MFCs could be grouped together with hydrogen as an alternative renewable energy for the future in place of petroleum (Logan et al., 2006; Mohd Yusoff et al., 2012). In addition, research and development in MFCs just enter an initial stage of their complex understanding application due to the biological process; especially in the anode compartment, bioelectrochemistry and engineering (Eom et al., 2011; Logan et al., 2006). MFCs were reported as biosensor, desalination of sea water, and hydrogen production (Sun et al., 2011)

Fuel cells in general convert chemical energy into electrical energy. However, in MFC the fuel source is microbially degradable organic matter. Microorganisms act to catalyse the release of electrons from organic matter and transfered to various electron acceptors such as oxygen, nitrate, and so on. Bioelectricity generation through MFC become greatly important since a breakthrough of knowledge regarding a series of respiratory enzymes in the bacteria

cells, it known as catalyser to degrade organic matter and producing electron. In addition, bacteria are self-replicating and self-sustaining to maintain their growth and productivity.

Figure 1.4 shows typical mediator less two-chamber microbial fuel cell completed with proton exchange membrane (PEM) or cation exchange membrane (CEM). This membrane allowed proton released during biodegradation process translocate only from anode to cathode compartment. Equation 1 and 2 are the example of the equilibrium state of biochemical reaction in anodic and cathodic chambers during MFC process. Boidegradation occurs in anodic compartment helped by microorganism and secreted electron and protons (eq 1). Only water produces in cathodic compartment in combination of proton and electron acceptor, O_2 (eq 2).

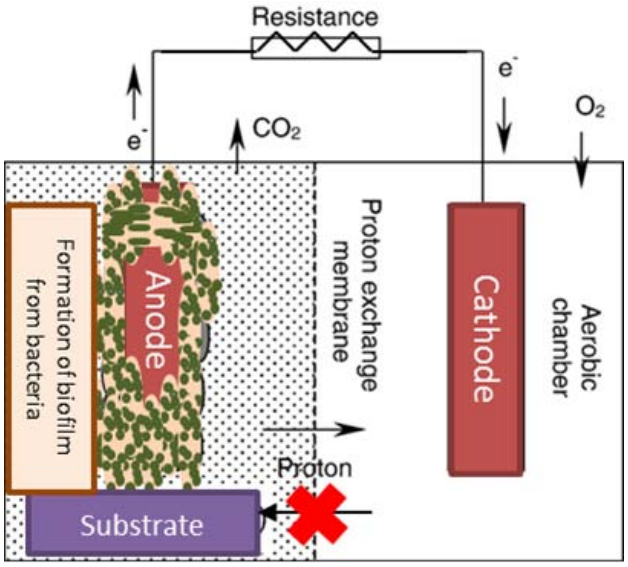


Figure 1.4: Schematic diagram of a typical two-chamber microbial fuel cell (Du et al., 2007).

Anodic reaction : $CH_3COO^- + 2H_2O \xrightarrow{microbes} 2CO_2 + 7H^+ + 8e^-$ (1)

Cathodic reaction : $O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$ (2)

There are several argument of the mechanism how the the electron transfer from degradation process to anode electrode and finally oxidized in cathode chamber. Direct electron transfer through outer-surface c-type cytochromes (redox macromolecules) (1.5a). Another suggestion is, biofilm forming on the electrode one of the factor that contributes to the electron transfer (1.5b). Other ways of electron transfer are through microbial nanowires, mediators or soluble electron shuttles (1.5 c and d). These phenomena was thoroughly described by Lovley (2008) (figure 1.5). However, concrete understandings of the electron transfer are still in debate and under research progress.

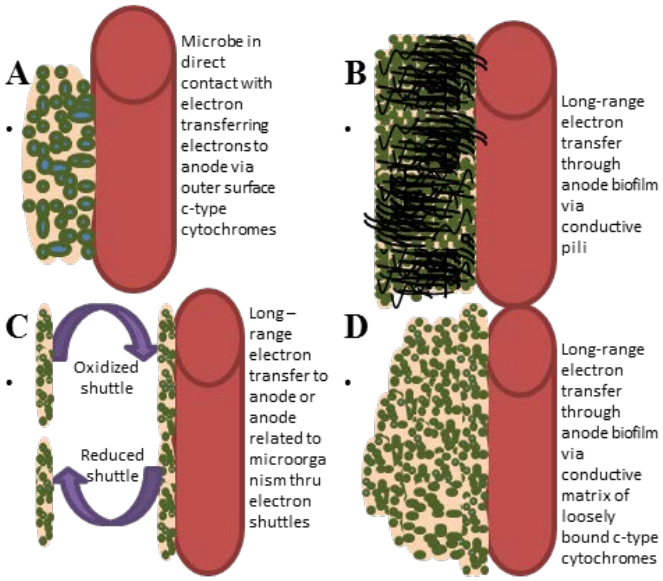


Figure 1.5: Reported mechanisms for electron transfer to the anode of microbial fuel cells. Red dots represent outer surface cytochromes (a), black lines represent nanowires (b), arrow indicates electron shuttled by mediators (c) and the blue sky indicates the possible extracellular matrix which forming biofilm that contains c-type cytochromes conferring conductivity (d) (Lovley, 2008).

In MFC, the microorganism that contribute electrons to fuel cell proposed with different terms such as exoelectrogens (Logan, 2007), electrogenic microorganism, anodophiles (Park and Zeikus, 2003) and electrochemically active bacteria (Jong et al., 2011). These bacteria were

observed dominant during MFC process such as *Shewanella putrefaciens (Du et al., 2007), Desulfitobacterium hafniense* (Milliken and May, 2007), *Geobacteraceae sulferreducens (Bond and Lovley, 2005), Gammaproteobacteria* and *Deltaproteobacteria* (Lovley, 2008). The bacteria committee involve in MFC not only depend on the exoelectrogens but also require another degradation type of bacteria. These bacteria are expected to ferment the bigger molecules to a simple monomers, then electrode-reducing microorganisms can oxidize and transfer the electron to the anode (Lovley, 2008).

Chapter 2

Uncharacterized Genes in *E. coli* Related to Biohydrogen Production

Framework

Reported, there are several hydrogenase genes in *Escherichia coli* that responsible for hydrogen metabolism. Nevertheless, comprehensive works have not been done on the responsible of single gene in relation to hydrogen production. In this study, uncharacterized genes of *E. coli* were used for hydrogen production screening experiment. Single gene deletion from Keio library was used as targeted strains. Sixteen positive response of single mutants that obtained through hydrogen membrane screening were used for further investigation. The hydrogen production ability from each strain was confirmed through fermentation using glucose or formate as substrate. Organic acids produced were measured and finally transcriptional analysis was performed using specific primers.

Abstract

Screening of hydrogen related genes from 3985 isogenic mutants was carried out using hydrogen membrane screening methods. Positive strains were chosen for the confirmation through fermentation analysis. Glucose and formate were used as substrate for hydrogen production assay. Eleven strains namely *yhjY, ydjA, sufD, yehP, yqiG, ydfW, phnN, yhfX, ypdJ, yieL* and *yhbP* were consistently delivered a hydrogen deficient phenotype from glucose and format as substrate. Organic acids analysis from fermentation broth had shown acetic acid and lactic dominated in all strains. Meanwhile formic acids appeared at the majority of the mutants except from *yhjY*, *sufD* and wild type. The presence of formic acid in the broth after fermentation

indicated that the mutants have lost their ability to consume formate for hydrogen produced from glucose degradation.

1. Introduction

The merits of biohydrogen as a fuel source remains undisputed due to its higher energy content compared to hydrocarbon fuels (Argun and Kargi, 2011; Maeda et al., 2008a). The necessity of replacing fossil fuels also have been discussed extensively since petroleum prices have increased dramatically and there is continued anxiety about the level of greenhouse gases (GHG) in the atmosphere (Mathews and Wang, 2009). Critically, Kim reported only water vapor is produced once biohydrogen is combusted, and no pollutants evolve which may contribute to the GHG phenomena (Kim et al., 2009).

Beside the hydrogenase enzyme, FHL complex is one of the essential components for biohydrogen generation in *E. coli.* The FHL complex consists of formate dehydrogenase (FDH-H) encoded by *fdhF* (Maeda et al., 2008b), 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 (Sanchez-Torres et al., 2009a).

During biohydrogen production through fermentation, organics acids and ethanol have been reported as excess by-products derived from the anaerobic process of biohydrogen production (Arooj et al., 2008; Li et al., 2007). Overabundance of unnecessary or accessory products can be increased and decreased by metabolic engineering techniques based on the anaerobic pathway (Datsenko and Wanner, 2000). Numerous studies have shown 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 (Maeda et al., 2008b; Sanchez-Torres et al., 2009a). 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 2.1. Parent strain *E. coli* BW 25113 and the Keio mutants were obtained from the Genome Analysis Project in Japan through their Keio collection (Baba et al., 2006a). All the mutants were mutated at the specific location respected to their allele, respectively. The strains were routinely grown at 37°C using lysogenic broth containing 1% Bacto Tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl (LB medium) with 100 μg/mL kanamycin or 30 μg/mL chloramphenicol where appropriate. The cultures were grown at 37°C for 15 to 18 h. Cell growth was measured by UV/VIS spectrophotometric (JASCO V-530) at the optical density at 600 nm (OD_{600}). The total cell protein values were quantified using the relationship of 0.22 mg mL^{-1} $OD_{600}{}^{-1}$ (Maeda et al., 2007b).

Table 2.1: *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 *pgi*	BW25113 *Δpgi::kan* Km^R	(Baba et al., 2006a)
BW25113 *yjdJ*	BW25113 *ΔyjdJ::kan* Km^R	(Baba et al., 2006a)
BW25113 *mppA*	BW25113 *ΔmppA::kan* Km^R	(Baba et al., 2006a)
BW25113 *mtr*	BW25113 *Δmtr::kan* Km^R	(Baba et al., 2006a)
BW25113 *yncB*	BW25113 *ΔyncB::kan* Km^R	(Baba et al., 2006a)
BW25113 *yhjY*	BW25113 *ΔyhjY::kan* Km^R	(Baba et al., 2006a)
BW25113 *ydjA*	BW25113 *ΔydjA::kan* Km^R	(Baba et al., 2006a)
BW25113 *sufD*	BW25113 *ΔsufD::kan* Km^R	(Baba et al., 2006a)
BW25113 *yehP*	BW25113 *ΔyehP::kan* Km^R	(Baba et al., 2006a)
BW25113 *yqiG*	BW25113 *ΔyqiG::kan* Km^R	(Baba et al., 2006a)
BW25113 *ydfW*	BW25113 *ΔydfW::kan* Km^R	(Baba et al., 2006a)
BW25113 *phnN*	BW25113 *ΔphnN::kan* Km^R	(Baba et al., 2006a)
BW25113 *yhfX*	BW25113 *ΔyhfX::kan* Km^R	(Baba et al., 2006a)
BW25113 *ypdJ*	BW25113 *ΔypdJ::kan* Km^R	(Baba et al., 2006a)
BW25113 *yieL*	BW25113 *ΔyieL::kan* Km^R	(Baba et al., 2006a)
BW25113 *yhbP*	BW25113 *Δ yhbP::kan* Km^R	(Baba et al., 2006a)

2.2 Fermentation and biohydrogen assay

Cells suspensions from the overnight culture (1 mL) and fresh complex medium containing glucose or formate (9 mL) (Rachman et al., 1997) 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 (Maeda et al., 2008b). 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 (Maeda et al., 2007d).

2.3 Organic acids analysis

Minimal glucose 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 I.D x 300 mm L) X 2 column) as reported previously (Shinagawa et al., 1997).

2.4 Strains verification

The two mutants used in this study were verified by polymerase chain reaction (PCR). Two pairs of specific primers were used (Table 2.2). PCR amplification was done by using KOD plus reagent (TOYOBO CO., Ltd), the mixture consists of 2 µl 10 x Buffer for KOD plus, 2 µl dNTPs (2mM), 1.6 µl $MgSO_4$ (25mM), 0.3 µl each primer (20mM), 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 15 sec min

followed by annealing 52°C for 15 sec and elongation at 68°C for 2 min, the cycle was pre-denatured at 95°C for 5 mins.

Table 2.2: Primers designed according to the targeted genes in *E. coli* for strains verification

Targeted gene primer name	**Sequence**	**Characteristic**
ydjA-confirm	f- 5'- CGGTATCGACTACCTGATGAAAG -3' r- 5'- CAGAGATGTCGTAGCCGTATTTC -3'	Confirmation of *ydjA*
yhjY-confirm	f- 5'- GTAATGACCCAACCGGTACT-3' r- 5'- GTAACCCTCATTCCGTGAAG -3'	Confirmation of *yhjY*
ydfW -confirm	f- 5'- ACGCTGGAAGGCTATCGACACCAGG -3' r- 5'- AGACTGACTCAGCGCGTTTCTGGC -3'	Confirmation of *ydfW*
sufD -confirm	f- 5'- TACGCACTACCAACGCATTC -3' r- 5'- CCGAACGGGCATTAATAAAC -3'	Confirmation of *sufD*
yehP-confirm	f- 5'- CGCATCAGGTGCTTGAGCATTATC -3' r- 5'- GTAGACTGAGTGGTGGCACAAAG -3'	Confirmation of *yehP*
phnN -confirm	f- 5'- GACCAGAGCAACCGCTTTAC-3' r- 5'- CAGATGGTAGCGCATGTTTG -3'	Confirmation of *phnN*
yhfX -confirm	f- 5'- GGATGGCTGCTAACGAAGAG -3' r- 5'- CCCTACGCCAAAGCTATCAATC -3'	Confirmation of *yhfX*
ypdJ -confirm	f- 5'- GACTCTCGGTTGGATCATCTG -3' r- 5'- GTGTTGCATGAGTGCTATCTCC -3'	Confirmation of *ypdJ*
yieL -confirm	f- 5'- AGGCTGAAATCTGGTGGTAATG -3' r- 5'- TACCAGGGCTTACCTTTAACCAG -3'	Confirmation of *yieL*
yqiG-confirm	f- 5'- TATGGAATCCCCAAGCAAAG -3' r- 5'- TCATTATGATACGGGTACGATCC -3'	Confirmation of *yqiG*
yhbP -confirm	f- 5'- CGGCGGTACGGATCAAGTAAAGAAACCAGG-3' r- 5'- GGGCGCAAACTGACCGCAGTTAAACC -3'	Confirmation of *yhbP*
K1	k1-5'- CAGTCATAGCCGAATAGCCT -3'	Confirmation of kanamycin
K2	k2- 5'-CGGTGCCCTGAATGAACTGC -3'	

3. Results and Discussion

3.1 Escherichia coli and uncharacterized gene

There are many species of bacteria favorable for biohydrogen production through fermentation such as *Clostridium* sp., *Enterobacter* sp., *Escherichia* sp, *Klebsiella* sp., (Chen et al., 2006; Chong et al., 2009; Datsenko and Wanner, 2000). For example, Chong has shown that *Clostridium butyricum* is an efficient strain for biohydrogen production in dark fermentations using palm oil mill effluent as carbon and nitrogen sources (Chong et al., 2009). On the other hand, *E. coli* is one of most notable bacteria that reported in biohydrogen study especially due to their complete genome accessibility (Baba et al., 2006b; Maeda et al., 2008a). The strain K-12 of *E. coli* is isolated in 1922 from a convalescent diphtheria patient (Bachmann, 1972). *E. coli* is fast-growing, nonsporulating, and well-characterized in biochemical and genome functions (Redwood et al., 2008). MG1655 and W3110 are the two commonly used strains in laboratory experiment as wild type across the world. Both of the strains were *E. coli* K 12 isolates but their sequences were not identically same. Higher length updated in W3110 with 4 646 332 nt, while MG1655 was 4 639 675 nt. A different length due to insertion sequence (IS) elements and absence of a defective phage in the W3110 genome (Riley et al., 2006).

There were about 4333 open reading frames (ORF) in *E.coli* K-12, however less than half (40%) of the identified ORF were reported as unknown function, so called y-genes (Miki et al., 2008; Tohsato et al., 2010). The y-genes are classified as uncharacterized genes since their role are poorly understood. Instead of that, some of y-genes had been studied and reported as associated to adhesive function and possibly contributed to bioflm-formation (Korea et al., 2010). Information obtained from Ecogene database, among Y-genes, there are 116 genes known as pseudogenes (Zhou and Rudd, 2013). From reported ORFs in *E. coli* genome MG1655, 3985 single gene deletion mutants were established known as Keio collection (Baba et al., 2006b). The

Keio collection is one of the best efforts in molecular biology study and the credits have to be shared with Detsenko and Wanner who had invented the one-step inactivation of chromosomal genes technique in early 2000 (Datsenko and Wanner, 2000). On the other hand, complement with Keio mutants, a Complete Set of *E. coli* K-12 ORF Archive (ASKA) was constructed. ASKA clones expression is under the control of an IPTG-inducible promoter. The establishment of ASKA is essential to elucidate functional analysis not only to uncharacterized genes but to verify known function protein reported earlier (Kitagawa et al., 2006). Complementation experiments become more straightforward with recovery function of deleted protein and concrete explanation of "moonlighting proteins" the protein that has two or more distinct functions (Jeffery, 1999).

3.2 Screening and hydrogen confirmation ability in glucose and formate as substrate

The 16 trains obtained from membrane screening were confirmed their hydrogen ability through fermentation using 100 mM complex glucose medium. Eleven strains were believed that inheriting hydrogen-deficient phenotype, *yhjY, ydjA, sufD, yehP, yqiG, ydfW, phnN, yhfX, ypdJ, yieL and yhbP* figure 2.1. Almost no hydrogen observed from all these strains except *yhjY* that showed about 30% compatred to wild type as indicator. The result obtained clearly showed that some of the mutants probably have a problem in glucose degradation for hydrogen production. On the other hand, another five strains *pgi, yjdJ,mppA,mtr* and *yncB* showed similar amount with wild type. It indicates that probably the absence of these genes function did not contribute to the hydrogen metabolism pathway. According to glycolytic pathway, formate is essential precursor in glucose degradation for hydrogen production. Hence, formate was used as substrate for hydrogen production in positive strain obtained from fermentation result.

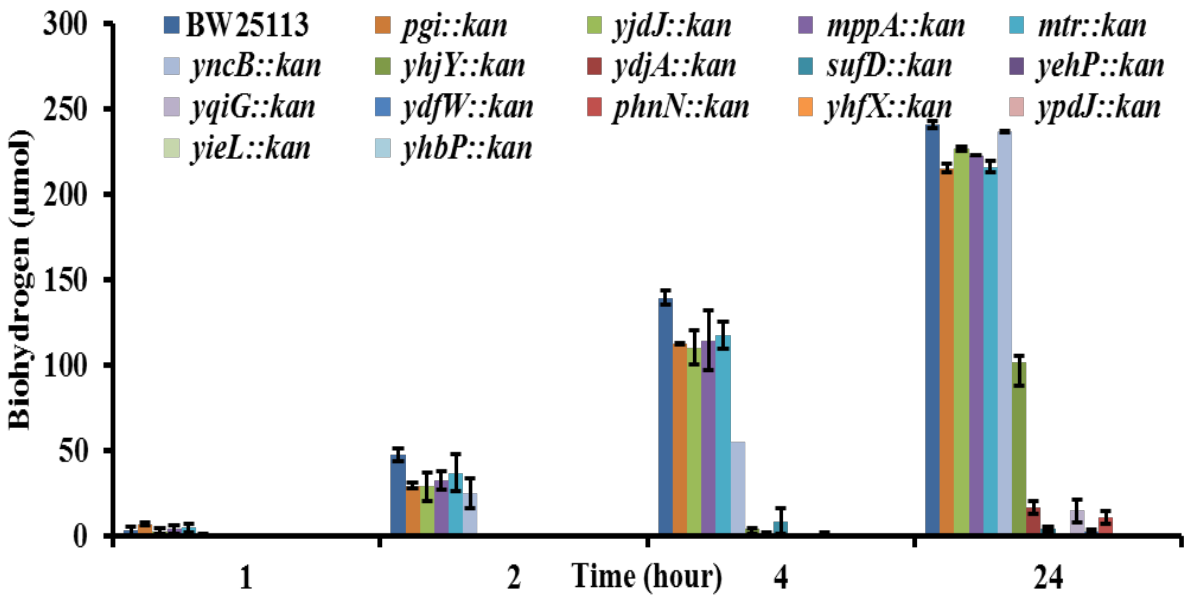


Figure 2.1: Biohydrogen amount produced from 100 mM of glucose using uncharacterized genes

The positive strains were elected as candidates for another fermentation using formate as initial substrate. All the fermentation is constant with previous experiment except 100 mM of glucose was replaced with 100 mM of sodium formate. Very interesting result was obtained through out fermentation process. Most of the targeted stains showed a consistent activity compared to glucose as substrate, whereas no hydrogen observed at the end of the fermentation process *ydjA, ydfW, sufD, yehP, yhfX, ypdJ, yieL* and *yhbP* (figure 2.2). Theses strains consistently showed hydrogen deficiency in glucose and formate as substrate. It is important to conclude that the mutants not only inheriting deficiency in glucose consumption but also they cannot utilize formate as precursor for hydrogen evolution. In contrast, another three mutant's *yhjY, phnN* and *yqiG* delivered an opposite result with others. Hydrogen presented in these strains, however the hydrogen amount does not show higher increment. The hydrogen productivity relatively lower than wild type about (60 -70 % lower). In addition, *yhjY* shows slightly similar pattern to previous experiment with glucose as a substrate, whereas, about 50%

hydrogen obtained observed from the mutant in both glucose and formate as substrates. It indicates that probably *yhjY* has a limitation in order to consume glucose and formate towards hydrogen production.

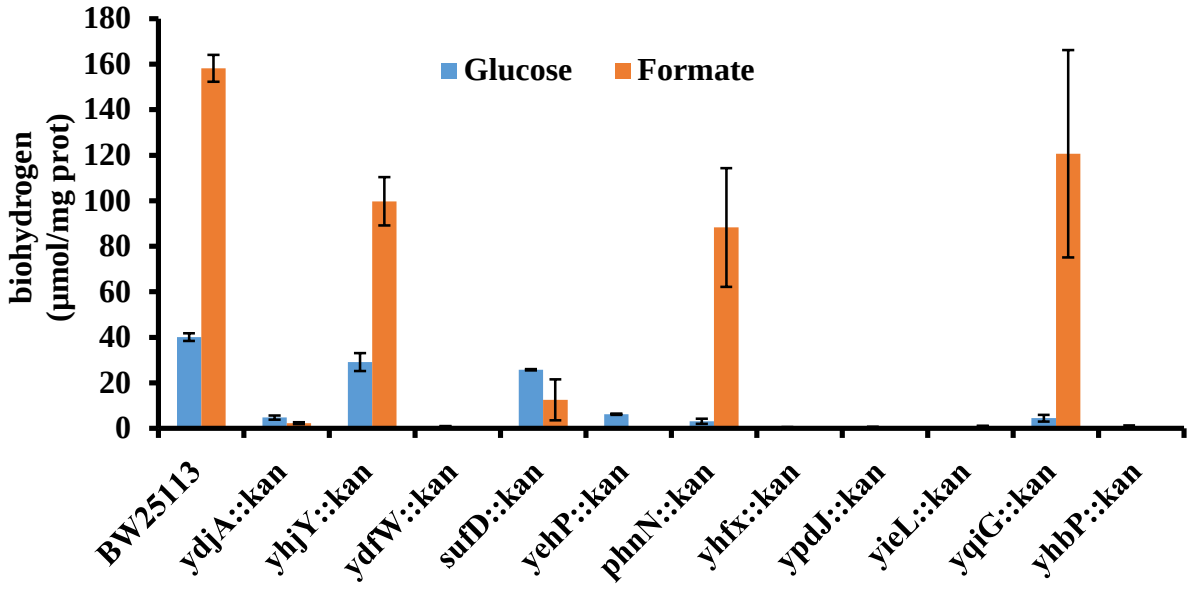


Figure 2.2: Biohydrogen produced from 100 mM of sodium formate using different strain of Keio mutants

3.3 Organic acids analysis from the mutants using minimal medium

Sample from end of 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 (Figure 3.3). Elaborated previously, acetic acid play essential role during biohydrogen production with higher mole hydrogen produced from a mole of acetic (Leite et al., 2008; Noike et al., 2002). 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* and *sufD* strains with 13.1 mM and 15 mM respectively. In other strains, amount of lactic acid is relatively similar to wild type

between 6-8 mM. Reported by Yasin *et al*., (2011) lower pH due to accumulation of lactic acids might interrupt bacteria internal pH and consequently it will suppress bacterial cells growth. Higher lactic acid will quash pH value and affecting biohydrogen evolution (Baghchehsaraee et al., 2011).

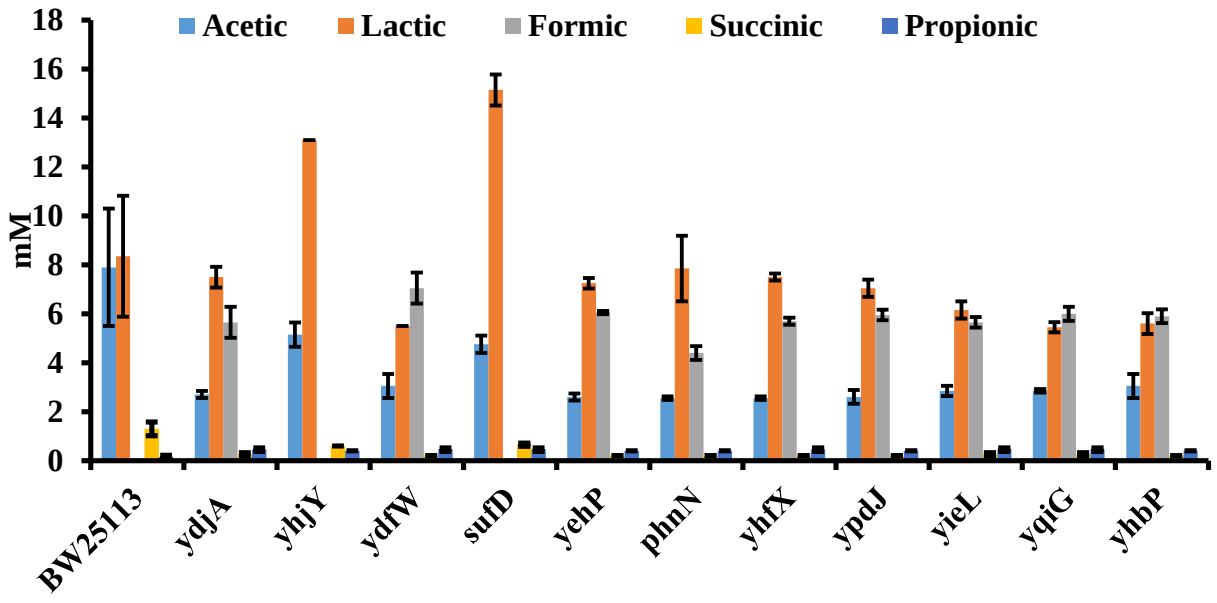


Figure 2.3: Organic acid composition detected using HPLC after a 24 h fermentation with minimal glucose for the BW 25113 (parent strain), ydjA and yhjY strains.

On the other hand, formic acid has revealed extra information especially in format degradation behavior. Formic acid hardly detected from parent strain it shows that formic acid has been utilized for biohydrogen production during the process at similar phenomenon was exhibited by *yhjY* and *sufD.* Indeed, absence of formic acid in *yhjY* and *sufD* wer 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) (Vardar-Schara et al., 2008). Meanwhile, formic acid was observed in all mutants at similar amount between 5 – 6 mM at the

end of fermentation. It demonstrated that the mutants had a difficulty to covert format to hydrogen. so it believe it is one of the indicators which showed the inefficient responsible genes for formate conversion to hydrogen The format accumulation observed in from organic acids analysis is in agreement with previous fermentation using formate as substrate.

Reported hydrogenase 3 is responsible for hydrogen production from formate (Maeda et al., 2008a). *ydjA* failed to consume formic acid most probably due to inefficient gene responsible for formate conversion. Meanwhile; through *yhjY* and *sufD* strains, minute hydrogen has shown in the end of fermentation although no formic acid detected, probably another genes were accidently activated and has consumed formic acid to another by-product instead of hydrogen.

On the other hand, lactic acid is one of organic acids produced during fermentative hydrogen production processes (Debabrata and Veziroglu, 2001). According to metabolic pathway of glucose degradation towards hydrogen production, lactate is one of intermediates produced through pyruvate synthesis (Sinha and Pandey, 2011). Based on data obtained, most of pyruvate seems has been consumed for lactate production pathway instead of consumed for format production and finally for hydrogen production (Hallenbeck, 2009). As a wrap up, only lactic acid and formic acid have triggered sensible effect on hydrogen metabolism in both mutants equated with parent strain

4. Conclusions

Eleven genes in *E. coli* show a positive relation towards metabolism in glucose and formate as substrate. The genes named as *yhjY, ydjA, sufD, yehP, yqiG, ydfW, phnN, yhfX, ypdJ, yieL* and *yhbP*. Some of them are uncharacterized genes which undetermined function was noted. From fermentation broth analysis, acetic acid and lactic are dominant in all strains, meanwhile formic acid appeared at the majority of the mutants except from *yhjY, sufD* and wild type,

respectively. The presence of formic acid in the broth after fermentation indicated that the mutants have lost their ability to consume formate for hydrogen produced from glucose degradation.

Chapter 3

Uncharacterized *Escherichia coli* Proteins YdjA and YhjY Related to Biohydrogen Production

Framework

A continuity study was carried from previous finding, in the first approach, two genes were chosen (*ydjA* and *yhjY*) to be performed for further investigation especially in molecular biotechnology sight. The genes were undergone in transcriptional analysis using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR is used to qualitatively determine gene expression through complementary DNA (cDNA) reverse transcripts from RNA. In this study, RNA of these genes was isolated at optimum amount of hydrogen produced from complex glucose medium fermentation. Specific primers were designed complement to the targeted genes (*fhlA, hycE, ldhA, hycA, hypF).* The expression level was quantified through relative quantification for qRT-PCR ($2^{-\Delta\Delta CT}$).

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

Glucose is converted to pyruvate and NADPH through glycolysis pathways. Afterwards, 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 (Mathews and Wang, 2009). In biohydrogen production, all the processes are fundamentally dependent upon the presence of a hydrogen-producing enzyme and its activity. This enzyme activity has been identified as the critical limiting factor in hydrogen production (Hallenbeck and Benemann, 2002). 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 (Trchounian and Trchounian, 2009). H_2ase has been classified into three groups, [NiFe]-, [FeFe]- and [Fe]-hydrogenases based on the metal contents of their active sites (Vardar-Schara et al., 2008), these enzymes catalyze the conversion of hydrogen to the protons and electrons, through redox reaction ($H_2 \leftrightarrow 2H^+ + 2e^-$) (Mathews and Wang, 2009).

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 (metallocchaperones for NiFe insertion*)*, HycI, encoded the endopeptidase and SlyD responsible for nickel insertion. Similarly, [FeFe]-hydrogenases require HydE, HydF and HydG proteins to complete the biosynthesis (Maeda et al., 2011; Maeda et al., 2007d). *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 (Abo-Hashesh et al., 2011; Mathews and Wang, 2009), and hydrogenase 4 (Maeda et al., 2008b; Trchounian and Trchounian, 2009). Among them, hydrogen 3 has a dramatic effect in hydrogen synthesis. Hydrogen 3 is encoded by the *hyc* operon and is comprised of the large subunit *hycE* and the small subunit is encoded by *hycG* (Maeda et al., 2007b).

Many efforts were done to promote higher biohydrogen from glucose using *E. coli* through either bioprocess or molecular engineering approaches. In term of metabolic engineering, some of the important genes were modified by redirect the hydrogen metabolism pathway, enhance substrate consumption or by reduce a wasted precursors toward hydrogen production (Mathews and Wang, 2009). Metabolic engineering is one of the potent ideas since it can be applied not only for synthetic substrate but applicable to the other biomass substrate.

Thus, in this study two genes *ydjA* and *yhjY* from previous work were chosen for further investigation for transcriptional and complement analysis. A specific primer was used in transcriptional analysis based on the result from hydrogen assay and organic acids analysis.

2. Materials and Methods

2.1. Bacteria strains and growth condition

The ASKA plasmid strains (*ydjA*, *yhjY*, *fdhF*, *hypC*, and *fhlA*) were obtained from the Genome Analysis Project. The strains routinely streak in lycogenic broth luri bertani in the presence of 30 µg/mL chloramphenicol. All the parameters and condition are same with previously described in beforehand.

2.2. Complementation analysis

Complementation was done through plasmids isolated from ASKA clones. Different ASKA clones harboring *ydjA, yhjY, fdhF, hypC,* and *fhlA* were used. The transformed stains were induced with 1 mM isopropyl-D-1-thiogalactopyranoside (IPTG) at initial of fermentation (Table 3.1). Plasmids were isolated 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 electroporation using the Bio-Rad Gene Pulser II unit (1.8 kV, 25µF, 200-500Ω). The transformed cells were incubated in 1 mL of LB for at least one hour at 37°C before spreading on LB plates with specific antibiotic selection. Overnight transformed cells were used for the inoculum for the biohydrogen assay as described previously. 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 electroporated strains (Maeda et al., 2007b).

Table 3.1**:** *E. coli* strains and plasmids used in this study.

Strains and plasmids	**Genotype/relevant characteristics**	**Reference**
ASKA JW2701	Harboring *fhlA* Cm^R	(Baba et al., 2006a)
ASKA JW2968	Harboring *hypC* Cm^R	(Baba et al., 2006a)
ASKA JW0886	Harboring *pflB*Cm^R	(Baba et al., 2006a)
ASKA JW4040	Harboring *fdhF* Cm^R	(Baba et al., 2006a)
ASKA JW1754	Harboring *ydjA* Cm^R	(Baba et al., 2006a)
ASKA JW5659	Harboring *yhjY* Cm^R	(Baba et al., 2006a)

2.3. Pellet isolation

RNA was obtained from cells pellet 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 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 cells pellets was immersed in 100 ml of dissolved dry ice in ethanol for ten seconds and stored at -80ºC prior to RNA isolation.

2.4 Total RNA isolation and quantitative RT-PCR

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.,* (2004). 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 3.2). 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 ($2^{-\Delta\Delta CT}$) (Pfaffl, 2001).

Table 3.2: Primers designed according to the target genes in expression analysis

Name/targeted gene/function	Sequence	Relevant characteristics
fhlA Transcriptional activator	f- 5' -GTGTATTGCAGGAACAGGAGTTTG-3' r- 5'- GAATACGTTCAGGCGGTAATAGAG-3'	activator of FHL complex (Baba et al., 2006a).
fdhF formate dehydrogenase-H	f-5'- GGATTTCTACGGTGCGACTTAC -3' r-5'- GGTACTCGTCGGTGAGTTTGTC-3'	enzymes catalyse the reaction $HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$ (Maeda et al., 2008a).
hypB GTPase	f- 5'- CACTGGACGATAACGGTATTCTG -3' r- 5'- AAGTTGAGATACGGCAACAGGT -3'	Nickel liganding into hydrogenase large subunit (Maeda et al., 2008a).
hypF Carbamoyl phosphate phosphatase	f- 5'- ATCTTCACGGCGATGTCTGT -3' r- 5'- CTGTGCTCTGGCGTATAGTGAA -3'	Required during assembling of Ni–Fe metallocenter (Maeda et al., 2008b).
hycE Large subunit	f- 5'-ATCAGCTGACTGTCACCGTAAAG -3' r- 5'-GTAATCCAACACTTAGTGCCCTTC -3'	Large subunit of hydrogenase 3 (Hyd-3) (Sanchez-Torres et al., 2009a).
ldhA lactate dehydrogenase	f- 5'- GCTAACTTCTCTCTGGAAGGTCTG -3' r- 5'- GACATACTCCACACCGAGTTCC -3'	converting pyruvate to lactate (Maeda et al., 2008b).
rrsG Housekeeping gene	f- 5'-TATTGCACAATGGGCGCAAG -3' r- 5'-ACTTAACAAACCGCCTGCGT -3'	Housekeeping gene for expression data (Lee et al., 2009).
hycA Repressor gene	f- 5'- CTACTGCAATTCGCTGGTTCAG -3' r- 5'- CGACGTAATACTCGATGGTGTG -3'	FHL complex repressor gene (Kim et al., 2009).
pflB	f- 5'- CGGTATCGACTACCTGATGAAAG -3' r- 5'- CAGAGATGTCGTAGCCGTATTTC -3'	Coded for pyruvate formate lyase (PFL) (Wu et al., 2009)

3.0 Results and Discussion

3.1. Literature survey on ydjA and yhjY genes

BW 25113 *ydjA::kan* and BW 25113 *yhjY::kan* have been appointed as candidates to be explored for their specific gene response to biohydrogen metabolism. Reported, *ydjA* in *Bacillus subtilis* is grouped in the *ydiR, ydiS* and *ydjA* operon. It has resided in prophage 3 region in *B. subtillis* and it functions to support activity of DNA restriction enzyme. In the meantime, *ydjA* also exhibited some homology with DNA restriction enzyme of *Lactococcus* plasmid PTR2030 (LlaI-3) and this enzyme known as methyltransferase enzyme (Maeda et al., 2008a). *ydjA* holding an accession number of EG11134 in EcoGene database and ECK1763 obtained from K-12 gene database. The gene located at 1846149 – 1846700 bp and coding for about 183 amino acids (Choi et al., 2007; Choi et al., 2008). *ydjA* has been described as one of the genes that coded for nitroreductases (NTR). NTR commonly used in bioremediation technology especially when dealing with hazardous nitroaromatic compounds. NTRs polypeptide were encoded by more than 210 amino acids, however *ydjA* gene consists only 190 amino acids in total, thus it has classified as one of the smallest NTRs has been found to date (LinWu et al., 2009; Maeda et al., 2008a). 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 known as lipase and embedded in outer membrane cells. Translated *yhjY* protein was 25.1 kDa in weight, and its sequence located between *yhjX* and *tag* gene in *E. coli* genome accordingly. In the study, an expression level of outer proteins have 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 found in inclusion bodies and does not related to glycolysis pathway at any stage (Trchounian and Trchounian,

2009; Vardar-Schara et al., 2008). In addition, *yhjY* is coding for about 232 amino acids and the gene located at 3710259 – 3710957 bp in *E.coli* gene bank. *Yhjy* holding a EcoGene accession number EG12269 and ECK3535 in K-12 Gene accession number, respectively (Datsenko and Wanner, 2000).

3.2 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 bymembrane screening. 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 it is a nitroreductase (Choi et al., 2008) 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 glucose by *ydjA* and *yhjY* mutants were thoroughly measured.

Figure 3.1 shows the amount of biohydrogen and final biomass difference during fermentation. The wild-type strain was able to produce biohydrogen as soon as after one-hour 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 fold less hydrogen was produced by the*n yhjY* strain.

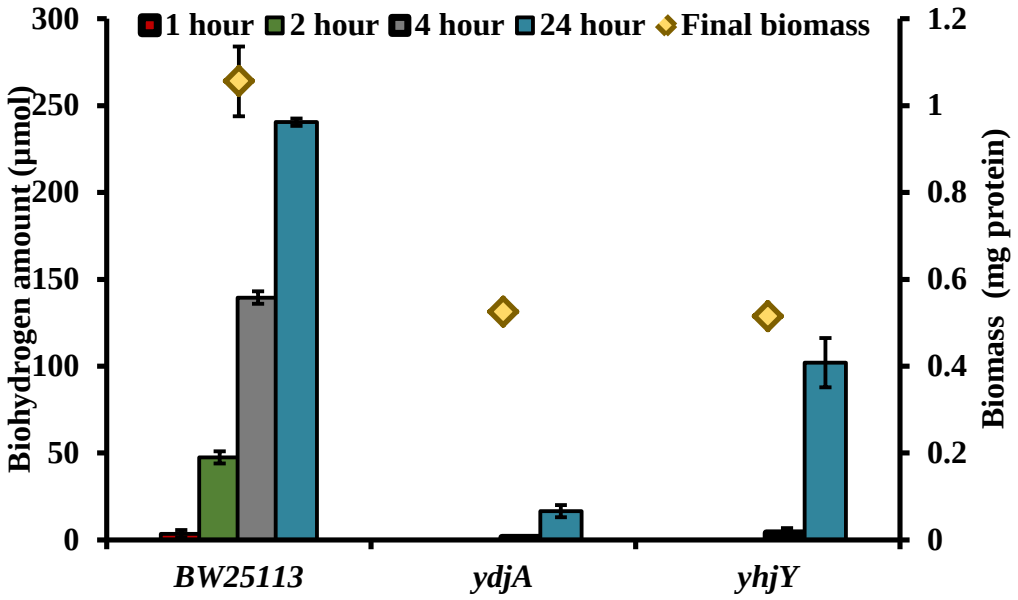


Figure 3.1: Time course of biohydrogen amount (column) and optical density (OD) difference base on protein content (scatter) after a 24 h fermentatiom with 100 mM of glucose in BW 25113 (parent strain), *ydjA* and *yhjY* strains.

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 on *ydjA* and *yhjY* alleles. In general, mutation has bounded the ability of mutant for biohydrogen generation through glucose degradation. According to table 3.3, 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 has measured both in glucose and formate fermentation from mutants compared to parent strain. Ten fold lower has observed from *ydjA* and about two fold given by *yhjY* in glucose fermentation. Moreover, same mode delivered from formate degradation with 168, 3 and 67 µmol/mg protein obtained from parent strain, *ydjA* and *yhjY*, respectively. It is obviously given the deficiency of these strains to 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 condition using complex glucose whereas similar rate has shown from *yhjY* and parent strain at 1.14 ± 0.06 and 1.3 ± 0.1 (1/h), respectively. Intriguingly, lower growth rate 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 should be conceived that the hydrogen production rate (HPR). HPR has given parallel response to the growth rate of all strains, the production rate of *ydjA* 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 (Maeda et al., 2008a). 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.

Table 3.3**:** Hydrogen production rate, biohydrogen productivity, and final pH of each strain from the fermentation of glucose and formate after 24 hour 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
	[a]glucose			[b]formate		
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

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 byproduct. Meanwhile, mutants' 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 has observed.

3.4 Influence of pH and hydrogen generation

Van Ginkel and Logan have proven pH value is an immense factor affecting biohydrogen evolution due to undissociated forms of acetic or butryic acid during fermentation. The pH value between 4.5 – 5.5 has revealed tremendous result of inhibition on the bacteria and suggested higher pH would be optimum atmosphere to raise biohydrogen yield (Van Ginkel and Logan, 2005). Summarised by Herbert 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 (Fang and Liu, 2002). In another report by Morimoto and her colleagues, pH was not control 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 dropped to acidic conditions pH 3.0-4.0 due to formation of acids throughout the fermentation process (Morimoto et al., 2004).

In this study, all fermentation experiments were started at neutral pH around 6.9 – 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 control accurately; it is depend on medium preparation itself, basically the initial pH for both medium were around 6.8 and 7.3 in glucose and formate fermentation, respectively. The descent effect of pH during fermentation is agrees with mixed acid fermentation; whereas, organic acids are one of the byproducts being presented during hydrogen metabolism (Morimoto et al., 2004; Shin et al., 2004; Zheng and Yu, 2005). On the other hand, final pH in glucose and formate have 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 (Morimoto et al., 2004). Meanwhile, form 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, acetate acid reported as substantive byproducts in dark fermentation, which delivers maximum yield at 4 moles of hydrogen from each mole of acetate, stoichiometrically (Fang et al., 2005). Nevertheless; other products such as acetic,butyric, lactic and alcohols also

contribute in the process despite demonstrate modest effects (Kim et al., 2009). 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 (Mohd Yasin et al., 2011).

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 3.3[b]). 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 has shown in the broth at the end formate fermentation (Van Ginkel and Logan, 2005).

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 (Liu et al.). 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 compare 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 Transcription analysis

In hydrogen metabolism, there are a few essential genes 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 (Maeda et al., 2008b), FHL complex regulator components *fhlA* and *hycA* (Sanchez-Torres et al., 2009a), hydrogenase 3 with their big subunit coded by *hycE (Maeda et al., 2008b).* Also another components in FHL complex, *fdhF* (Maeda et al., 2011). 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, *fdhF* gene is one of the important genes functioning in conversion of formate to biohydrogen (Vardar-Schara et al., 2008). This is important analysis to verify role of *fdhF* in mutant during biohydrogen production. On the other hand, *fhlA* responsible to the activation of FHL complex in biohydrogen metabolism, therefore; excision of this gene abolished transcriptional activation of FHL complex consequently inhibit biohydrogen evolution as by-product (Sanchez-Torres et al., 2009a; Trchounian and Trchounian, 2009). Inactivation of *fhlA* will cause depletion on biohydrogen productivity and disrupt transcription of *fdhF* and the *hyc* operon (Maeda et al., 2008a; Sanchez-Torres et al., 2009a). In addition, FHL complex

requires accessory proteins for maturation process such as *HycI* protease, *hycABCDEF* for assembling of Ni – Fe metallocenter (Maeda et al., 2008b), 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 fold increase of biohydrogen yield and enhances large subunit expression level (*hycE)* (Vardar-Schara et al., 2008). Indeed; malfunction of these genes (*fhlA, hypC* and *fdhF)* absolutely abolish hydrogen metabolism, nevertheless; only one mutant showed positive correlation through complement experiment with plasmid harboring specific alleles.

RNA was successful isolated from pellets of each strain. Seven primers as target genes were used to elucidate expression difference between WT 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 (Yoshida et al., 2006). Meanwhile, *rrsG* gene was used as housekeeping gene while parent strain played as reference gene to normalize the expression data in C_T value (Häggblom et al., 2002).

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-fold followed by *fhlA, hypF* and *fdhF* at almost 2 fold repression, respectively (Figure 3.2). In contrast, *hycA* showed high expression at 4-fold 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 (Kim et al., 2009). Meanwhile, complementary effect observed from *fhlA* expression level, whereas, two fold repression has delivered against parent strain. The repression in *fhlA* is conceived due to higher expression level observed from *hycA* gene. The role of HycA regulator seems very important since Sanchez-Torres has reported that over expression of *hycA* has delivered 1 to 4 fold reduction in biohydrogen production (Sanchez-Torres et al., 2009a).

Consequently, impetus expression level from *hycA* and repression in *fhlA* have 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 has shown suppression effect in *fhlA* gene which responsible as activator gene for FHL complex (Self et al., 2004). Prevailing knockout of activator regulator of FHL complex definitely threaten the efficiency of the regulation system (Sanchez-Torres et al., 2009a).

According to organic acids analysis, lactic acid accumulation in *ydjA* strain was nearly equal to parent strain; however, no relevant reason has obtained from gene analysis from *ldhA* as targeted gene. Through transcription gene analysis, it prevails 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 demonstrated from *yhjY* strain. Repression has observed from *hycE, fhlA* and *hypB* genes, 1.5, 1.7 and 2.7 fold, 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 showed no formic acid accumulation; hence, the expression of *fdhF* revealed reliable factor support 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.

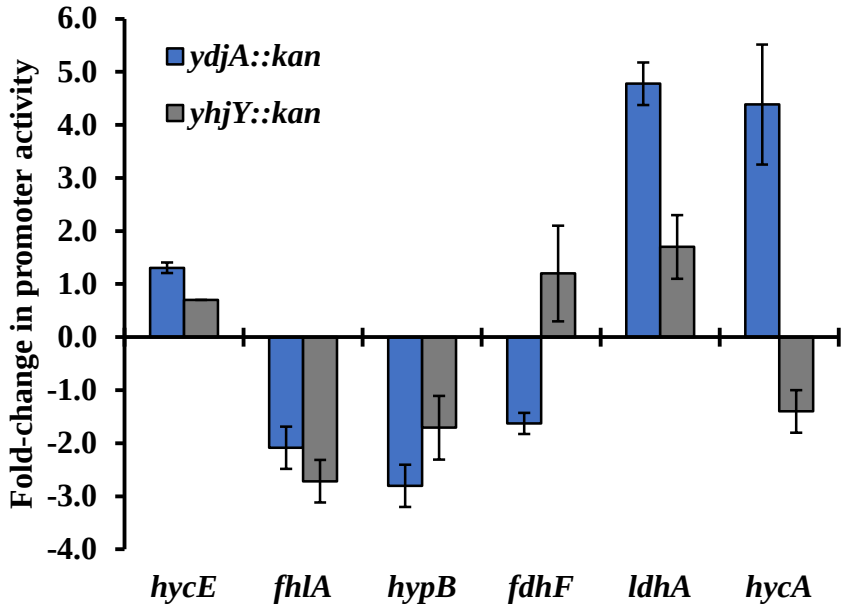


Figure 3.26: Fold change in promoter activity during transcriptional analysis obtained from targeted genes (*hycE, fhlA, hypB, fdhF, ldhA, hycA* and *pflB*) using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) against *ydjA* and *yhjY* RNA as a template.

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 (Maeda et al., 2007b).

3.7. Complement test analysis thru 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 *yjY* 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 produce 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. The *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 more than one essential genes has 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.

Chapter 4

Metabolic Engineering to Enhance Hydrogen Production Using YhjY in *Escherichia Coli*

Framework

Previously reported, a septuple engineered strain of *E. coli* had successfully constructed and increased the hydrogen yield compared the wild type. However, the increment was lower than the theoretical yield. Consequently, based on the information obtained from previous experiment on the transcriptional analysis of *yhjY*, the protein was used as targeted gene to be overproduced in the wild type and the engineered strains. It assumes that presence of YhjY in the strains will be elevated the hydrogen productivity and yield.

Abstract

We previously demonstrated that an uncharacterized protein of *Escherichia coli*, YhjY is essential for hydrogen production. In this study, YhjY was used to enhance bacterial hydrogen production from glucose as a carbon source. The production of YhjY promoted higher growth under anaerobic conditions and increased glucose consumption for hydrogen production. Based on transcriptional analysis, inactivation of YhjY repressed *pgi* and *pflB* by 13-fold and 5-fold, respectively. Pgi and PflB are required for converting glucose-6-phosphate into fructose-6-phosphate and for converting pyruvate into formate and acetyl-coenzyme-A during glycolysis. Hence, YhjY is proposed that stimulates glycolysis. An engineered strain with 8 gene changes including production of YhjY had a 200% increase in hydrogen productivity and an increased yield of 1.89 mol H_2/mol glucose which is 2 fold higher than the wild type strain.

1. Introduction

Stringency of non-renewable energy and fossil fuels depletion crisis led to the debate of renewable energy sources. Hydrogen is one of alternative renewable energy sources along with methane (Hassan et al., 2008), biodiesel (Talebian-Kiakalaieh et al., 2013), electricity from microbial fuel cells (Mohd Yusoff et al., 2013) and bioethanol (Roslan et al., 2011). Hydrogen is a clean fuel because its combustion only produces water vapor, and it has a high energy content compared to other hydrocarbon fuels (Maeda et al., 2007d). As an additional advantage, hydrogen can be produced from numerous renewable substrates (Mohd Yasin et al., 2011; Ntaikou et al., 2008; Yusoff et al., 2009).

Escherichia coli is the best-characterized bacterium and represented basic gene information of prokaryotic bacteria (Maeda et al., 2011; Riley et al., 2006) in which facilitates its use in metabolic engineering studies for hydrogen production. Theoretically, 2 mol of hydrogen can be produced from 1 mol of glucose in *E. coli* (Seol et al., 2012). Maeda and his colleagues constructed one of the best-engineered *E. coli* strains to enhance hydrogen production (a septuple-engineered strain). The strain was constructed by inactivating seven genes that reduce the metabolic flux to hydrogen: *hyaB* and *hybC* which encode the large subunits of two hydrogenases that remove hydrogen, *hycA* which encodes the repressor of the formate hydrogen lyase (FHL) system, *fdoG* which encodes the α subunit of formate dehydrogenase-O that prevents formate from being converted to hydrogen, *ldhA* which encodes D-lactate dehydrogenase that convert pyruvate into lactate, *frdC* which encodes fumarate reductase that may prevent conversion of phosphoenolpyruvate to pyruvate but instead of succinate and *aceE* which encodes pyruvate dehydrogenase that convert pyruvate to CO_2 (Maeda et al., 2007b). However, due to limitations based on substrate consumption, the best engineered strain produced only 1.3 mol of hydrogen per mole of glucose (Maeda et al., 2007b).

We previously screened 3985 isogenic mutants for hydrogen production and identified that YhjY is required for hydrogen production (Mohd Yusoff et al., 2012). This motivated us to utilize YhjY to improve hydrogen production using metabolic engineering. Specifically, we sought to use YhjY to improve growth on glucose as a means to improve the yield of hydrogen on glucose since improving yield is vital for industrial applications (Blankschien et al., 2010).

2. Materials and Methods

2.1. Bacterial strains and cell growth

All the experiments were conducted with at least two independent biological replicates. *E. coli* BW25113 *yhjY::*kan (Keio collection) (Baba et al., 2006b) and the ASKA clones (Kitagawa et al., 2006) were obtained from the National Institute of Genetics, Mishima, Shizuoka, Japan. The parent strain *E. coli* K-12 BW25113 was obtained from the Yale Coli Genetic Stock Center and the septuple-engineered *E. coli* strain was from our lab collection constructed by (Maeda et al., 2007b). All the strains used in this study are described in table 4.1. The strains were routinely grown at 37°C using lysogenic broth containing 1% Bacto Tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl (LB medium) with 100 μg/mL kanamycin or 30 μg/mL chloramphenicol where appropriate. Cell growth was measured by UV/VIS spectrophotometric (JASCO V-530) at the optical density at 600 nm (OD_{600}) with at least two biological replications independent cultures for each strain.

Table 4.1: *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 *yhjY*	BW25113 *ΔyhjY::kan* Km^R	(Baba et al., 2006b)
BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE*	BW25113 *ΔhyaB hybC hycA fdoG ldhA frdC aceE*	(Maeda et al., 2007b)
ASKA JW3985	Harboring *pgi* Cm^R	(Baba et al., 2006b)
ASKA JW0886	Harboring *pflB* Cm^R	(Baba et al., 2006b)

[a] Km^R is kanamycin resistance.
[b] Cm^R is chloramphenicol resistance.

2.2 Inoculum preparation and hydrogen assay

Hydrogen was assayed as described previously (Maeda et al., 2007b). Prior to the hydrogen assay, overnight cultures was prepared by inoculating a single colony into 50 ml LB medium and were sparged for 5 minutes with nitrogen to eliminate oxygen for anaerobic cultivation. One or two milliliters of the overnight cultures were inoculated into 9 or 18 ml of fresh complex glucose medium and sparged in 34 or 68 mL vials and crimp-sealed with butyl rubber stoppers. The hydrogen assay was conducted at 37^{o}C, 120 rpm, for 2 to 24 h. The hydrogen generated in the head space (50 µl) was measured using gas chromatography (Agilent Technologies Inc., Santa Clara, CA) with a thermal conductivity detector and capillary column (L×I.D: 15 mm × 0.53 mm, Agilent Technologies Cat# 19095P-MS5) with oven temperature, front inlet and detector at 70°C, 100°C and 200°C, respectively. For hydrogen productivity, the total cell protein were quantified based on cell turbidity at OD_{600} using the relationship of 0.22 mg mL^{-1} $OD_{600}{}^{-1}$ (Maeda et al., 2007c). The low partial pressure assay was carried out between BW25113/pCA24N against septuple-engineered strain/YhjY in using complex glucose. Low partial pressure assay was adopted from (Maeda et al., 2007b).

2.3 Overexpression assay analysis

ASKA plasmid pCA24N-YhjY or the empty vector pCA24N were transformed into BW25113 and the septuple-engineered strain, respectively. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used at 1 mM at the initial stage of fermentation process along with inoculation to induce the plasmids.

2.4. Glucose consumption

Sufficient amount of broths were sampled in each experiment and the sampling times for glucose consumption were varied at any appropriate investigation. Glucose concentration was assayed using glucose kit (Glucose C2 , Wako Pure Chemical Industries, Ltd, Japan cat# 439-90901).

2.5. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated as described previously (Mohd Yusoff et al., 2012; Sanchez-Torres et al., 2009a), and 50 ng of total RNA was used as the template for the transcriptional analysis using qRT-PCR**.** The housekeeping gene *rrsG* (16S rRNA) was used to normalize the expression data. *pgi* and *pflB* transcription were analyzed using a StepOne Real-Time PCR system with the Power SYBR green RNA-to C_T 1-Step kit (Applied Biosystems, Foster City, CA). The primers used were *pflB* forward 5'-CGGTATCGACTACCTGATGAAAG-3' and reverse 5'-CAGAGATGTCGTAGCCGTATTTC-3'. For *pgi,* forward and reverse primers were 5'-CTCTGGCGAGAAGATCAACC-3' and 5'-TGCCGGTATAACCTTTCCAC-3', respectively. At least two independent biological replicates were evaluated for each strain and the total RNA from parent strain was used as the reference template in all the analysis. The expression level was quantified through as $2^{-\Delta\Delta CT}$ (Pfaffl, 2001).

3.0. Results and Discussion

3.1 Deletion of yhjY *reduces hydrogen production and glucose consumption*

Our previous study determined that uncharacterized protein YhjY is important in hydrogen metabolism (Mohd Yusoff et al., 2012). Deletion of *yhjY* from parent strain BW25113 repressed hydrogen production 2.5 fold. However, the complementation analysis did not yield the expected outcome (Mohd Yusoff et al., 2012). Hence, it was believed that YhjY was not directly interrupting hydrogen synthesis but probably interfered with metabolism.

In order to elucidate the role of YhjY, glucose consumption throughout the fermentation process was studied. Figure 4.1 shows the hydrogen production and glucose consumption of the *yhjY* mutant compared with BW25113. Glucose uptake was reduced by 17% for the *yhjY* mutant. After 10 h of fermentation, 40 µmol H_2/mg protein was produced by the wild-type strain whereas the *yhjY* mutant produced less than 5 µmol H_2/mg protein. These results clearly indicate that the mutant has diminished glucose consumption and it is probably related to the glycolytic pathway. Also reported, during the hydrogen assay, the *yhjY* mutation had little impact on the specific growth rate since this parameter does not change appreciably compared to the wild type (1.14 ± 0.06/h and 1.3 ± 0.1/h, respectively (Mohd Yusoff et al., 2012).

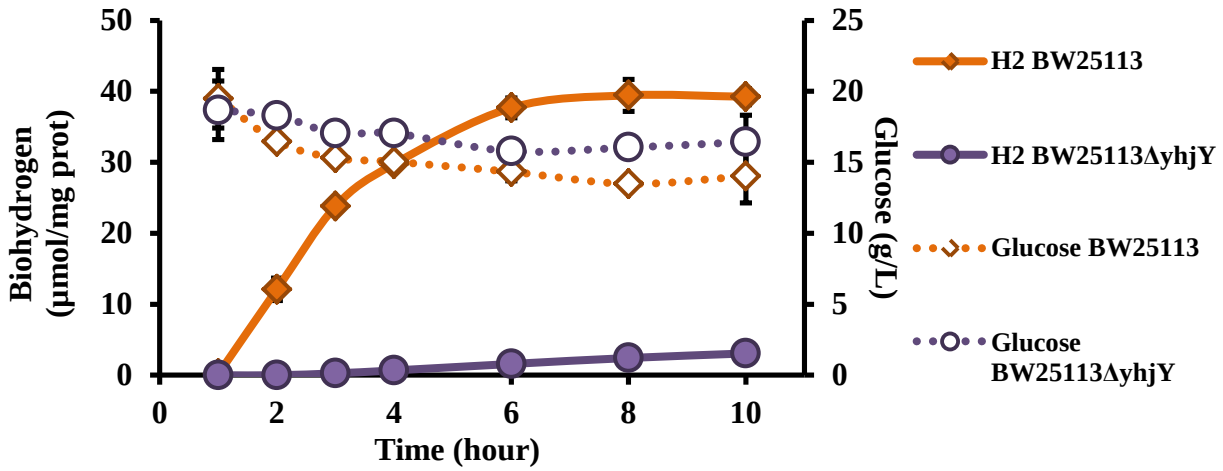


Figure 4.1: Hydrogen productivity and glucose uptake for the yhjY mutant. Hydrogen produced by the wild-type strain (full diamond, solid line), Hydrogen produced by the yhjY mutant (open circle, solid line), glucose uptake by the

3.2 Production of YhjY increases hydrogen and glucose consumption

Given that the *yhjY* mutation reduces hydrogen production, an experiment was conducted to evaluate the ability of YhjY to increase hydrogen production. Henceforth, the *E. coli* BW25113 strain producing YhjY via pCA24N-YhjY will be described as "YhjY" and the strain with the empty vector pCA24N will be indicated as "control". A separate set of assays were conducted in parallel for the hydrogen assay and glucose consumption using the same inoculum. As shown in figure 4.2, YhjY increased both hydrogen productivity and increased glucose consumption relative to the control. The glucose consumption in wild type was 40 % higher compared to the mutants. It is most likely that YhjY stimulated glycolysis and its metabolites were utilized for hydrogen production.

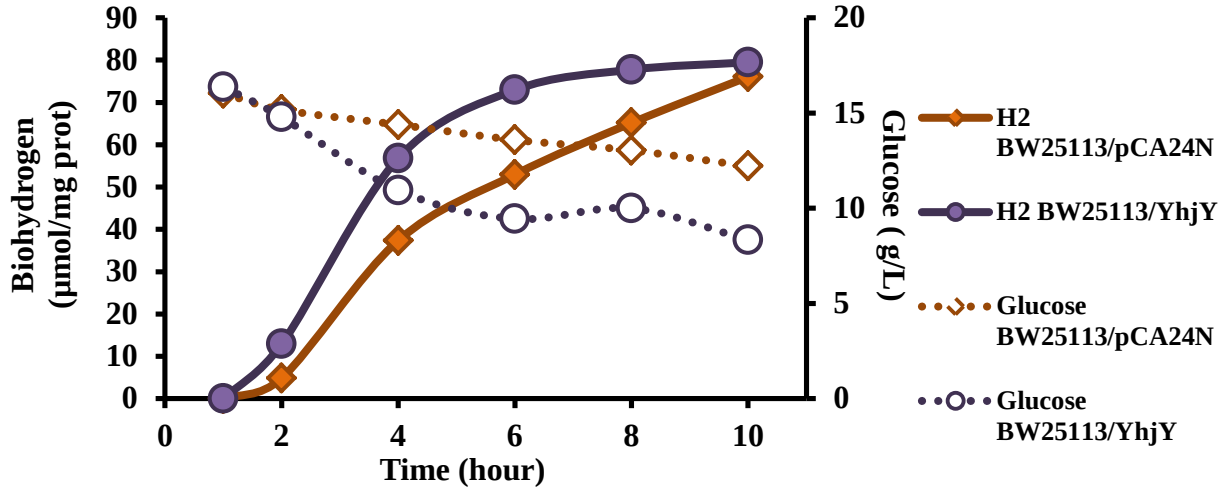


Figure 4.2 Hydrogen productivity and glucose uptake for producing YhjY . Hydrogen produced by the wild-strain with the empty vector (full diamond, solid line), Hydrogen produced by overproduction of YhjY (open circle, solid line), glucose uptake from the empty vector control (full diamond, dot line), and glucose uptake from YhjY (open circle, dot line).

3.3 YhjY increases growth.

By varying IPTG from 0 to 10 mM, it was found that, hydrogen production was optimum at 1 mM IPTG to induce *yhjY* and this condition was used for the remainder of the results. The growth patterns of the mutant and wild type strain were studied under aerobic and anaerobic conditions using complex glucose medium. In general, significantly more growth was obtained with YhjY compared to the control, especially during anaerobic growth (Figure 4.3). By producing YhjY, the specific growth increased to 1.17 ± 0.01 from 1.0 ± 0.1/h for anaerobic growth and from 0.97 ± 0.02 to 0.92 ± 0.08/h for aerobic growth. The increased growth during anaerobic conditions is particularly important for hydogen production since the higher biomass density increases metabolites (Wang et al., 2012) for hydrogen production.

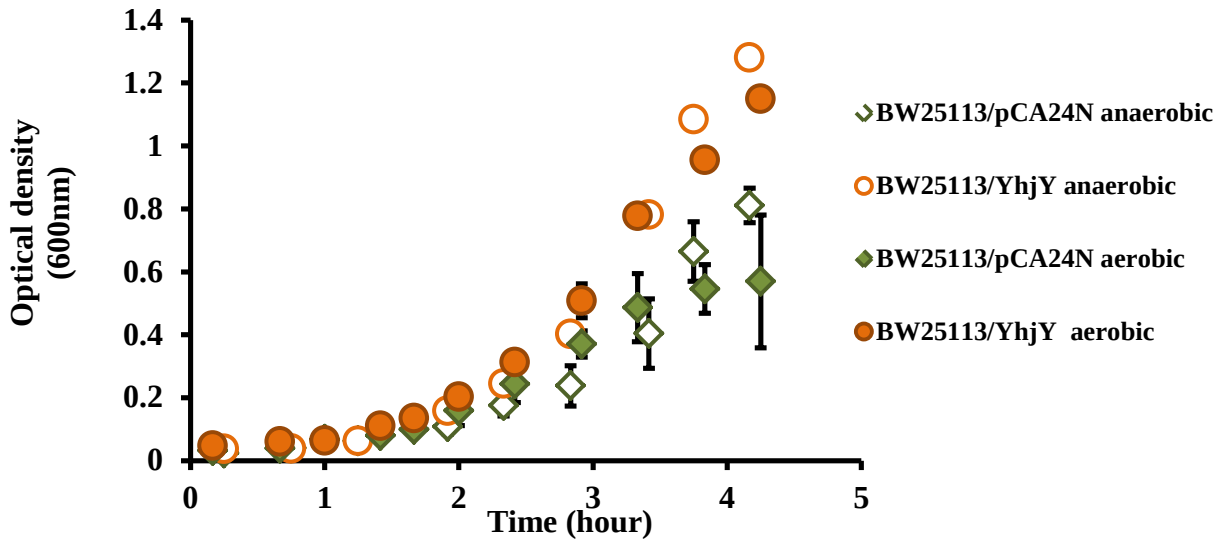


Figure 4.7: Anaerobic and aerobic growth production of YhjY in the wild-type strain. BW25113 with the empty vector, anaerobic growth (open diamond) aerobic growth (close diamond), BW25113 with overproduced YhjY, anaerobic growth (open circle) and aerobic growth (close circle).

3.4 Hydrogen productivity of overexpressed strains from glucose and formate.

In *E. coli*, the pyruvate formate-lyase system produces hydrogen from pyruvate and includes complex FHL regulation ($HCOO^- + H_2O \leftrightarrow H_2 + HCO_3^-$) (Chou et al., 2008; Sanchez-Torres et al., 2009a). Formate is one of the intermediates for hydrogen derived from glycolysis. Theoretically, two moles of pyruvate are synthesized from one mole of glucose, and a mole of pyruvate yields a mole of hydrogen (Hallenbeck, 2009). To determine the hydrogen yield on glucose with production of YhjY, the same amount of glucose or formate (100 mM) was used for the hydrogen assay, and the hydrogen production was determined for 24 h. Higher hydrogen productivity was obtained by producing YhjY compared to the control with both substrates. The different yields obtained from glucose and formate are due to the by-products produced. In general, there are several organic acids produced from glucose and this is the main factor that will give a lower amount of hydrogen from glucose compared to formate (Vrije et al., 2007). We

found that producing YhjY increased the final yield almost 2 fold in comparison to the control with both substrates (**Figure 4.4).** This result shows that YhjY not only stimulates glycolysis but also increases formate conversion to hydrogen, which results in a higher yield of hydrogen after 24 h.

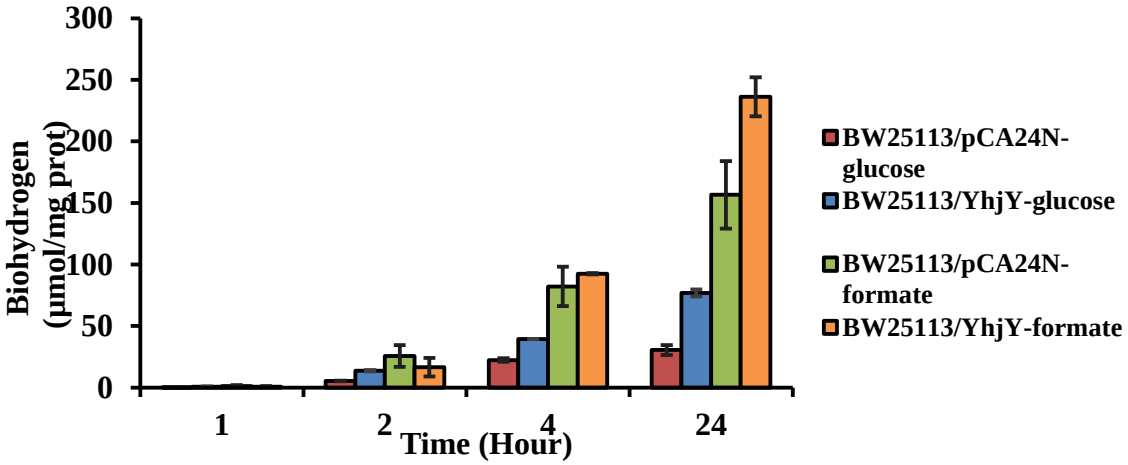


Figure 4.4: Hydrogen productivity via overproduction of YhjY in BW25113 with different carbon sources. Hydrogen from complex glucose medium and complex formate medium with BW25113/pCA24N and with BW25113/YhjY.

3.5 Transcription analysis of the mutant yhjY versus wild-type

Two glycolysis related genes, *pgi* and *pflB,* were chosen as the target genes for the transcription analysis. The *pgi* encodes glucose-6-phosphate isomerase (Pgi) and is required for glycolysis and disruption of *pgi* decreases bacterial growth (Kurumbang et al., 2010). *pflB* encodes pyruvate formate-lyase which is known as a central enzyme of anaerobic glucose metabolism in *E. coli*. This enzyme converts pyruvate into formate and acetyl-coenzyme-A (Sanchez-Torres et al., 2013; Shams Yazdani and Gonzalez, 2008). PflB increases to 30% of soluble protein during anaerobic growth (Sawers and Bock, 1988). Therefore, we used these two genes to further probe the mechanism behind the deficient phenotype of the *yhjY* mutant.

From the transcriptional analysis, both *pgi* (13-fold) and *pflB* (6-fold) are repressed in the *yhjY* mutant (Figure 4.5). This repression pattern corroborates the assumption that YhjY plays an important role in stimulating glycolysis. Since PflB converts pyruvate to formate that ends up as hydrogen, it would be expected that PflB production should increase hydrogen production. However, direct production of PflB did not increase hydrogen from complex glucose as substrate (Mohd Yusoff et al., 2012). Thus, it is believed that the repression observed in this study with the *yhjY* deletion is due stimulation of formate conversion to hydrogen; i.e., the step upstream/downstream of that catalyzed by PflB.

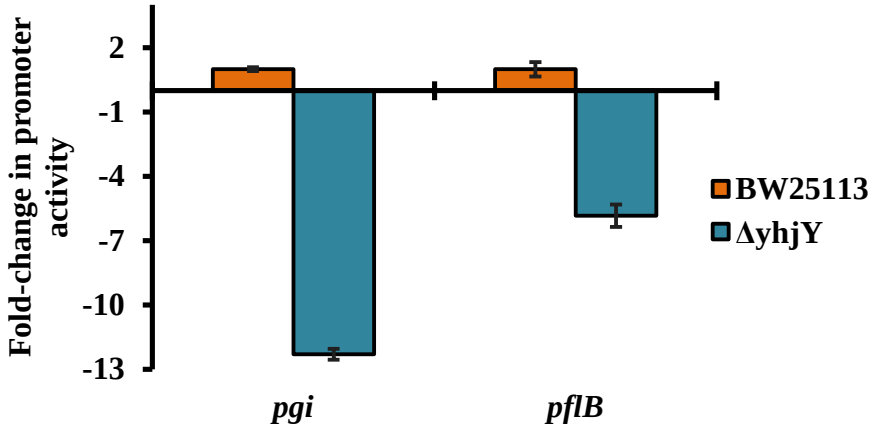


Figure 4.8: Fold change in promoter activity obtained from quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis using target genes *pgi* and *pflB* for *yhjY* and BW25113.

3.6 Enhanced rapid production of hydrogen using an-engineered strain

The previously constructed septuple-engineered strain had 4.6 fold increased hydrogen productivity and a 200% increase in yield on glucose (Maeda et al., 2007b). However, the strain had relatively shown slow growth in comparison to the wild type strain in complex glucose medium and under anaerobic conditions. Here, YhjY was introduced to the septuple-engineered to stimulate growth and hydrogen production. Adding YhjY increased the hydrogen yield dramatically about 2 fold from 1.0 ± 0.3 to 1.89 ± 0.03 mol H_2/mol glucose compared to control.

This yield was 1.5-fold higher than reported septuple-engineered strain (Maeda et al., 2007b).

Hydrogen productivity was also increased in YhjY from 35 µmol H_2/mg protein/h compared to the control with 18 µmol H_2/mg protein/h (Figure 5). The productivity seems increased almost 200% compared control. The enhanced hydrogen production was seen in the first 3 h and increases with time in comparison with control. Therefore, we successfully demonstrated higher hydrogen productivity obtained from glucose fermentation using an-engineered strain with 8 gene changes (BW25113 *hyaB hybC hycA fdoG ldhA frdC aceE/*YhjY).

4.0 Conclusions

YhjY is essential for hydrogen production since in the absence of YhjY, glucose consumption is reduced by 50%. Production of YhjY increased both hydrogen productivity and yield by increasing glucose uptake and growth. YhjY stimulates growth by inducing both *pgi* and *pflB.* PflB is necessary for conversion of pyruvate into formate and acetyl-coenzyme-A while Pgi catalyzes the conversion of glucose-6-phosphate into fructose-6-phosphate during glycolysis. Our results suggest that YhjY stimulates glycolysis to boost hydrogen metabolism efficiency and productivity.

Chapter 5

The curiosity a cryptic function of pseudogene in *Escherichia coli* genome towards hydrogen production

Framework

Follow up from screening experiment of uncharacterized genes in *E. coli* genome using hydrogen membrane sensor, some of pseudogenes in *E.coli yqiG*, *ydfW* and *ypdJ* happened to give a positive response towards hydrogen generation. The *ydfW* gene was chosen for further investigation for its hydrogen deficiency. The pseudogenes have been described as pseudogenes and reported as junk genes which not participate as functional protein in any essential metabolism of *E. coli* regulation system.

Abstract

The function of pseudogenes in *E. coli* genome still in curiosity and most of the researchers still believe the pseudogenes are nonfunctional genes. In this study, the pseudogene *ydfW* has shown a great character in relation to hydrogen production activity. The microarray analysis given biggest evident that the absence YdfW function influences the activity of FDH-N synthesis. The presence of FDH-N function believed will re-direct the formate degradation to CO_2 instead of hydrogen itself. The consumption of formate will leave lower formate threshold for the activation of FHL complex.

1. Introduction

In *E.coli* genome, there are still many of the uncharacterized genes that are exciting to be discovered. Interestingly, there are about 118 genes are classified as pseudogenes. There were many manuscripts discussed about these uncharacterized genes in *E. coli* so do the pseudogenes (Casjens, 2003; Wang et al., 2010). However to date, none of the extraordinary data and evidence brought through to prove the cryptic function of pseudogenes translated for essential proteins in *E. coli* metabolism. No doubt, the illumination of pseudoegenes will break through a lot of cryptic function of uncharacterized genes in *E. coli* genome as well as in another microorganism. However, the biggest challenges for the pseudogenes study it is how to explain these genes happened to know as junk genes and suddenly the pseudogens actually caring a cryptic magnification function in *E. coli* genome especially in hydrogen metabolism. Pseudogene *ydfW* was found that given a positive response during hydrogen membrane screening of uncharacterized genes. Thus mutant *ydfW* was chosen to be studied for more information and understanding.

Primary information of the structural and functional annotation of *ydfW* in *Escherichia coli* K-12 was obtained from EcoGene (http://ecogene.org). The gene sequence in the EcoGene was referred to the *E.coli* K-12 strain MG1655 (Zhou and Rudd, 2013). The details about ydfW are as elaborated in table 5.1.

Table 5.1: General information of pseudogenes *ydfW*

Specification	Description
EcoGene Accession Number	EG13835
K-12 Gene Accession Number	ECK1561
MG1655 Gene Identifier	**b1567**
Alternate Gene Symbols	*ydfW'/intK'*
Description	Pseudogene, integrase fragment, Qin prophage
CDS position	1645198 - 1645380
Amino acid size	60aa

A previous version of an intact IntK(YdfW) reported consist of 75 aa has been replaced with the 60 aa intK'(ydfW') C-terminal pseudogene. An intact version of 437 aa IntK is found in *E. coli* ATCC 8739 (EcolC_2069, NCBI: 169755010). The position of YdfW has been re-notified from genebank U00096.2 in correspond to the EcolC_2069 in ATCC 8739, thus 75 aa was replaced with 60 aa. The position of the YdFW sequence matched at the end of the C-terminal EcolC_2069.

Figure 5.1 shows, an analysis data obtained from STRING analysis (Known and Predicted Protein-Protein Interactions) of EcolC_2069. The conclusion from the analysis described that a region starts at the position 422 and ends at position 437 known as low complexity region (fig. 5.1: pink). Meanwhile, there were no features detected in the region from region 1 to 128 (fig. 5.1: blue) and region 255 to 421 (fig. 5.1: green). A region from 129-254 coded for integrase core domain (fig. 5.1: black). Thus, based on the predicted analysis, the YdfW region that matched from 377 to 437 fell in the non-feature and low complexity regions. It would describe that the predicted 60 aa from *ydfW* sequence was no function reported yet.

Figure 5.1: The SMART diagram represents a summary of the requested sequence of EcolC_2069. Domains with scores less significant than established cutoffs are not shown in the diagram. (*Pfam* is a database of protein families that includes their annotations and multiple sequence alignments generated using hidden Markov models).

In addition, from protein BLAST search, 60 aa of YdfW were predicted as either transposase, integrase fragment, or integrase core domain proteins. Whole sequence 100% hits of 180 aa coded a transposase of *E. coli* DEC9C and the rest are hit for integrase related proteins. On the other hand, (Wang et al., 2010) has reported that Qin prophage located at 163031-1650767 in *E.coli* genome K-12 MG1655. Thus, *ydfW* known become a part of Qin phage fragment since it located within that range. Based on Motif protein analysis any protein function that relates to binding or regulation system were picked. Both protein of intact IntK (437 aa) and YdfW' (60aa) were analyzed. Unfortunately, none of the predicted protein functions matches for binding role or any binding related family. Rely on current information, it difficult to assume the appropriate size and role of YdfW in the hydrogen metabolism or for any other functions.

Henceforth, in the study, a preliminary investigation was concentrated on pseudogene *ydfW* obtained during hydrogen membrane screening of uncharacterized genes (chapter 2).

2. Materials and Methods

2.1 Hydrogen fermentation assay for pellets isolation.

Two strains BW25113 and BW25113 *ydfW*::kan were streaked on an LB plate with kanamycin 50 µg/mL, where appropriate. One-day single colonies were inoculated in 25 mL of LB and cultured at 37^{o}C, 250 rpm overnight (12-16h). The OD of overnight cultures was measured at OD_{600} with appropriate dilution. A 1 mL of overnight cultures were inoculated into 100 mM of complex glucose. Fermentation was carried out under anaerobic condition at 37°C according previously mentioned in the second chapter.

2.2 Cells pellets and total RNA isolation

Broth samples from three hour fermentation were used for cells pellets and total RNA isolation. At least two independent biological samples were collected for each strains. Wild type was used as a control for the expression observation in the analysis.

2.3 RNA quantification and integrity checked.

A 2µl of RNA sample was diluted with 400 µl TE buffer pH 8, the mixture re-suspended gently and thoroughly in micro centrifuge 1.5 ml. The diluted RNA samples were determined using spectrophotometer at absorbance A_{260} and 400 µl TE buffer with 2µl RNase free water as a blank in quartz cuvettes. The OD 1.0 at A_{260} equals to 40µg/mL RNA. The quality check determine based on ratio of A_{260}/A_{280}, the ratio around 1.8 – 2.1 it indicate an acceptable quality of RNA sample.

Approximately 1 µg of RNA samples were mix with 2µl RNA later, 2µl of 6X loading dye and RNase free water up to 10 µL. A 10µl of the mixture s were loaded in 1.4% agarose gel. Elecrophoresis was carried out 15-20 mins 107 mV.

2.3 Microarray analysis of BW25113 versus BW25113 ydfW*::kan*

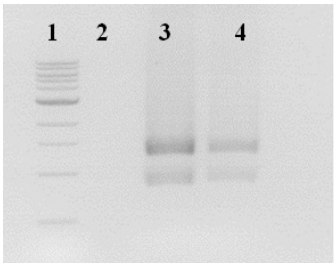
cDNA synthesis, cDNA fragmentation and microarray analysis were carried out according to the laboratory protocol of Biofilms and Biotechnology Laboratory, under guidance of Prof. Thomas K. Wood at Penn State University, Pennsylvania State, USA.

3.0 Results and Discussion

3.1 RNA and cDNA analysis of BW25113 versus BW25113 ydfW::kan

The total RNA was successfully isolated from the hydrogen assay with acceptable quantity and integrity (Figure 5.2). Two distinguish bands clearly observed from the 1.4% gel electrophoresis analysis. The integrity of RNA is one of rigid criteria to be proceed for the next step of the analysis.

Figure 5.2: Total RNA samples run on a 1.4% agarose gel. Ten microliter of marker was loaded in the well.



Legend:
1- Marker 1 kb DNA ladder (500 ng)
2- Blank lane
3-Total RNA of BW25113
4- Total RNA of BW25113Δ*ydfW* 1

The accurate estimation of fragmented cDNA is mendotary before the sample will be used for the array analysis. The smaller fragment will give higher scaling factor compared to wild type and tends to influence the transcriptional analysis result. The acceptable size of the fragmented cDNA is around 50 – 200 bp according to the 100 bp DNA marker (figure 5.3).

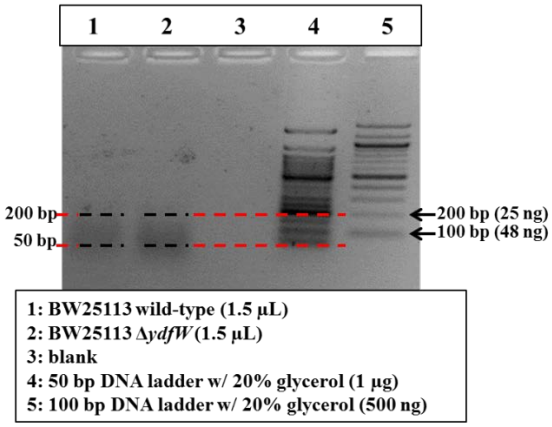


Figure 5.3: The fragmented cDNA of BW25113 and mutant *ydfW* in 2.0% agarose gel.

3.2 Microarray analysis of BW25113 versus BW25113 ydfW::kan

Interestingly, with all the doubts, the YdfW role seems important in hydrogen production. According our microarray result, mutant *ydfW* has upragulated *fdnG, fdnH* and *fdnI* expression at 34, 21 and 14 fold, respectively (fig. 5.4). These genes are the Formate dehydrogenase N (FDH-N) subunit. FDH-N is a membrane-bound enzyme comprise in *E. coli* that is synthesized when the bacterium grown anaerobically with nitrate as exogenous electron acceptor. It has been reported the absence of FDH-N function will leave the fermentation broth with higher intracellular formate and *fhlA* in FHL complex will be activated (Maeda et al., 2007a), hence, higher activity of FDH-N should be effectively competitive with FHL system and as a result lowered the hydrogen production. FDH-N consisted of 3 subunit (*fdnG, fdnH and fdnI)* as observed these genes are sharing same operon. Assumed that, in the absent of YdfW, the fdnGp operon was turned ON and induce the higher expression level. It obviously showed that higher expression level observed from *fdnG* that closer to the operon followed by *fdnH* and *fdnI* (fig. 5.4).

On the other hand, reported, FDH-N was synthesized in the availability of nitrate under anaerobic condition (Suppmann and Sawers, 1994), then probably the formate has been consumed by FDH-N and the relative amount of formate remaining is lower than threshold that necessary to activate the transcriptional regulator *fhlA*. Higher expression levels of FDH-N components are observed from mutant *ydfW*. The synthesis of FDH-N is activated in the presence of nitrate under anaerobic condition. However, no direct and/or concrete evidence is found that the relation of YdfW to hydrogen metabolism yet. The BLAST of the 60 aa reported as YdfW in K12 MG1655. The result shows, the protein matches to the transposase of *E. coli* DEC9C and other integrase related protein of Qin phage. No MOTIF was found that shows the

fragment is associated to the DNA binding proteins or regulators. Three version of YdfW were found (75 aa, 60 aa and 49 aa). The exact size and role of YdfW in hydrogen metabolism is yet still far from concrete evidence.

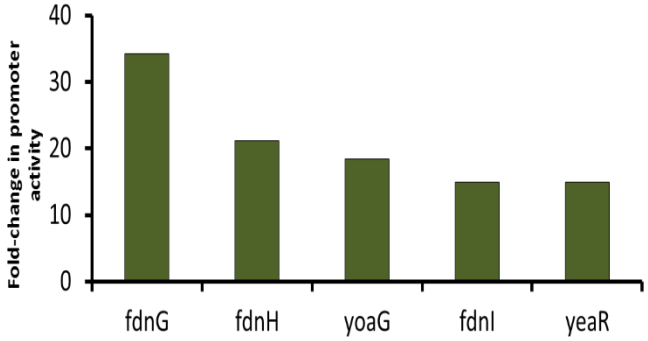


Figure 5.4: Fold change in promoter activity during hydrogen production obtained from microarray analysis of *E. coli* genome.

3.3 Possible function and assumption of YdfW function towards hydrogen production

The possible assumption probably, in the absence of YdfW, the strain could not consumed the nitrate presence in the medium. The higher capacity of nitrate in the fermentation broth will induce the formation of FDH-N. The presence of FDH-N activity will interrupt the formate consumption instead of for hydrogen it will re-direct to the formation of CO_2. The amount of nitrate was higher in the *ydfW* broth compared to the wild-type. The data obtained was complement with our hypothesis. In addition, described by (Wang and Gunsalus, 2003) in the presence of nitrate (added into the fermentation broth) around 4-8 mM, the expression of *fdnG* gradually increased more than 20-fold compared to the nitrate amount lower than 4 mM. It was obvious evidence that the expression of *fdnG* was induced by the presence of nitrate.

3.4 The behavior of hydrogen metabolism related genes in pseudogenes ydfW

Based on microarray analysis, no significant changes observed from hydrogen related genes in *E.coli* (**fig. 5.5**). The fold change was below than 3.0 for the activator regulator (*fhlA*), maturation protein (*hypF*) and big subunit of hydrogenase 3 (*hycE*). However about 3-fold observed from hydrogen repressor regulator (*hycA*). As conclusion, probably the hydrogen metabolism activity not significantly observed in the mutant, even though, *hycA* was upragulated in mutant, but reported by (Sanchez-Torres et al., 2009b) the overexpressed strain with *fhlA* has affected a hydrogen production and another hydrogen genes related (*fdhF*, *hyp*), however the hydrogen production less affected by the repressor HycA.

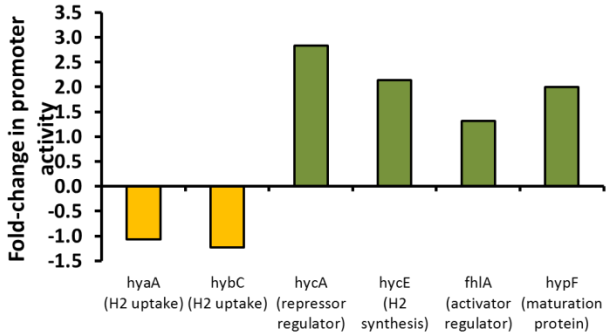


Figure 5.5: Fold change in promoter activity of hydrogen metabolism related genes during hydrogen production obtained from microarray analysis of *E. coli* genome.

4.0 Conclusion

The pseudogene *ydfW* has shown a great character in relation to hydrogen production activity. The microarray analysis given biggest evident that the absence YdfW function influences the activity of FDH-N synthesis. The presence of FDH-N function believed will re-direct the formate degradation to CO_2 instead of hydrogen itself. The consumption of formate will leave lower formate threshold for the activation of FHL complex.

Chapter 6

Electricity Generation from Waste Activated Sludge Using Microbial Fuel Cells Application

Framework

The utilization of hydrogen fermentation broth is expected to be ideal strategy for bioelectricity generation. Hydrogen fermentation effluent is rich with organic acids and other smaller carbohydrate monomers. Hence, application of MFC is a complement idea for the production of hydrogen and electricity. However, in this study an application of MFC was used to utilize waste activated sludge for electricity generation. We successfully proved that the utilization of the effluent of wastewater for electricity generation. Thus, the combination of hydrogen production using best engineered strain and application of MFC by utilizing the effluent would create a huge impact for the bioenergy generation study.

Abstract

Microbial fuel cell reported as an efficient system for the industry application especially for the wastewater treatment and power generation. Here, we consider our study as the proof of concept, to setup the MFC system using biomass waste as inoculum and carbon source, henceforth the system will be applied for another substrate for electricity generation. Therefore, at this stage, two identical MFCs chamber were set up and the startup operation was performed using acetate synthetic wastewater for the nutrient source. Later, untreated waste activated sludge was replaced as substrate in the system. Similar pattern of voltage trend observes in both MFCs system that feed with the waste activated sludge. It indicates, a successfully MFC system is

practically operated using waste activated sludge as inoculum and substrate for electricity generation.

1. Introduction

The microbial fuel cell (MFC) technology has rapidly grown as a potential renewable power supply system that may utilize organic materials as a fuel. MFC converts biochemical energy especially from the degradation of organics into electrical energy by the catalytic reaction of microorganisms (Pant et al., 2010). MFCs could be considered together with hydrogen as promising renewable energy for the future (Logan et al., 2006; Mohd Yusoff et al., 2012). Rises of MFC would provide a sustainable wastewater treatment and management system toward efficient and sustainable energy sources.

The accumulation of saturated waste sludge and the sludge disposal are also critical issues connecting to an increased environmental burden that is why this experiment was planned in consideration (Khursheed and Kazmi, 2011). The utilization of biomass materials in MFCs would become a beneficial approach to treat sludge and provide an alternative energy resource. The efficient degradation of biomass during the MFCs process is important to enhance power generation and therefore, pretreatment might be necessary to promote value-added productivity into the sludge and subsequently increase the performance of MFCs using organic material as their substrate (Bougrier et al., 2008).

Electricity or bioelectricity generation through MFC become greatly influenced since through a series of respiratory enzymes in the bacteria cells, it known as catalyser to degrade organic matter and producing electron. In addition, bacteria are self-replicating and self-sustaining to maintain their growth and productivity. In MFC, some bacteria known as electrogens such as *Shewanella putrefaciens (Du et al., 2007), Desulfitobacterium hafniense*

(Milliken and May, 2007), *Geobacteraceae sulferreducens (Bond and Lovley, 2005),*) that able to release electrons outside the cells. The released electrons are transferred to a terminal electron acceptor (TEA) such as oxygen, nitrate and sulfate in cathode side.

In this study, we investigated the optimized way to use waste activated sludge as a substrate in our MFC system for sludge reduction and electricity generation. Process performance of pretreated sludge using ozonation or microwave digestion was compared. On top of that, microbial community analysis was carried out to understand how bacteria contributed to the energy production during each MFC process. The knowledge obtained will be essential for the development of advanced in-situ waste sludge treatment and power generation in treatment plants.

2. Materials and Methods

2.1 Inoculum and substrates used for the MFC

Raw sludge for the inoculum and substrates was taken from the oxidation ditch in a local Wastewater Treatment Plant in Xiamen China. In general the sludge used consisted of 1-3 g/L chemical oxygen demand (COD), 29-31 g/L total solid, 11-15 g/L suspended solid and 0.1 -1.2 g/L total carbohydrate. Raw sludge was stored at 4°C prior to use. As inoculum, settled sludge was acclimatized using glucose medium. 50% v/v of sludge was added in 500 mL Erlenmeyer flasks containing basal medium as follows (g/L): glucose 3, NH_4Cl 0.5, $NaHPO_4$ 0.25, Na_2HPO_4 0.25, $MgCl_2$ 0.3, $CaCl_2$ 0.005, $ZnCl_2$ 0.015, $CuCl_2$ 0.0105, $MnCl_2$ 0.015, and $NaMoO_4$ 0.001, pH 7.5. Prior to the incubation, the flasks used were purged with nitrogen gas for at least 15 min in the solution to eliminate oxygen in the medium. The incubation was conducted under the anaerobic condition at room temperature (25°C). The acclimation process was kept for more than two weeks in Erlenmeyer flasks.

2.2 Microbial fuel cell construction and operation

MFC reactors were fabricated using PVC. Two identical chambers (anodic and cathodic chambers, 60 × 60 × 70 mm) were adjacently attached and physically separated by proton exchange membrane, Nafion (Merch Ltd., Lutterworth, UK). Both chambers consisted of an identical carbon cloth (70 × 50 mm) as electrodes. The electrodes were connected through Ti wires to form the full electric circuit and loaded with an external resistance of 1000 Ω.

During start-up operation, 50% of acclimatized sludge was inoculated into the anodic compartment and purged with nitrogen, the inoculum was supplemented with acetate medium (Park et al., 2000) up to 200 mL working volume to enrich the electrogens on the anodes. Slow mixing about 100 rpm was applied by using the magnetic stirrer in order to customize homogenous environment for bacterial reaction. Meanwhile, 50 mM of fresh phosphate buffer at pH 7.0 was filled in the cathodic compartment in each experiment and continuously purged with air as electron acceptor. After stable state operation was obtained, the MFCs system was fed with 50 ml – 100 ml settled waste activated sludge as substrate.

2.3 Data collection and analytical methods

Output voltage was measured in millivolt (mV) over time using multimeter LabJack USB Data Acquisition Module model U12. Recorded data were used to groom a performance graph and quantify maximum power produced through the degradation process and electricity generation. The productivity analysis and calculation were carried out through equations reported by previous researchers (Cheng and Logan, 2007)

3.0 Results and Discussion

3.1 Startup operation of microbial fuel cells using waste activated sludge as inoculum.

Substrate competency and effectiveness were evaluated based on their physical characteristic and organic contents that contribute to the power generation. The voltage produced was evaluated along with current density, COD removal effect and microbial community involved during the MFCs operation. These parameters were examined in order to observe the crucial aspects of sludge treatment for its application in MFC operation. Identical aspects and measures were conducted in each experiment in order to gain representable data for the comparison.

Startup MFC operation was started using waste activated sludge as inoculum and feed with artificial wastewater containing acetate as substrate. During the process, voltage generated was captured every 10 minutes. Figure 5.1 shows amount of voltage captured from two identical MFCs for electricity generation using synthetic wastewater medium. Hardly to observe a presence of voltage at the initial stage of the operation especially at initial first 5 days. Probably this period called an adaption time and assimilation process od the sludge with new environment and time to generate an attachment to the carbon cloth as anode electrode. However, voltage gradually increased and maximum voltage was observed around 250 mV during the operation, the voltage gradually increase in both MFCs system and the voltage keep stable within 5 days at before slowly lost the voltage value after two weeks operation time. The voltage values showed fluctuation during the star-up process due to the instability and inconsistency of the MFC system and the time for the bacteria attachment to the electrode.

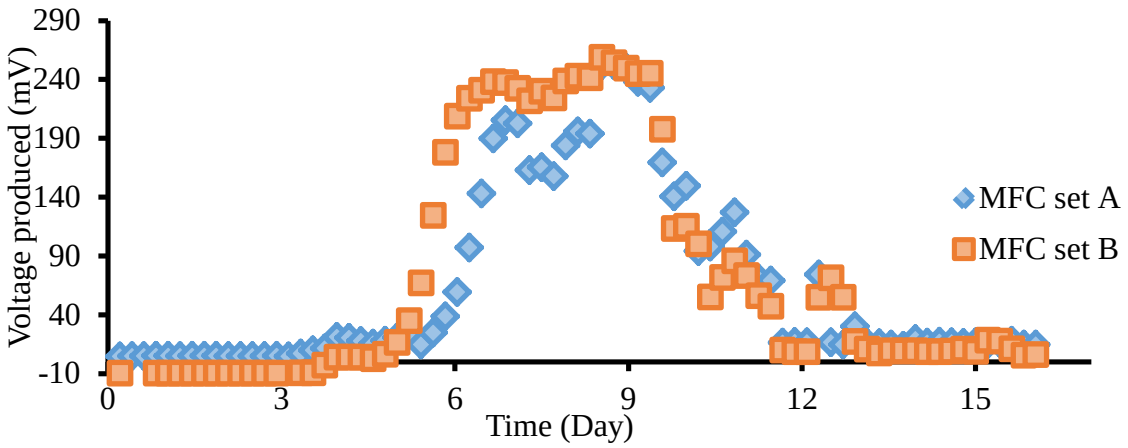


Figure 5.1: Voltage produced during MFC startup operation using acetate synthetic wastewater as substrate

Lower voltage observed after two weeks' time probably the bacteria consumed all the substrate consist of acetate during the MFC operation. However, small fluctuation shoots up intermittently probably due to the electron release from a layer of bacteria on the carbon cloths. We finally managed to prepare two identical MFCs system using waste activated sludge as inoculum. These systems now ready to be fed with new substrate for further investigation.

3.2. Microbial operation using waste activated sludge as substrate

The MFCs system that successfully setup using synthetic medium were used in the operation. Both MFCs systems were fed with waste activated sludge as substrate. Since very active bacteria dominanted in the MFCs system, immediate voltage was observed at the initial stage of the operation. The voltage kept increasing and fluctuating throughout the process. Average voltage within 80 – 100 mV was obtained during more than two weeks operation (figure 5.2).

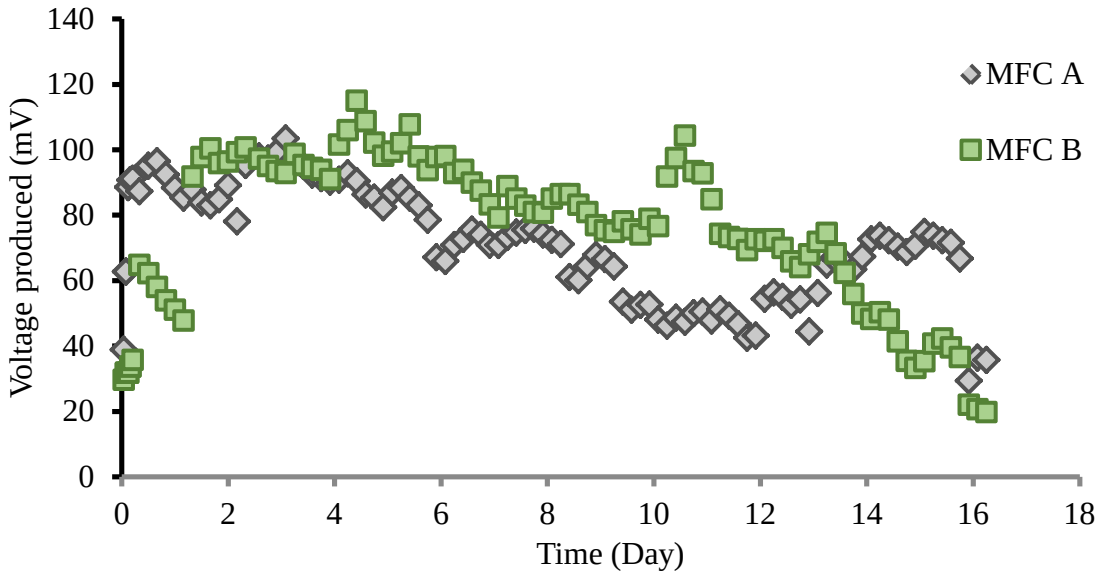


Figure 5.2: Voltage produced from two identical microbial fuel cells system feeding with waste actibvated sludge as substarte.

In addition, the voltage produced in both MFCs system did not show consistently identical. It is probably due to complex organic material in the sludge. Furthermore, in the process, non-sterilized sludge was used and probably some microorganism originally retained in the sludge merely influenced the MFC system. However, the trend and voltage amount produced relatively similar. Two identical MFCs system were successfully produced an electricity from un-treated waste activated sludge as substrate.

4.0 Conclusion

Two identical MFCs chamber are set up and startup operation is performed using waste activated sludge as inoculum using acetate synthetic wastewater for the nutrient source. The stable state operation of the MFCs using synthetic medium is obtained after two weeks operation time. An untreated waste activated sludge is replaced as substrate after the stable state of startup operation occurs. Similar pattern of voltage trend observes in both MFCs system that feed with

the waste activated sludge. It indicates, a successfully MFC system is practically operated using waste activated sludge as inoculum and substrate for electricity generation.

Chapter 7

Influence of Pretreated Activated Sludge for Electricity Generation in Microbial Fuel Cell Application

Framework

Previous chapter has shown the electricity was generated from waste activated sludge as inoculum and substrate. In this chapter, different pretreatment was applied to the waste activated sludge before feed into MFC for electricity generation. Pretreatment sludge was designed in order to compare with raw sludge. In many wastewater treatment study, pretreated sludge was commonly used either to reduce solid content in sludge, to improve dewater ability or to reduce pollutant in term of chemical oxygen demand. Although the other study has not shown the clear impact of different pretreatment to produce electricity through MFCs Therefore, in this study, we investigated ozonation and microwave digestion as pre-treatment of activated sludge for electricity generation in MFCs. On top of that, microbial community analysis was carried out to understand how bacteria contributed to the energy production during each MFC process.

Abstract

Influence of different pre-treated sludge for electricity generation in microbial fuel cell (MFC) was investigated in this study. Pre-treatment has shown significant improvement in MFC electricity productivity especially from microwave treated sludge. Higher COD reduction in the MFC has been revealed from microwave treated sludge with 55% for total and 85% for soluble COD, respectively. Nonetheless, longer ozonation treatment did not give additional advantage compared to the raw sludge. On the other hand, samples from anodes were analyzed using the 16S rRNA gene-based pyrosequencing technique for microbial community analysis. There was

substantial difference in community compositions among MFCs fed with different pretreated sludge. *Bacteroidetes* and *Firmicutes* were abundant bacterial phyla dominated in anodes of higher productivity MFCs. These results demonstrate that using waste sludge as the substrate in MFCs could achieve both sludge reduction and electricity generation, and proper pretreatment of sludge could improve the overall process performance.

1. Introduction

Ozonation and microwave digestion processes have been applied as pretreatment of the sludge (Eskicioglu et al., 2006). In many wastewater studies, ozonation process is one of the commonly used approaches to reduce solid contents of waste sludge. It is a simple process, easy to perform, little space required and less hazardous to human (Wu et al., 2008). Ozonation process will lead to cell membrane disruption and cell lysis. Destructed cell will release organelles and other cell components and consequently increase soluble organic substance in the solution (He et al., 2006).

On the other hand, microwave has been used to pretreat lignin-based materials for bioethanol production (Lu et al., 2011). High temperature and pressure are important criteria in order to wound up the firm lignin structure to furnish digestible structure for further application (Budarin et al., 2010). Microwave irradiation has shown great advantages due to flexible temperature control, rapid heating, and low overall cost and resulting higher soluble chemical oxygen demand (SCOD) content compared to raw sludge (Ayrilmis et al., 2011). Thus, efficient pretreatment through microwave degradation process would be essential to fasten the process such as hydrolysis known as a rate-limiting step in anaerobic degradation. Meanwhile, microwave treated sludge has resulted in accumulation of readily biodegradable compounds for the bacterial growth such as carbohydrate, protein and fat (Thungklin et al., 2011)

Considering the issues of operation and capital cost, pretreatment process might be not a good idea to raise a profit for the industry at certain conditions and applications. Chu et al., (2009) reported, an ozonation process was a major obstacle to be implemented in a big scale industry. However, the efficiency and optimum parameter would be the most important criteria needs to be evaluated for the persistent treatment process. On the other hand, when the operation cost considered with the starting operation such as the equipment and facilities, sludge-ozonation process may not be economical viable in the industry. Nevertheless, from their study, with appropriate control parameters, the electricity price dropped to 3.0 cent/kW h from the current market price 5.4 cent/kW h. (Yeom et al., 2002) also reported, the appropriate dosage of ozonation process was essential to deliberate the cost and the efficiency of the treatment towards less wastewater effluent discharged. This was supported by (Sakai et al., 1997). Main problem for the disposal of industrial sludge was due to biodegradability, pathogen destruction, dewaterability and bulk amount sludge treated per batch of operation, as stated by (Eskicioglu et al., 2007). These factors represented major capital and operational cost in a sewage treatment plant. However from the study, they found that microwave treated sludge was the most efficient treatment that can solve most of the wastewater disposable problems.

In addition, the utilization of microwave has delivered sufficient potential in industrial applications include environmental engineering field. It was unusual situation that cost of heating process is lower than electricity cost, especially due to low conversion of efficiency. Reported elsewhere, the efficient microwave generator was essential to overcome the energy problem, and lower tonnage material per operation was recommended to balance the cost, product recovery and sludge disposal. Thus MW treatment will deliver advantages over the conventional process

(Haque, 1999). In their study, it showed that microwave could recover 15-50% of operation cost compared conventional operation.

In this study, we investigated ozonation and microwave digestion as pre-treatment of activated sludge for electricity generation in MFCs. On top of that, microbial community analysis was carried out to understand how bacteria contributed to the energy production during each MFC process. The knowledge obtained will be essential for the development of advanced in-situ waste sludge treatment and power generation in treatment plants.

2. Materials and Methods

2.1. Inoculum and substrate used for the MFC

Same inoculum source and preparation used as described in previous chapter. For the substrate, four different types of substrate were performed in MFC as follows: untreated sludge (RW), 2 h ozonation treated sludge (2hO), 4 h ozonation treated sludge (4hO), and microwave digested sludge (MW). For the ozonation process, 200 mL of sludge was exposed for two and four hours with ozone, individually according to (He et al., 2006). Ozonation treatment was done using ozone generator icano3 model CFY-3 (Zhejiang, China), and the oxygen flow rate was maintained at 0.5 mL/min using air flow meter. On the other hand, microwave treatment was conducted using the microwave digestion system, MARS-X, CEM Corp. (NC, USA, 0–1250 W, 2450 MHz frequency). Twenty mL of slurry sludge samples was poured into the pressure-sealed vessel costumed with the machine. Slow cooking profiles at 1.4°C/min were applied up to maximum temperature of 175°C (Eskicioglu et al., 2009). All the treated samples were kept in 4°C prior to use in MFCs and for further chemical analysis.

In each operation cycle, supernatant from previous process was withdrawn before substituted with the new substrate. All the experiments were carried out at least in two

independent experiments with particular substrate and operation continued until stable condition was obtained.

2.2 Analytical methods

Chemical oxygen demand, carbohydrate, total suspended solids (TSS), and volatile suspended solids (VSS) were measured according to the Standard Methods, APHA (APHA, 2005). For soluble sample measurement, a broth sample was collected and span down at 10,000 rpm for 5 min using centrifuge (5418, Eppendorf). The supernatant was used as sample for analysis. All the samples were kept in 4°C prior analysis. The Coulombic efficiency (CE) was calculated as described by Logan, chapter 4, page 48-50 (Logan, 2007) and also reported by Durruty (2012) (Eq.1).

$$CE = \frac{8 \cdot \int_0^t I \cdot dt}{F \cdot V \cdot \Delta COD} \qquad (1)$$

Where 8 is a constant used for COD (grams per electron in oxygen), *I* is the harvested current from MFC, *F* is the Faraday constant (96,485 C/mol), V is operation volume in anode compartment and ΔCOD is change of COD over time (t).

2.3 Microbial community analysis

2.3.1. DNA extraction, PCR amplification and pyrosequencing

Samples for DNA extraction were obtained from attached microorganisms on the anode at the end of each MFC operation. Genomic DNA was isolated using the FastDNA SPIN kit for soil (Qbiogene-MP Biomedicals, Irvine, CA, USA). The V4-V5 region of bacterial 16S rRNA genes was amplified using the primer 515F (454-adapter A + barcode + 5'-GTGCCAGCMGCCGCGG-3'**)**, and primer 907R (454-adapter B + 5'-

CCGTCAATTCMTTTRAGTTT-3’**)**. Each forward primer contained a unique 10 bp barcode which was used to distinguish each sample. The PCR mixture (50 μL) consisted of 25 μL Failsafe Premix F (Epicentre Biotechnologies, Madison, WI, U.S.A.), 0.4 μM of each primer, 2.5 U of Ex Taq DNA polymerase (Takara, Dalian, China) and 1 μL template. PCR was carried out under the following conditions: an initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 90 s, and a final extension at 72°C for 7 min. Each sample was amplified in triplicate, pooled and purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, U.S.A). Equal amounts of all purified amplicons were pooled and sent to the Beijing Genomics Institute (Shenzhen, China) for pyrosequencing using 454 FLX Titanium technology. The results were deposited into the NCBI short reads archive database (accession number: SRX193676).

2.3.2. Sequences analysis

Pyrosequencing data were processed using QIIME v1.3.0 by following the procedure described in the previous study (Caporaso et al., 2010). Briefly, sequences were filtered according to the following thresholds: minimum read length of 100 bp, minimum average quality score of 25, maximum homopolymer of 6 bp, no ambiguous bases (N) in the entire sequence and non-assigned barcodes or mismatches in the primer sequence. The sequence errors were further corrected using Acacia v1.52 (Bragg et al. 2012). Operational taxanomic units (OTUs) were identified with a cutoff of 97% identity using uclust (Edgar, 2010), and alpha and beta diversity were further analyzed using corresponding Python scripts in QIIME. A representative sequence from each OTU was then selected and assigned to taxonomic rank using RDP classifier with a confidence threshold of 50%.

3.0 Results and Discussion

3.1. Substrate structure and morphology

The scanning electron microscope (SEM) demonstrated that all pretreated samples showed distinct physical surface structures compared to the RS sample (Fig. 1). The difference of the surface morphology probably would affect MFC performance especially in bacteria degradation activity. Abrasive surface with loose structure might have higher surface area and allow for adequate space for microorganism attachment and activity. Fig. 1a shows morphology of raw sludge observed under 100K magnification, and it clearly shows an intact and flat surface of sludge samples. The rigid structure would give lower surface area for bacterial attachment and as a result, require a longer degradation process as supported by Angelidaki and Sanders (Angelidaki and Sanders, 2004). In contrast, different features have been observed from ozonation (2hO and 4hO) and microwave (MW) treated sludge. Based on 2hO images, small porous particles were observed as a cluster of solid grains, which clumped together and formed a bunch of granule (Figure 6.1a). Visibly, it is hard to distinguish the difference between 2hO and 4hO samples, respectively. Longer ozonation exposure does not show substantial difference in the structure and shape, coherently (Figure 6.1b and 6.1c). On the other hand, the MW sample has shown dissimilar structure and size among all samples examined. The MW sample consisted of a mixture of small particles with globular and tubular structure in shape. Many pores have been spotted, which indicated that the loose structure was formed and would give higher surface contact for bacteria (Figure 6.1d). From the surface morphology examined, MW and ozonation treated sludge have shown distinct characters, which could be pre-indicators to the substrate performance in MFCs beforehand.

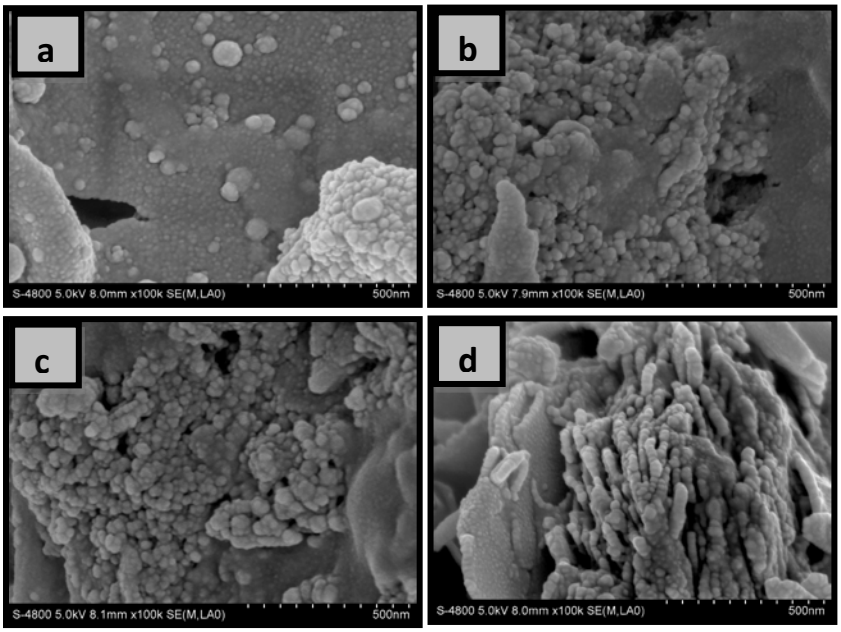


Figure 6.1: Image of sludge samples captured from scanning electron microscope with 100K magnification. (a) indicates raw sludge without any treatment, (b) indicates ozonation treated sludge at 2 h exposure time, (c) indicates ozonation treated sludge at 4 h exposure time and (d) microwave treated sludge at 1.4°C/min up to maximum temperature of 175°C.

3.2 Influence of pretreatment processes to the organic contents

The pretreatment processes have brought changes on the physical structure and the organic content of sludge. Figure 6.2 demonstrated the COD contents in the raw sludge, after pretreatment processes and after MFC operation. In average, after each particular pretreatment process, almost 21% of total COD (TCOD) reduced in MW samples followed by 4hO and 2hO samples with 17 and 11% reduction, respectively. In addition, after a typical cycle of MFC operation, 40% reduction was observed in TCOD from RS samples, whereas more than 50% reduction of TCOD achieved from pretreated samples. On the other hand, MW samples showed the highest SCOD increment about 3.5-fold followed by both 4hO and 2hO samples with 1.3-

fold compared with RS samples which had the lowest amount of SCOD. Almost the entire SCOD was consumed during the MFC process from all substrates applied. The highest consumption of SCOD was detected from MFCs fed with MW and 2hO samples, which showed more than 80% reduction. Therefore, most of the residual TCOD after MFC operation was particulate COD, although the results also indicated that smaller percentage of particulate COD was removed possibly by sludge-lysing bacteria (Li et al., 2009). In summary, the pretreatment processes, especially the microwave digestion, have increased accumulation of SCOD after pretreatment and more reduction in TCOD after MFC operation.

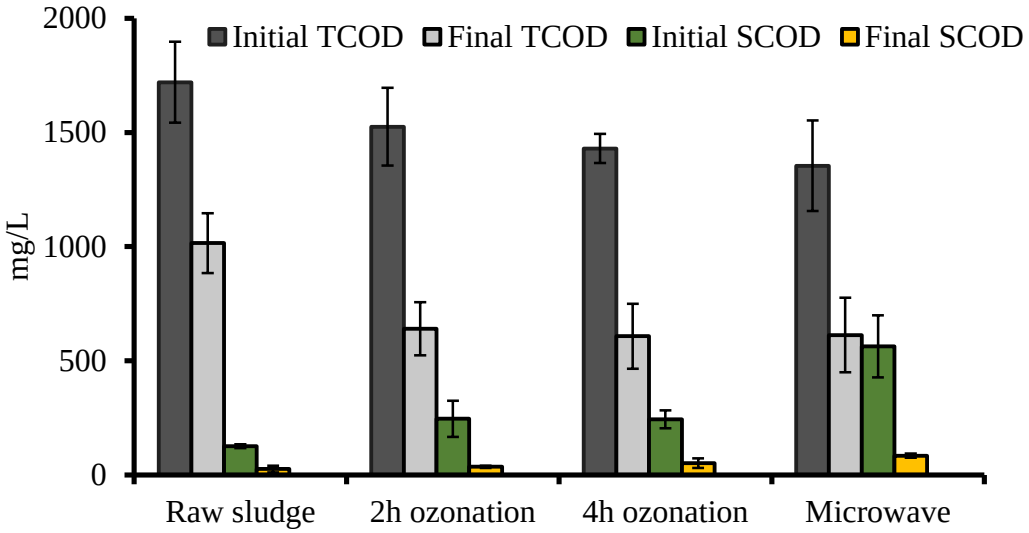


Figure 6.2: Amount of total and soluble chemical oxygen demand (TCOD and SCOD) in raw sludge and treated sludge samples before and after MFC operation. Grey with dot bar indicates TCOD at initial MFC process, dark grey bar indicates TCOD after ten days of MFC process, light grey bar indicates SCOD at initial MFC process and white bar indicates SCOD after ten days of MFC process.

3.3 Comparison of voltage production in MFCs

Distinct patterns of voltage production were obtained during the MFC operation from different types of substrate applied (Figure 6.3). Typically, bacteria expeditiously oxidized

soluble substrate in the sludge and at the same time it converted chemical energy that stored in chemical bonds of organic compounds into electrical energy (Du et al., 2008).. As shown in Fig. 3, at initial stage of each MFC cycle, the voltage increased and remained stable for a period of time, and later it gradually diminished with time. This is because some of nutrients have been consumed and led for the biomass growth and electricity generation. For the MFC with MW samples, the voltage slowly increased up to more than 160 mV and kept at stable state with small fluctuation up to more than 10 d of operation time. Lower voltage observed in MW at the initial stage of MFC probably due to the condition of the sludge and the pH of the treated with MW is slightly acidic compared to ozonation treatment. The bacteria were undergone adaptation with new MW treated sludge. Most of the MFC operated at the neutral pH to give optimum growth and activity of the bacteria (Liu et al., 2005). The voltage value remained higher compared to other MFCs, and it was probably due to the availability of organic contents, i.e. SCOD in the sludge substrates. Higher SCOD in the substrate would be beneficial during MFC operation since it may provide more easily accessed carbon sources, nutrients and supplements to the electrogens. For MFCs fed with 2hO and 4hO samples, the voltage increased up to more than 150 mV and 120 mV, respectively when new substrates were introduced in MFCs. The voltage produced finally decreased over time in both samples. Nevertheless, even similar treatment was applied, longer ozonation contact time, although still positive, did not give much better influence to voltage generation as can be seen in Fig. 3. This result is similar to a previous study which investigated the effect of ozonation pretreatment on anaerobic digestion (Eskicioglu et al., 2009). They found that higher ozone doses resulted in less improvement on methane production. Admittedly, some pretreatments would improve the amount of biodegradable organic content present in substrates but on the other side, it also will commit unintended drawback, such as accumulation of disfavored chemicals or reduction of the essential nutrients for the bacteria

(Laguerre et al., 2011). Overall, the MFC operation using MW and 2hO samples as substrates showed better performance than that using RW samples.

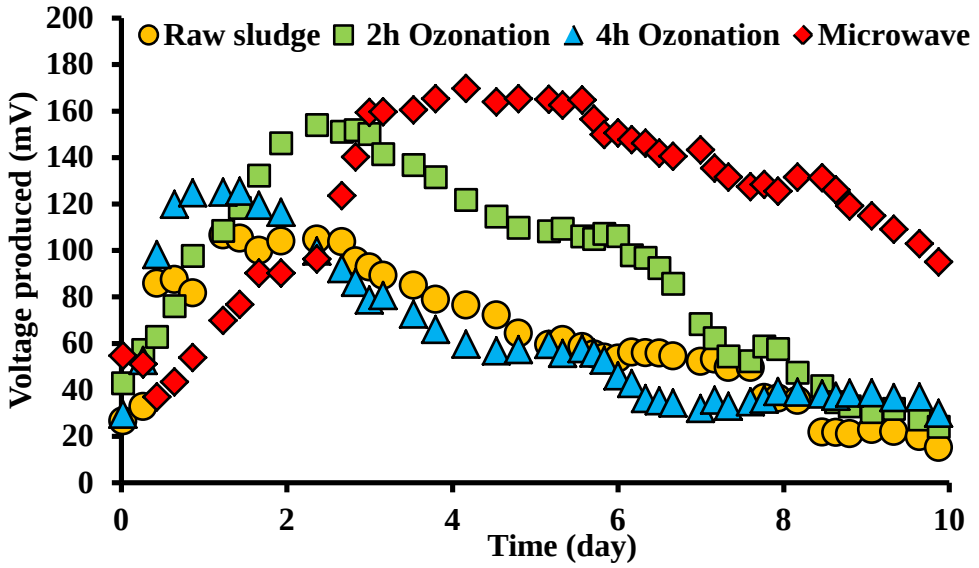


Figure 6.3: The distinct patterns of voltage produced during MFC operation using different types of treated and untreated sludge as substrates. Yellow round symbol indicates raw sludge, green square symbol indicates 2 h ozonation treated sludge, sky blue triangle symbol indicates 4 h ozonation treated sludge and red diamond symbol indicates microwave treated sludge.

3.4 Comparison of Power density, current density and Coulombic efficiency

Power density, current density and Coulombic efficiency have been reported as appropriate parameters to be used for comparison between each performance of MFCs (Pant et al., 2010). Considering we were using complex medium as substrate, COD was used as value to be integrated for reduction and columbic efficiency measurement, (Logan et al., 2006). Table 6.1 shows general values of MFC performance obtained throughout the process. Power density and current density were obtained based on electrode size of anode used during the MFC process, which was assumed as the active electrochemical surface (Ieropoulos et al., 2005). The surface area of electrode is important in MFCs due to the capacity of contact and reaction area between

bacteria, sludge and electron transfer. Corresponding to our data, the highest current density was observed from the MFC fed with MW samples at 0.35 mA/m^2 and the lowest was obtained from the MFCs with RS and 4hO samples, which had equal performance of 0.17 mA/m^2. Power density indicates the power generated from an effective electrode covered by bacteria. In this study, the highest power density was obtained from the MFC with MW samples at 42 mW/m^2 followed by the MFC with 2hO samples at 21 mW/m^2. The MFC with 4hO samples again gave similar performance as the MFC with RS at 10 mW/m^2. Along with previous results, MFCs that had higher consumption rate of organic contents would increase the electricity production except for the MFC with 4hO samples. The CE was calculated based on TCOD removal and current generation. The highest CE was observed from the MFC with MW at 5.9% followed by the MFC with 2hO at 3.5%. The lowest CE was obtained from the MFC with 4hO at 2.6%, and it is less than the MFC with RS at 3.0%. The data obviously showed that the MW sample was a more prolific substrate for electrogens toward power generation and the 4hO sample was a less effective substrate for electrogens although it contained higher SCOD content compared to the RS sample. Even the value obtained literally low compared to reported MFC related manuscript, but we found that such pretreatment has increased the voltage produced from raw sludge. There are many optimum factors were overlooked in this process (pH, temperature, internal resistance, mediators, cathode development) the optimum parameters might increase the productivity and performance of MFC system (Logan et al., 2006; Oh et al., 2004; Pandit et al., 2012). Internal resistance also one of the important criteria to enhance voltage productivity as reported by (Zhang et al., 2012) lower internal resistance resulted higher power yielded from MFC. However, in our study, internal resistance was not measured and it was assumed the internal resistance was higher since from the observation, open circuit voltage almost 3-folds higher than loaded voltage measured through multimeter.

Table 6.1: General parameters of MFCs derived throughout the MFC operation from four different types of substrate used.

	RS	2hO	4hO	MW
Maximum voltage obtained (mV)	105±15	154±20	125±12	170±5
Current density (mA/m^2)	0.17±0.02	0.25±0.03	0.17±0.01	0.35±0.01
Power density (mW/m^2)	10±2	21±6	10±1	42±3
Coulombic efficiency (%)	3.0	3.5	2.6	5.9

Abbreviation: RS, raw sludge; 2hO, 2 h ozonation treated sludge; 4hO, 4 h ozonation treated sludge and MW, microwave treated sludge.

3.5 Diversity, composition and structure of anode microbial community

In the observation, it was assumed that difference in electricity production was influenced by the different substrate used and probably it led to the changes of the microbial community during MFC processes (Sun et al., 2011). Therefore, in this study, the microbial community analysis was carried out in order to observe their affiliation and relationship to the power generation. The anode microbial communities from four MFCs were investigated using 16S rRNA gene-based pyrosequencing. Overall, 24,029 high quality reads were obtained after sequence quality filtering (RS, 3,403; 2hO, 9,757; 4hO, 4,302 and MW, 6,567) (Table 2). α-Diversity indices were calculated with 3,000 re-sampling reads per sample as unequal sample sizes may have an effect on diversity comparison. In total, 2,169 OTUs were identified based on the 97% identity cut-off, with 896, 867, 924 and 788 OTUs recovered from RS, 2hO, 4hO and MW, respectively. The Shannon (H') index was highest for 2hO and lowest for MW. The values are comparable to the microbial diversity observed from other anode biofilms from MFCs (Zhang et al., 2012). However, these results were higher than those from previous studies based on 16S rRNA gene clone libraries (Kiely et al., 2011), suggesting the diversity of microbial

community in the anode of MFCs could be determined at a high-resolution level by high-throughput sequencing methods. Indeed, our anode samples have a greater number of rare species (such as singletons), resulting in higher diversity and lower evenness in the complex microbial communities. The rare species may be missed by conventional molecular methods. However, the number of observed OTUs only represented 42.1% to 52.7% of the total number of predicted richness (Chao1) in the present study (Table 2), implying that more sampling efforts are needed to get a full picture of anode microbial community. Figure 6.4 demonstrated the taxa of four anode microbial communities at the phylum or class level, with 23 bacterial and one archaeal phyla were identified. Generally, *Proteobacteria* (38.4%), *Bacteroidetes* (30.5%), *Firmicutes* (15.9%), *Chloroflexi* (8.1%), *Actinobacteria* (5.6%), *Planctomycetes* (2.7%), *Acidobacteria* (1.0%) and *Nitrospira* (0.88%) were the top eight most abundant phyla in total, while the remaining phyla account for less than 0.5% of the total sequences, and most of them were not shared by all four anode samples. According to figure 6.4, there was clear difference in community compositions among different samples at the phylum or class level. For example, the relative abundances of *Bacterioidetes* and *Firmicutes* were higher in the treated sludge except 4hO had relatively low abundance of *Bacterioidetes*. Meanwhile, the percentage of *Betaproteobacteria* was significantly lower in the treated sludge than that in the untreated sludge. The clear difference in microbial community compositions between the untreated (raw) and treated sludge feeding MFCs was probably due to the substrate biodegradability resulted from different sludge treatments.

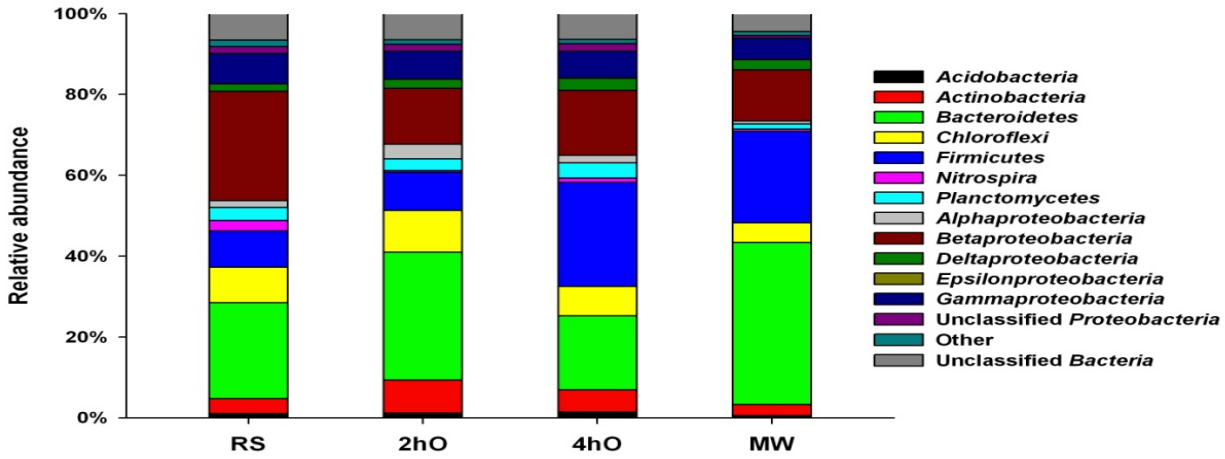


Figure 6.3: Taxa of four anode microbial communities at the phylum or class level. (a) Relative abundances of dominant bacterial taxa in different MFC samples. Minor phyla accounting for < 0.5% of total sequences are summarized in the group 'other'. (b) Heat map of the cosmopolitan genera which occurred in all MFC samples (Only classified genera were shown here, and unclassified genera were listed in the Table S2 of supplementary material). The scale bars shows the relative abundance (log scale) of each genera within a sample.

The higher abundance of *Bacterioidetes* in two higher efficiency MFCs (2hO and MW) raised the possibility that some bacterial populations from phylum *Bacterioidetes* may play a critical role in electricity generation or efficient anode function. This result is consistent with previous findings, where *Bacteroidetes* dominated in the anode biofilms of a cassette-electrode MFC (Shimoyama et al., 2009). Interestingly, in a long-term MFC operation, *Bacteroidetes* were enriched in anode biofilms more than those of initial inoculum during 300 d operation (Ishii et al., 2012). Further pure culture studies may shed light on the putative *Bacteroidetes*-like electrogens and their physiological characteristics.

The high β-diversity among anode microbial communities was confirmed by the Venn diagram analysis. Venn analysis demonstrated that, on average, 57.2 ± 1.30% of genera (163 ± 7 genera) were shared between any two samples, 42.8 ± 1.03% of genera (137 ± 5 genera) were shared between any three samples, and 34.8% of genera (120 genera) were common to all four

anode samples (Figure 6.5). At the OTUs level, 23.4 ± 0.77% of OTUs (330 ± 13 OTUs) were shared between any two samples, 11.5 ± 0.46% of OTUs (209 ± 9 OTUs) were shared between any three samples, and 7.3% of OTUs (158 OTUs) were common to all four anode samples. Furthermore, there were 10.8 ± 2.88% of genera (26 ± 9 genera) and 39.9 ± 1.05% of OTUs (346 ± 16 OTUs) were unique to single samples. These results indicated that more unique species or OTUs-level phylotypes appeared in each anode samples, which increase the β-diveristy among our samples. This finding is consistent with the results of Beecroft et al. (2012).

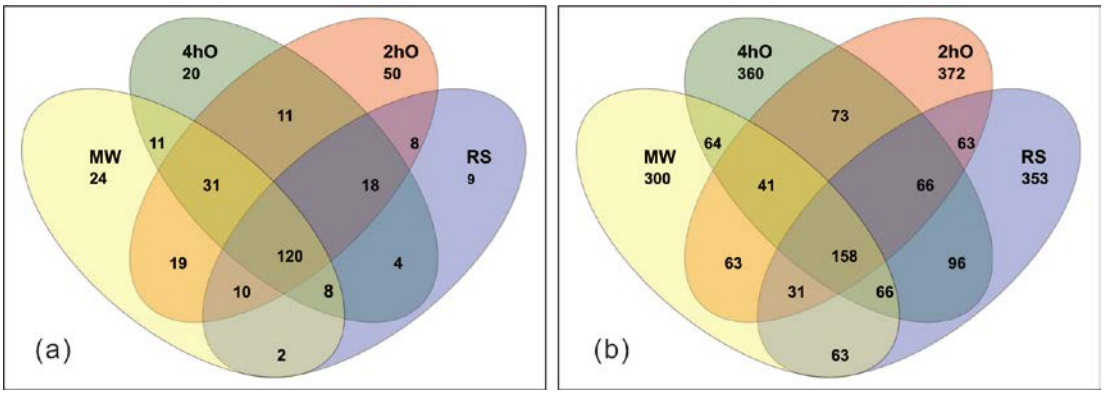


Figure 6.4: Venn diagrams show the number of genus (a) and OTUs (b) shared between four MFC samples.

Although the number of cosmopolitan genera and OTUs were relatively low compared with the total number of genera and OTUs, they accounted for 93.1% and 49.9% of the total community at the genera and OTUs level, respectively, suggesting that the potential important roles of these populations in maintaining the MFC process. As shown in Fig. 4b, the member of cosmopolitan bacterial communities included 66 classified genera which belong to *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Gemmatimonadetes*, *Nitrospira*, *Planctomycetes*, *Alpha-*, *Beta-*, *Delta-*, *Gammaproteobacteria*, and *Spirochaetes*. Several well-known exoelectrogens could be identified in these cosmopolitan bacterial communities of four

anode samples, such as *Clostridium*, *Desulfobulbus* and *Pseudomonas* (Park et al., 2001; Rabaey et al., 2004). Some other exoelectrogens including *Arcobacter* and *Geothrix*, were recovered from several samples with low abundance (Bond and Lovley, 2005; Fedorovich et al., 2009).

3.6 *Economic viability analysis*

There are not many studies deliberately described the treatment cost towards MFC operation. However, one of the recent published papers has discussed between MFC versus conventional anaerobic digestion (Durruty et al., 2012). Conceiving mentioned benefits of MFC and conventional anaerobic treatments (Chu et al., 2009; Durruty et al., 2012; Eskicioglu et al., 2007; Sakai et al., 1997), it described that both treatments had given great advantages toward operation and capital cost over conventional method. Interestingly, MFC promotes product recovery such as electricity and less polluted effluent.

Economic viability in comparison with conventional method such as anerobic digestion for methane production, MFC has played a complement role as describe by Durruty et al., (2012). In their study, methanogenic bacteria was used as inoculum and proved that MFC was an appropriate system for low COD wastewaters. As suggested low COD content of wastewaters were not favorable for methanogenesis reaction. Their study has developed a MFC using effluent from methanogenic reactor and called MFC post-treatment. Combination between MFC and conventional anaerobic treatment had increased the energy recovery and treatment efficiency compared to stand-alone process. Thus, the MFC operation is one of the complement processes if the total treatment cost comes in the state of imbalanced

However, in our study, we could not provide firm economic study and seems impossible since our MFC operation was carried out with lab scale and using low end treatment system for

industrial capability. As suggestion, we recommend the study might be extended to the industry scale capacity, which the industry that utilizing heating and ozonation processes for business as usual and applied for the sludge treatment and MFC application and the this time economic study come to the actual place.

4. Conclusion

Pretreatment of sludge has resulted in changes of sludge morphology and SCOD accumulation. Microwave was considered as the better pretreatment process to the sludge for MFC application in our study. Extended ozonation of sludge did not show much improved effect compared to untreated sludge. According to the 16S rRNA gene-based pyrosequencing analysis, difference in microbial community compositions among MFCs was observed. *Bacteroidetes* and *Firmicutes* were abundant bacteria dominated in the anode biofilm of higher efficiency MFCs. From our result, an appropriate pre-treatment of sludge will produce better substrate for MFC application to achieve both sludge reduction and electricity generation.

Chapter 8

Exclusive conclusion and suggestion

1.0 Exclusive conclusion

Through the study, a few uncharacterized genes in *E. coli* are found that associates to hydrogen metabolism. Some of the genes are gone through further investigation to elucidate their role in hydrogen production. On the other hand, the targeted gene obtain from the experiment are utilized to enhance hydrogen production and finally we constructed a octuplet metabolic engineered strain that given higher productivity and yield compared to wild type and reported engineered strain.

Along with hydrogen study, microbial fuel cells system is set up using waste activated sludge as inoculum and substrate for bioelectricity generation. Later, electricity generation is enhanced by applied the pre-treated sludge as their substrate. It is proven that MFC can be used as system that can consumes wide spectrum of substrate for electricity generation.

Thus, it is a great idea to integrate the knowledge and application of hydrogen production generation and electricity generation in sequential system. Whereas, effluent wasted from fermentation for hydrogen production can be applied to the MFC for electricity generation. Simultaneously, dual bioenergy are generated (hydrogen and electricity) from single substrate.

2. Suggestion

The application of the constructed metabolic engineered strain has to bring forward for utilization using environmental sample instead of synthetic medium. The consideration might be useful for the industrial application rather than bench experiment.

Specific role of each uncharatcterized genes will be a novel finding that may contribute a tremendous knowledge for the *E. coli* genome information and characterization. On the other way, it may help to enhance the hydrogen productivity and efficiency.

In MFC study, further pure culture studies will be needed to shed light on the putative *Bacteroidetes*-like electrogens and their physiological characteristics that contributes to electricity generation. Detail explanations of the how the electron generated and transferred from anodic chamber to cathode might be necessary by using single culture with sterilized sample might be useful for the bench study. The biofilm formation also an interesting studies to understand how to enhance electricity generation from MFC system.

Achievements

1. Grant award

1) **Mohd Yusoff, M. Z.** and Maeda, T. Research grant from Japan Science Society (JSS) through Sasakawa Scientific Research Allocation, Identification and Determination of Functional Prominent Genes Related to *Escherichia coli* for Hydrogen Production (24-521), April 2012 – March 2013.

2. Publication

1) **Mohd Yusoff, M. Z.,** Maeda, T., Sanchez-Torres, V., Ogawa, H. I., Shirai, Y., Hassan, M. A., Wood, T. K., 2012. Uncharacterized *Escherichia coli* proteins YdjA and YhjY are related to biohydrogen production. *International Journal of Hydrogen Energy*. 37, 17778-17787. (Impact factor: 4.054)

2) **Mohd Yusoff, M. Z.,** Hu, A., Feng, C., Maeda, T., Shirai, Y., Hassan, M. A., Yu, C.-P., 2013. Influence of Pretreated Activated Sludge for Electricity Generation in Microbial Fuel Cell Application. *Bioresource Technology*. 145, 90-96. (Impact factor: 4.98)

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3) **Mohd Yusoff, M. Z.,** Hashiguchi, Y., Maeda, T., Wood, T. K our Products from *Escherichia coli* Pseudogenes Increase Hydrogen Production. *Biochemical and Biophysical Research Communications. Accepted 3 September 2013.* (Impact factor: 2.406)

4) **Mohd Yusoff, M. Z.,** Hashiguchi, Y., Maeda, T., Mohd Yasin, N.H., Shirai, Y., Hassan, M. A., Wood, T. K**.** Metabolic Engineering to Enhance Biohydrogen Production Using YhjY in *Escherichia Coli*. *Submitted to Metabolic Engineering 16 August 2013.*

3. Contribution

1) Sanchez-Torres, V**., Mohd Yusoff, M. Z.,** Nakano, C., Maeda, T., Ogawa, H. I., Wood, T. K., 2013. Influence of *Escherichia coli* hydrogenases on hydrogen fermentation from glycerol. *International Journal of Hydrogen Energy*. 38, 3905-3912. (Impact factor: 4.054)

4. Conference attended

1) **Mohd Yusoff, M. Z.,** Maeda, T., Sanchez-Torres, V., Wood, T. K., Shirai, Y., Ogawa, H. I., Hassan, M. A., 2011. Functional Analysis of an Important Gene Related to Biohydrogen Production in *Escherichia coli*. BioMicroWorld2011, Torremolinos, Malaga, Spain. (14-16 September 2011**).**

2) **Mohd Yusoff, M. Z.,** Maeda, T., Ogawa, H. I., Hassan, M. A., Shirai, Y., 2012. Elucidation of Uncharacterized Genes Associated to Biohydrogen Production in *Escherichia coli* through Molecular Biotechnology Approaches. JSPS seminar. Universiti Putra Malaysia, Malaysia (30 March 2012).

3) **Mohd Yusoff, M. Z.,** Feng, C.-J., Hu, A., Yu, C.-P., Maeda, T., Shirai, Y., Ogawa, H. I., Hassan, M. A., 2012. Microbial Fuel Cells using Activated Sewage Sludge for Electricity Generation. Conference, International Biotechnology Symposium 2012, Daegu, Korea (16-22 September 2012).

4) **Mohd Yusoff, M. Z.,** Maeda, T., Hashiguchi, Y., Wood, T. K., Shirai, Y., Ogawa, H. I., Hassan, M. A., 2012. Unrealized function of *Escherichia coli* Genes *sufD* and *yehP* in Biohydrogen Evolution. The Society for Biotechnology, Symposium, Kobe, Japan (23-27 October 2012).

5) **Mohd Yusoff, M. Z.,** Hashiguchi, Y., and Maeda, T., 2013. New Functional Protein, YhjY, in *Escherichia Coli* known to be essential protein in biohydrogen metabolism 9th Asia Pacific Conference on Sustainable Energy and Environmental Technologies, Narita Japan. (5-8 September 2013).

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APPENDIX