Biohydrogen production from organic wastes using dark and photo fermentations

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**BIOHYDROGEN PRODUCTION FROM ORGANIC
WASTES USING DARK AND PHOTO
FERMENTATIONS**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2016

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WASTES USING DARK AND PHOTO
FERMENTATIONS**

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School of Civil and Environmental Engineering

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SUMMARY

Hydrogen is considered a viable and alternative fuel because of its renewability and clean burning nature. Integrated dark and photo fermentations systems were developed for biological hydrogen production from organic waste.

Hydrogen production potential and characteristics of four dark fermentative bacteria (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii*, and *E. aerogenes*) were investigated using glucose as substrate. Among the four different bacteria, *C. butyricum* had higher hydrogen yield and production rate compared to the other three bacteria. In addition, it was found that the distribution of volatile fatty acids was different among the fermentative bacteria. In food waste fermentation study, the comprehensive characteristics of biodegradable food waste were quantitatively analyzed. The effect of the initial pH and autoclaving on hydrogen production was investigated as well. *C. butyricum* efficiently converted food waste to hydrogen and organic acids after autoclaving food waste with pH adjustment at 7.0. Artificial mixed-culture was assorted to study the synergy among different bacteria on hydrogen production and system stability. The mixed bacteria culture *C. butyricum*, *C. pasteurianum*, and *C. beijerinckii* had a significant positive effect on the enhancement of hydrogen production. Results showed *C. butyricum* and *C. beijerinckii* well enhanced hydrogen production and improved system stability in dark fermentation using glucose and food waste as substrates.

Purple non-sulfur photosynthetic bacteria *R. palustris* were able to convert the main organic acids from food waste fermentation (acetate, butyrate and lactate) into hydrogen under incandescent lighting system. Although pH self-adjustment was observed in most fermentative conditions, pH control was extremely important for photo fermentative hydrogen production. A mutant strain *R. palustris* NifA* was studied towards hydrogen production in the existence of ammonium. By adding small amount of yeast extract, hydrogen production was duplicated while the lag phase was reduced. As well, non-growing cells highly improved substrate conversion efficiency in the photo fermentation. Substrate conversion efficiency

of 78.3% was achieved in the nitrogen starvation study by non-growing cells. Besides, the lighting efficiency was highly improved because of the stable low cell density.

Based on the above findings, sequential dark and photo fermentation processes were developed using *C. butyricum* and *R. palustris* NifA*. An overall hydrogen yield of 7.01 mol/mol glucose was achieved, which was 58.5% of the maximum theoretical hydrogen yield. From system analysis, it was observed that the hydrogen yield was highly affected by the dark fermentation due to its lower substrate conversion efficiency. The sequential study also revealed that initial inoculum concentration had less effect on hydrogen production compared to pH, and initial substrate concentration. On the other hand, a combined dark and photo fermentative bacteria group CR1 (OD ratio 2:1 of *R. palustris* NifA* : *C. butyricum*) continued to produce hydrogen with higher overall hydrogen yield after several generation cultivation. RNAseq study was applied to unveil the mechanism of the complex co-culture system. Although a large proportion of genes were consistently regulated in both the co-culture system and pure cultures, nitrogen fixation and nitrogenase gene expression of *C. butyricum* was clearly distinctive. This was the first transcriptome study on co-culture of dark and photo fermentative bacteria. It provided insights into the quantitative and qualitative transcriptome in the co-culture consortium. The transcriptome findings could help to optimize the integrated process for hydrogen production and system stability enhancement. The successful application of this integrated system in food waste conversion to hydrogen would be economic and reliable.

PUBLICATIONS

Hu C.-C., Giannis A., Chen C.-L., and Wang J.-Y. (2014). Evaluation of hydrogen producing cultures using pretreated food waste. *International Journal of Hydrogen Energy*.

Hu C.-C., Giannis A., Chen C.-L., Qi W., and Wang J.-Y. (2013). Comparative study of biohydrogen production by four dark fermentative bacteria. *International Journal of Hydrogen Energy*.

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Hu C.-C., Giannis A., and Wang J.-Y. (2015). Biological hydrogen production from food waste by using integrated dark and photo fermentation. The 6th International Congress of Energy and Environmental Engineering and Management. 22-24 July, Paris, France.

Hu C.-C., Wang J.-Y., Giannis A., and Chen C.-L.. (2014). Biohydrogen production from food waste. The 7th International Conference on Environmental Science and Technology. 9-13 June, Houston, USA.

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Hu C.-C., Giannis A., Chen C.-L., Qi W., and Wang J.-Y. (2012). Biohydrogen production from glucose by different dark fermentative bacteria. The 2012 Asian Biohydrogen and Bioproducts Symposium (ABBS). 9-12 November, Chongqing, China.

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ACRONYMS

AHB	Annular hybrid bioreactor
AMPTs	Automatic Methane Potential Test System
ASBR	Anaerobic sequencing batch reactor
ATP	Adenosine triphosphate
CoA	Coenzyme A
COD	Chemical oxygen demand
CDW	Cell dry weight
C/N	Carbon to nitrogen ratio
CSTR	Continuous stirred tank reactor
DNA	Deoxyribonucleic acid
DFE	Dark fermentation effluent
Eq.	Equation
Fd ⁺	Ferredoxin (reduced form)
Fd ²⁺	Ferredoxin (oxidized form)
FHL	Formate:hydrogen lyase
FID	Flame ionization detector
FW	Food waste
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HPR	Hydrogen production rate
HRT	Hydraulic retention time
HydA	Fd-dependent hydrogenase
MBR	Membrane bioreactor
MSW	Municipal solid wastes
NADH	Nicotinamide adenine dinucleotide
NAD ⁺	Nicotinamide adenine dinucleotide
NFOR	NAD(P)H:ferredoxin oxidoreductase
OD	Optical density
PCR	Polymerase chain reaction
PFL	Pyruvate:formate lyase

PFOR	Pyruvate:ferredoxin oxidoreductase
PNSB	Photosynthetic non-sulfur bacteria
RNA	Ribonucleic acid
SCOD	Soluble chemical oxygen demand
SHPR	Specific hydrogen production rate
SCE	Substrate conversion efficiency
TCD	Thermal conductivity detector
TS	Total solids
TSS	Total suspended solids
UASB	Upflow anaerobic sludge blanket
VFAs	Volatile fatty acids
TVFA	Total volatile fatty acids
VS	Volatile solids
VSS	Volatile suspended solids

Chapter 1 Introduction

1.1 Background

The global energy demand has demonstrated a great increase during last few decades, together with the fast depletion of fossil fuels and huge amount of greenhouse gas emissions. As a result, exploration of alternative clean energy sources has been a hot topic for researchers all over the world. Hydrogen is considered a viable alternative fuel for the future because of its renewability and clean burning nature (Nath and Das 2004b). It is also a promising “energy carrier” with high-energy yield (142.35 kJ/g) that is 2.75 times greater than hydrocarbon fuels (Logan 2004, Lin *et al.* 2007). Furthermore, it is also widely used as raw material for the synthesis of ammonia, alcohol and aldehydes, for the hydrogenation of various petroleum products, and for desulfurization and reformulation of gasoline in refineries (Fang *et al.* 2002a). Around 50 million tonnes of hydrogen are traded annually globally with an expected growth rate of 3.5% annually (Winter 2005, Timothy 2011). Based on the National Hydrogen program of the United States, the contribution of hydrogen to total energy market will be 8-10% by 2025 (Ravi Kumar Parihar and Upadhyay 2016).

Currently, 98 % of hydrogen in the market is produced through the expensive, energy intensive and unsustainable methods such as steam methane reforming (SMR), refining and water electrolysis, etc. (Singh and Wahid 2015). Biological hydrogen production from renewable sources has attracted more attention in recent years due to its economic and environmental-friendly characteristics (Nath and Das 2004a, Urbaniec and Bakker 2015). Biohydrogen production through biological processes mainly involves biophotolysis by algae or cyanobacteria, dark and photo fermentation. Biophotolysis only converts hydrogen from water with a relatively slow production rate and sometimes it is inhibited by oxygen. Dark and photo fermentation processes are considered more feasible due to simultaneous waste treatment and hydrogen production. A lot of bacteria have been isolated and investigated for their capability to produce hydrogen using a wide range of organic

wastes (carbohydrates, organic acids, etc.). However, the relatively low hydrogen yield in dark fermentation and limited hydrogen production rate in photo fermentation have put a brake on its development and marketing application.

An integrated system has been proposed to improve the biohydrogen production efficiency, where photo fermentative bacteria utilize organic acids from dark fermentation effluent to further convert carbohydrates to hydrogen (Lo *et al.* 2008a). In general, integrated systems involve sequential and combined dark and photo fermentation processes. To date, integrated systems have been assessed to be capable of hydrogen production enhancement with the highest hydrogen yields of 7.2 and 7.1 mol H₂/mol hexose reported in sequential and combined systems, respectively (Yokoi *et al.* 2002, Asada *et al.* 2006, Singh and Wahid 2015). However, compared with the economical target of 8 mol H₂/mol glucose and the theoretical maximum 12 mol H₂/mol glucose, more efforts and studies are still in demand. Another issue for the application of integrated systems is the system stability. As it is known, microorganism's environment is complicated and difficult to control. The integrated processes are also sensitive and complex since different functional bacteria are employed in the systems. Molecular biology tools are needed to apply for further insights study into the co-culture system in order to improve the system stability and hydrogen production (Hallenbeck and Ghosh 2012, Kumar and Chowdhary 2016).

In the urban environment, the amount of municipal solid waste (MSW) is growing faster than the rate of urbanization. MSW produced today is almost twice as much as 10 years ago (0.68 billion tonnes per year). While food waste takes a big portion of MSW in highly urbanized city such as Singapore, it is challenge in solid waste management. According to Ministry of Environment and Water Resources (MEWR) report (MEWR 2015), Singapore generated 0.79 million tonnes FW in 2015 with a relative low recycling rate of 13%, while the traditional treatment methods are landfill disposal or incineration. However, the odorous and easy-burning landfill gas is intolerable, and leachate contaminates underground water as well. Because of the high moisture content, burning efficiency of food waste in incineration plant is insufficient. Food waste is a complex mixture of organic

matters and carbohydrates, easily hydrolysable and biodegradable (Kim 2004, Kapdan and Kargi 2006, Ng *et al.* 2015). This study will provide an alternative option for renewable energy - hydrogen production from organic waste using an effective biological fermentation system. It will contribute to establish a sustainable renewable energy strategy and help on waste management.

1.2 Research objectives

The main objective of this research was to develop an efficient integrated dark and photo fermentation system for biological hydrogen production by using food waste as feedstock. To achieve this goal, the following tasks have been carried out in this study:

- ✚ Study and optimize the individual dark and photo fermentative hydrogen production processes by using pure and artificial mixed cultures. Systematically analyse dark fermentation effluent and then investigate its further utilization feasibility for photo fermentative bacteria producing hydrogen;
- ✚ Evaluate and optimize hydrogen production from food waste, characterization and pre-treatments methods of food waste were discussed;
- ✚ Develop and investigate the integrated dark and photo fermentations system for hydrogen production, including sequential and combined dark and photo fermentations system. A semi-continuous CSTR fermentor was recommended and studied;
- ✚ Fundamental research on the co-culture system was conducted by using molecular biology tools for potential biological hydrogen production enhancement.

1.3 Scope and organization

In order to comprehensively elaborate the development of the efficient integrated biological hydrogen production system, this thesis has been structured into seven chapters.

Chapter 2 is a detailed literature review on: 1) fermentative hydrogen production processes in terms of metabolic pathways and important factors affecting the hydrogen production; 2) organic waste to energy studies and application; 3) molecular biology technologies application in environmental engineering research.

Chapter 3 focuses on dark fermentation characteristics study by evaluating hydrogen production potential from several dark fermentative bacteria. The final effluent was comprehensively analyzed especially on the organic acids parts.

In Chapter 4 evaluates food waste was on biological hydrogen production. To enhance hydrogen production efficiency from food waste, pretreatment methods were applied. In addition, mixed culture system was proposed and investigated according to the bacteria characteristics study from Chapter 3.

Chapter 5 presents the photo fermentation study, including photo fermentative bacteria selection, lighting system development, and efficient optimization strategies. Mutant photo fermentative bacteria strain was applied for better utilization of dark fermentation effluent.

Chapter 6 evaluates the developed integrated dark and photo fermentation system. It also provides the important insights into the co-culture system from RNA sequencing study that gave rise to both qualitative and qualitative information on the transcriptome.

The last chapter summarizes the most important findings from this research. Some research suggestions on biological hydrogen production are recommended the future studies and applications.

Chapter 2 Literature Review

2.1 Introduction

Hydrogen is the lightest and most plentiful element in the universe. Hydrogen is a clean energy with water as the main product and a high heating value. As compared to other gaseous fuels like nature gas, hydrogen is harmless to the human and environment. Currently hydrogen production is mainly produced from fossil fuels, water and biomass. Steam reforming, coal gasification and water electrolysis are the main industrial methods for hydrogen production (Balat 2008, Ghimire *et al.* 2015), which are energy intensive and not environmentally friendly. On the opposite, biological hydrogen production processes are more sustainable, of which most of the operations are at the ambient temperature and pressure. As well, they could utilize the renewable energy resources such as waste as feedstock (Dutta 2014, Arimi *et al.* 2015)

In order to clearly understand the state of art for biological hydrogen production globally, a comprehensive literature review has been carried out and presented in this chapter. Biological hydrogen production processes are classified and reviewed in section 2.2, including bio-photolysis from water, dark and photo fermentation, integrated processes, as well as impact factors and bioreactors. Section 2.3 reviews food waste to energy processes. Finally, a summary is drawn and described in section 2.4.

2.2 Biohydrogen Production

Biological hydrogen production mainly includes bio-photolysis, dark fermentation and photo fermentation. Hydrogenase and nitrogenase are two major enzymes for hydrogen production in all the biological processes. Table 2.1 presents the advantages and disadvantages of these three main biological hydrogen production technologies. The technical barriers of each process are required to be overcome for practical applications. In general, both bio-photolysis and photo fermentation convert solar energy to hydrogen with a limited hydrogen production rate. Dark

fermentation has rapid H₂ production rate and simple operation in comparison to photosynthetic processes. Low hydrogen yield of dark fermentation is expected to be solved by the combination with photo fermentation, as photo fermentative bacteria could utilize organic byproducts in dark fermentation effluents for further hydrogen production.

Table 2.1 Comparisons of biohydrogen production technologies.

	Advantages	Disadvantages
Biophotolysis	Water and solar energy are sustainable sources; and products are simple of hydrogen and oxygen.	Photolysis enzyme-hydrogenase is oxygen sensitive; light conversion efficiency is low.
Dark fermentation	Hydrogen evolution rate is high; most of carbonhydrates could be used for substrate.	Total hydrogen yield is low; large amounts of organic byproducts inhibit the process.
Photo fermentation	The conversion effiecnecy from organic substrates to H ₂ is high.	ATP and hight energy electrons are needed; hydrogen evolution rate is low.

2.2.1 Biophotolysis of water by algae and cyanobacteria

Biophotolysis is a promising alternative biological process, as Krampitz (1972) first defined the term of “biophotolysis” that green plants or algae use solar energy to convert water to oxygen and hydrogen This approach would allow abundant production of hydrogen from the readily available resources - water and light. Biophotolysis includes direct (Eq. 2.1) and indirect (Eq. 2.2, 2.3) process by algae and cyanobacteria (Manish and Banerjee 2008, Oh *et al.* 2011).

2.2.1.1 Algae

Water molecules are dissociated by microalgae under sunlight, which is similar to the photosynthesis in plants, but algae adapts them for the generation of hydrogen gas instead of carbohydrates (Figure 2.1). Gaffron *et al.* (1940) first reported that

the adapted green alga *Scenedesmus obliquus* took up H_2 when CO_2 was present after being incubated anaerobically in the presence of H_2 in the dark. Afterwards, Kessler and Bishop et al. identified numerous species of algae that also evolved H_2 by photolysis under anaerobic conditions (Kessler 1974, Bishop *et al.* 1977). Other species of green, red, and brown algae have been reported with the similar observation (Dungey 1951, Antal *et al.* 2011, Azwar *et al.* 2014). Three pathways for hydrogen production in green algae have been reported. For water photolysis, microalgae directly utilize the energy of sunlight to extract electrons from water molecules in photosystem II. Electrons are then transported to the Fe-S protein ferredoxin (Fd) on the reducing side of photosystem I. Protons and electrons from reduced Fd generate H_2 molecule by the [FeFe]-hydrogenase that is in the stroma of the algal chloroplast (Seibert *et al.* 2008, McKinlay and Harwood 2010a, Srirangan *et al.* 2011).

Figure 2.1 Water biophotolysis for hydrogen production by algae.

2.2.1.2 Cyanobacteria

Cyanobacteria - that are also known as blue-green algae, cyanophyceae, or cyanophytes - can evolve hydrogen by direct and indirect biophotolysis. Various species and strains, within at least 14 genera of cyanobacteria under a vast range of culture conditions for hydrogen production have been assessed in last three decades (Benemann 1997, Ferreira *et al.* 2012). Cyanobacteria have simple nutritional requirements that can grow in air, water and mineral salts, with light as the only energy source. There are three different pathways in cyanobacteria for hydrogen production (Srirangan *et al.* 2011). Especially in indirect biophotolysis, it separates the O_2 and H_2 evolution reactions into two stages accompanied by CO_2 fixation and evolution. In the first step the photosynthetic organism grow in large quantities to obtain biomass rich in carbohydrates. The second step may vary in different species, but usually involves the hydrogen production by using the biomass from

the first step. In direct biophotolysis (Figure 2.2), N_2 -fixing cyanobacteria produce hydrogen mainly by nitrogenase instead of hydrogenase, however in several non- N_2 -fixing cyanobacteria, hydrogen evolution was also observed through hydrogenase. As well, hydrogen production is energetically burdened by nitrogenase so that significant ATP is required (Tamagnini *et al.* 2007, McKinlay and Harwood 2010a, Carrieri *et al.* 2011, Das *et al.* 2014).

Figure 2.2 Water biophotolysis for hydrogen production by cyanobacteria.

Although impressive results have been achieved in the water biophotolysis process for hydrogen production, some biological and engineering challenges should be considered (Antal *et al.* 2011, Ferreira *et al.* 2012, Azwar *et al.* 2014): (1) O_2 production in the process limits the hydrogenase activity that is the main enzyme for hydrogen production but extremely sensitive to oxygen. (2) In order to both produce and capture H_2 and O_2 , entire production area is required to enclose in photo-bioreactor, which is impractical to handle H_2/O_2 mixtures in large volumes or over large areas. (3) Solar conversion efficiency is low. Since 10 % of solar conversion efficiency is regarded as the economical level for the reducing power generated by photosynthesis, however, currently only 3-4 % of sunlight's available energy can be captured by photosynthetic organisms like higher plants. (4) Light intensity is sensitive to biohydrogen production. Although both H_2 uptake and evolution are enhanced by light, if the light intensity is increased beyond even moderate levels (>500 lux), activity ceases and normal oxygenic photosynthesis resumes. Researchers did the attempts to overcome these problems by: (1) sweeping out the produced oxygen to mitigate the inhibition for enzymes; (2) using both reversible and irreversible O_2 absorbers; and (3) using O_2 tolerant hydrogenase enzymes. Till now, all of these attempts have met with limited success, but the novel biomedical and genetic strategies developed and applied in the biophotolysis

system are promising, such as the hydrogenase protein and light-harvesting complexes engineering (Srirangan *et al.* 2011).

2.2.2 Dark fermentative hydrogen production

In dark fermentation, organic carbohydrates are converted to H₂, organic acids and CO₂ by fermentative bacteria (Figure 2.3). From the Eq. (2.4-2.6) negative free energy indicates that the reactions proceed spontaneously toward hydrogen formation without external energy input. The hydrogen yield depends on the products formed in the process. Theoretically, when acetic acid or acetone is the only product, a maximum hydrogen yield of 4 mol H₂/mol glucose can be achieved. If butyric acid is the only VFAs product, a maximum yield of 2 mol H₂/mol glucose is obtained. Formation of propionic acid consumes 1 mole H₂ per mole propionic acid. Other reduced compounds formation such as lactic acid, alcohols, ethanol and butanol also results in hydrogen yield decrease (Vardar-Schara *et al.* 2008). Besides, in practice hydrogen yields are lower than the theoretical calculation because glucose is also used for bacteria growth and maintenance (Argun and Kargi 2011).

Varieties of heterotrophic bacteria have been investigated to produce hydrogen by using a wide range of carbohydrates under anaerobic conditions in dark fermentation. In general, spore forming *Clostridium* species, facultative *Enterobacter* sp., *Citrobacter* sp., *Bacillus* sp., and some thermophilic bacteria are widely used (Kapdan and Kargi 2006, Valdez-Vazquez and Poggi-Varaldo 2009, Lee *et al.* 2011). Hydrogenase is the key enzyme in most of dark fermentative bacteria for hydrogen production (Benemann 1996, Mathews and Wang 2009).

Figure 2.3 Schematic diagram of dark fermentation.

2.2.2.1 Strict anaerobes

Clostridium sp.

Among the fermentative anaerobes, *Clostridia* have been well known and studied extensively, not only for their hydrogen production capability but also for their role in the industrial solvent production from various carbohydrates. *Clostridium* is a genus belonging to phylum of *Firmicutes*. *Clostridium* species are Gram-positive, rod shaped and capable of producing endospores. Most of cells are motile by using their peritrichous flagellum. They usually produce mixtures of organic acids and alcohols from carbohydrates or peptones. Some species can fix atmospheric nitrogen, but never carry out sulfate reduction. They are usually negative to catalase. For most species, pH 6.5-7 and temperatures 30-37 °C are the optimal conditions for growth. They are usually found in soil, sedimentation in oceans and fresh water, or intestinal of people or animals. Most species are obligate anaerobes and extremely sensitive to oxygen so that the presence of a trace amount of dissolved oxygen can completely stop the hydrogen production (Brenner *et al.* 2005, Hallenbeck 2005, Chen *et al.* 2006, Hsiao *et al.* 2009).

Two metabolism pathways were observed in the hydrogen production reaction by *Clostridium* species (Figure 2.4). The typical metabolic route is through the re-oxidization of NADH under acidogenic conditions in the presence of two major enzymes: NAD(P)H:ferredoxin oxidoreductase (NFOR) and Fd-dependent hydrogenase (HydA) (Mathews and Wang 2009, Wang *et al.* 2010a). In this reaction, NAD(P)H is formed during carbon metabolism. Fd_{ox} is reduced to Fd_{rd} by NFOR and then the electrons in Fd_{rd} are transferred to protons to form H_2 by hydrogenase (Eq. 2.7, 2.8, 2.10). In particular, hydrogen yield loss exists in real application as the NADH oxidation by NFOR is inhibited under standard conditions

and can only proceed at very low partial pressure of hydrogen (<60 Pa) (Hallenbeck 2005, Kraemer and Bagley 2007). The second pathway involves pyruvate:ferredoxin oxidoreductase (PFOR) and Fd-dependent hydrogenase (HydA) (Chen *et al.* 2006, Oh *et al.* 2011). The oxidative decarboxylation of pyruvate is catalyzed by PFOR to form acetyl-CoA and CO₂ under anaerobic conditions. Electrons are first transferred to Fd_{ox}, and then transferred to protons to form H₂ by HydA (Eq. 2.9, 2.10).

Figure 2.4 Metabolic pathways of *Clostridium* sp. for glucose fermentation (Sinha and Pandey 2011). (Substrate transformations are shown in solid lines, electron flow is shown in dashed lines.)

Various *Clostridium* species were investigated to produce hydrogen from a range of substrates via dark fermentation. Table 2.2 lists some isolated pure *Clostridia*

capable of hydrogen production. The hydrogen yields by *Clostridium* species vary between 1.1 mol H₂/mol hexose and 2.8 mol H₂/mol hexose due to different operation conditions and species per se.

Chen *et al.* (2008b) conducted control experiments with five isolated *Clostridium butyricum* strains and *Clostridium pasteurianum* strains to produce hydrogen from original starch. All the cultures could produce H₂ from hydrolyzed starch and displayed higher hydrogen production rates of 116 and 118 mL-H₂/g-VSS/h, for *Clostridium butyricum* CGS2 and *Clostridium pasteurianum* CH5, respectively. Meanwhile, the H₂ yields obtained from strain CGS2 and strain CH5 were 1.23 and 1.28 mol H₂/mol glucose, respectively.

Clostridium acetobutylicum ATCC 824 was tested by Zhang *et al.* (2006) using glucose as substrate. The H₂ production rate of 220 mL/h/L of reactor and specific H₂ production rate of 1270 mL/g glucose per liter of reactor was obtained. The H₂ yield was 15–27% based on the theoretical maximum 4 mol H₂/mole glucose in dark fermentation.

Hsiao *et al.* (2009) isolated three hydrogen-producing bacterial strains *Clostridium sporosphaeroides* F52, *Clostridium tyrobutyricum* F4 and *Clostridium pasteurianum* F40 from the seed fermentor. Their hydrogen productivities were determined using condensed molasses fermentation solubles as substrate. *C. pasteurianum* F40 was the dominant strain with a maximum hydrogen production rate of 7.7 mL/h at a CMS concentration of 50 g COD/L. *C. sporosphaeroides* F52 played a main role in degrading carbohydrate and glutamate with a lower hydrogen production rate of 1.0 mL/h. *C. tyrobutyricum* F4 also had a maximum hydrogen production rate of 3.1 mL/h at a CMS concentration of 50 g COD/L. Co-cultivation of these three strains as a symbiotic mixed-culture process could enhance hydrogen productivity.

The hydrogen productivities of more *Clostridia* such as *C. lentocellum* (Ravinder *et al.* 2000), *C. bifermentans* (Wang *et al.* 2003), *C. thermolacticum* (Collet 2004), and *C. saccharoperbutylacetonicum* (Ferchichi *et al.* 2005), were listed in Table 2.2.

2.2.2.2 Facultative anaerobes

Enterobacter sp.

Enterobacter species have attracted much attention in hydrogen production due to their high growth rate, easiness of cultivation and wide substrate range. *Enterobacter* are Gram negative, 0.6-1.0×1.2-3.0 µm straight rod shaped, and motile by 4-6 peritrichous flagella, which belong to the general definition of the family *Enterobacteriaceae*. Most strains are facultative anaerobes and give a positive Voges–Proskauer reaction and a negative methyl red test. They grow readily on ordinary media, with the optimum growth temperature of 30 °C. *Enterobacter* species are widely distributed in nature, and common in humans and animals (Brenner *et al.* 2005).

Enterobacter studied for hydrogen production (Table 2.2) include *Enterobacter aerogenes* (Tanisho *et al.* 1987, Zhang *et al.* 2009), *Enterobacter cloacae* (Kumar *et al.* 2001, Nath *et al.* 2006), *Enterobacter asburiae* (Shin *et al.* 2007), etc. Glucose is fermented with production of acids and gases in which the ratio of CO₂ to H₂ is generally 2:1. Gas production from glucose ceases at 44.5 °C. In addition, hydrogen yield could be improved through genetic modification (Jayasinghearachchi *et al.* 2009, Lu *et al.* 2009) and redirection of metabolic pathways (Rachman *et al.* 1997, Kumar *et al.* 2001, Nath and Das 2004a). Kumar *et al.* (2001) reported that hydrogen yield by *E. cloacae* was increased to 3.8 mol H₂/mol glucose by blocking the pathways of organic acid formation using NaBr and NaBrO₃.

Enterobacter species metabolize in a different process from *Clostridium sp.*, which is called the formic acid fermentation and employs two major enzymes: pyruvate:formate lyase (PFL) and formate:hydrogen lyase (FHL) (Hallenbeck and Benemann 2002, Hallenbeck 2005, Oh *et al.* 2011). Pyruvate from glycolytic cycle is split into acetyl-CoA and formate by PFL under anaerobic conditions (Eq. (2.11)). In the absence of suitable electron acceptors (such as Fd_{ox}²⁺) formate is then converted to H₂ and CO₂ by FHL (Eq. 2.12).

Due to the absence of specific coenzymes such as ferredoxin oxidoreductase, in *Enterobacter* metabolic process, NADH (that determines the anaerobic glycolysis) is re-oxidized to NAD^+ by using pyruvate or one of its derivatives as an electron and hydrogen acceptor. As a result, besides formate a large amount of mixed acids and alcohols (such as 2,3-butanediol and ethanol) are formed during the process of NADH consumption (Figure 2.5). These reduced compounds production limits hydrogen release in a gas form and results in a lowered hydrogen yield of less than 2 mol H_2 /mol glucose generally (Lee *et al.* 2011, Zhang *et al.* 2011).

Figure 2.5 Metabolic pathway of *E. aerogenes* for glucose fermentation (Zhang *et al.* 2011).

Citrobacter sp.

Another well-studied facultative anaerobe in dark fermentation is *Citrobacter* sp., which is Gram-negative, short rod-shaped and motile. *Citrobacter* is also a member of the family of *Enterobacteriaceae* (Jung *et al.* 1999). Most are chemoorganotrophic bacteria and not encapsulated. *Citrobacter* species use citrate, glucose, and sucrose as carbon and energy sources to produce VFAs and gases, in which the ratio of carbon dioxide to hydrogen is normally 1:1. When lactose is used as the carbon source, the reaction is slow. The methyl red test is positive but the Voges–Proskauer test is negative. They grow easily on ordinary media, having both

respiratory and fermentative type of metabolism. They are probably normal intestinal inhabitants that occur in the feces of humans and some animals, and also found in soil, water, sewage, and food. Sometimes some species are pathogenic and often isolated from clinical specimens as opportunistic pathogens (Brenner *et al.* 2005). The metabolic pathway for hydrogen production of *Citrobacter* sp is similar to that of *E. aerogenes*, which is also the fomite pathway (Figure 2.6).

Figure 2.6 Metabolic pathway of *Citrobacter* sp. for glucose fermentation (Oh *et al.* 2008b).

Brosseau (1980) investigated the hydrogen production by *Citrobacter intermedius* that was isolated from sewage sludge. They used a 14 L fermenter to do the batch tests and obtained the result that the optimum pH for hydrogen production was from 5.75 to 6.0, which gave a yield of 1.1 mol H₂/mol glucose. The optimum hydrogen productivity was 144 moles hydrogen per hour at pH 6.0.

Dr Park's group (Jung *et al.* 1999) isolated *Citrobacter amalonaticus* Y19 from an anaerobic wastewater sludge digester and characterized this chemoheterotrophic bacterium that could utilize CO with the production of H₂ and CO₂. They used glucose as carbon source to test its capability of fermentative H₂ production in batch cultivation (Oh *et al.* 2003a). By increasing culture temperatures in the range of 25–36 °C, faster cell growth and higher initial H₂ production rates were observed. However, final H₂ yields were almost constant irrespective of temperature. Cell

mass, ethanol, acetate and CO₂ were the major fermentation products and comprised more than 70% of the carbon consumed. Optimal specific H₂ production activity was observed at 36 °C and pH 6-7. The maximal H₂ yield and H₂ production rate were 2.49 mol H₂/mol glucose and 32.3 mmol-H₂/g-cell/h, respectively. Carbon metabolic-pathways were analyzed by using batch cultivation and an *in silico* metabolic-flux analysis. It revealed that initial glucose levels determined the major metabolites production. It was also reported to design the metabolic pathway for optimal hydrogen production. The metabolic pathway to achieve maximal H₂ yield of 8.7 mol H₂/mol glucose was identified (Oh *et al.* 2008, Oh *et al.* 2008b).

2.2.2.3 Other microorganisms

Besides the above three typical genera of hydrogen producing microorganisms, more bacteria were studied in dark fermentation in various conditions. In the thermophilic environment, *Thermoanaerobacterium* species are dominant among the hydrogen production microorganisms (Ueno *et al.* 2001, Zhang *et al.* 2003, Ahn *et al.* 2005). Zhang *et al.* (2003) reported that six species of *Thermoanaerobacterium* converted starch in wastewater into hydrogen at 55 °C. They got the maximum hydrogen yield of 92 mL H₂/g starch_{added} at wastewater pH 6.0, and the maximum specific hydrogen production rate of 365 mL-H₂/g-VSS/d at wastewater pH 7.0. Acetate and butyrate were dominant in the liquid byproducts. They also concluded that all *Thermoanaerobacterium* species had the optimal growth conditions of 55-70 °C and pH 5.2-7.8, and could degrade starch except *T. polysaccharolyticum*.

Hyper-thermophilic (> 80 °C) cultures seem to possess a superior ability to generate more hydrogen, but exhibit much lower hydrogen production rates generally ranging from 0.01 to 0.2 L H₂/L/h (Kanai *et al.* 2005, Lee *et al.* 2011). Schroder *et al.* (1994) reported the highest hydrogen yield was achieved by using extreme thermophiles *Thermotoga maritima* on glucose at 80 °C, which was close to the theoretical maximum of 4.0 mol H₂/mol glucose.

Other aerobes were also observed to be capable of hydrogen production. Oh *et al.* (2003b) isolated 7 *Aeromonas* spp. strains, 3 *Pseudomonas* spp. strains and 5

Vibrio spp. strains from anaerobic granular sludge. These species had the similar hydrogen yields of 1.02-1.2 mol H₂/mol glucose and their major fermentation by-products were ethanol and acetate. Jeong *et al.* (2008) studied the hydrogen productivity of *Bacillus megaterium* from glucose. The lag time of *B. megaterium* was independent of glucose concentration and as short as 2 h. Within the glucose concentration range of 5-20 g/L, hydrogen production rate was around 2-3.5 mL/h. Kalia *et al.* (1994) and Shin *et al.* (2004) also investigated the hydrogen productivity of *Bacillus* species.

Overall, the mesophilic bacteria clostridia have a relative higher hydrogen yield with range of 1.1 to 2.3 mol H₂/mol glucose. The wide-type facultative anaerobic bacteria (e.g. *Enterobacter* species) could only produce limited hydrogen less than 1 mol H₂/mol glucose, but the engineered strains achieved high hydrogen yield of more than 2 mol H₂/mol glucose. Hydrogen yields of thermophilic bacteria were more than 3 mol/mol glucose, however, the operation condition with the high temperature requirement was not economic or sustainable.

Table 2.2 Hydrogen production by different hydrogen producing microorganisms.

Microorganism	Substrate/ Carbon source	Culture operation	T	pH	Light intensity	Hydrogen yield	Hydrogen production rate	Reference
			°C		W/m ²	mol H ₂ /mol glucose	L H ₂ /L/h	
Dark fermentative bacteria								
<i>Clostridium butyricum</i> SC E1	Glucose	Continuous	30	-		2.3	-	(Kataoka <i>et al.</i> 1997)
<i>C. butyricum</i> IFO13949	Glucose	Continuous	36	-		2.3	1.15	(Yokoi <i>et al.</i> 1998b)
<i>C. butyricum</i> CGS5	Sucrose	Batch	37	-		1.39	0.21	(Chen <i>et al.</i> 2005)
<i>C. acetobutylicum</i>	Glucose	Continuous	30	-		1.08	0.22	(Zhang <i>et al.</i> 2006a)
<i>C. acetobutylicum</i>	Glucose	Batch	-	-		1.8	-	(Lin <i>et al.</i> 2007b)
<i>C. pasteurianum</i> CH4	Sucrose	Batch	37	7.5		2.07	0.57	(Lo <i>et al.</i> 2008)
<i>C. tyrobutyricum</i> F4	Condensed molasses fermentation solubles	Batch	35	-		15.1 mL H ₂ /g COD	0.05	(Hsiao <i>et al.</i> 2009)
<i>C. amygdalinum</i> strain C9	Starch	Batch	40	7.5		390 mL H ₂ /g starch	-	(Jayasinghearachchi <i>et al.</i> 2010)
<i>C. beijerinckii</i> L9	Glucose	Batch	-	-		2.8	-	(Lin <i>et al.</i> 2007b)
<i>C. acidisoli</i>	Sucrose	Batch	30	7		-	-	(Sun <i>et al.</i> 2010)
<i>C. sporosphaeroides</i> F52	Condensed molasses fermentation solubles	Batch	35	-		17.2 mL H ₂ /g- COD	0.02	(Hsiao <i>et al.</i> 2009)

Microorganism	Substrate/ Carbon source	Culture operation	T °C	pH	Light intensity W/m ²	Hydrogen yield		Hydrogen production rate	Reference
						mol H ₂ /mol glucose	L H ₂ /L/h		
<i>C. paraputrificum</i> M-21	Glucose	Batch	45	-	-	1.1	-	-	(Evvyernie <i>et al.</i> 2001)
<i>C. bifermentans</i>	Wastewater sludge	Batch	35	-	-	2.1 mmol H ₂ /g COD	-	-	(Wang <i>et al.</i> 2003)
<i>C. saccharoperbutylacetonicum</i>	Glucose	Batch	30	-	-	1.37	0.16	-	(Ferchichi <i>et al.</i> 2005)
	Lactose	Batch	30	-	-	1.41	1.12	-	
	Sucrose	Batch	30	-	-	1.42	0.20	-	
	Maltose	Batch	30	-	-	1.39	0.12	-	
	Fructose	Batch	30	-	-	1.2	0.14	-	
	Chesse whey	Batch	30	-	-	1.35	0.14	-	
<i>Enterobacter aerogenes</i> strain E.82005	Glucose	Batch	40.5	6	-	1	0.52	-	(Tanisho <i>et al.</i> 1987)
<i>Enterobacter aerogenes</i> HU-101 wt	Glucose	Batch	-	-	-	0.6	-	-	(Rachman <i>et al.</i> 1997)
<i>E. cloacae</i> D ₁₁	Glucose	Batch	36	-	-	3.8	-	-	(Kumar <i>et al.</i> 2001)
<i>E. cloacae</i> IIT-BT08	Glucose	Batch	36	-	-	2.2	0.45	-	(Kumar and Das 2000)
	Sucrose	Batch	36	-	-	3	0.66	-	
	Cellobiose	Batch	36	-	-	2.7	0.65	-	
	L-Arabinose	Batch	36	-	-	1.5	0.36	-	
	Fructose	Batch	36	-	-	1.6	0.44	-	
	Maltose	Batch	36	-	-	1.4	0.18	-	
	Potato starch	Batch	36	-	-	-	0.20	-	

Microorganism	Substrate/ Carbon source	Culture operation	T	pH	Light intensity	Hydrogen yield	Hydrogen production rate	Reference
			°C		W/m ²	mol H ₂ /mol glucose	L H ₂ /L/h	
	C.M. cellulose	Batch	36	-		-	0.09	
<i>E. asburiae</i> SNU-1	Glucose	Batch	37	7		0.54	0.40	(Shin <i>et al.</i> 2007)
<i>Citrobacter amalonaticus</i> Y19	Glucose	Batch	36	-		2.49	-	(Oh <i>et al.</i> 2003a)
<i>Citrobacter intermedius</i>	Glucose	Batch	34	6		1.1	-	(Brosseau <i>et al.</i> 1980)
<i>Citrobacter</i> sp. CMC-1	Glucose	Batch	34	6		1.82	-	(Mangayil <i>et al.</i> 2011)
<i>Klebsiella oxytoca</i> HP1	Bagasse	Continuous	38	-		1.6	0.35	(Wu <i>et al.</i> 2010)
<i>Klebsiella</i> sp. HE1	Sucrose	Batch	37	7.5		0.2	0.09	(Lo <i>et al.</i> 2008)
<i>Ethanoligenens harbinense</i> B49	Glycerol	Batch	35	3.5		2.26	-	(Xu <i>et al.</i> 2008)
<i>Halanaerobium saccharolyticum</i>	Glycerol	Batch	37	7.4		0.58	-	(Kivistö <i>et al.</i> 2010)
<i>Halanaerobium senegalensis</i>	Glycerol	Batch	37	7		1.21	-	(Kivistö <i>et al.</i> 2010)
<i>Bacillus licheniformis</i> JK1	Wheat slurries	Continuous	38-40	-		0.2	-	(Kalia <i>et al.</i> 1994)
<i>Clostridium thermocellum</i>	Delignified wood fibers	Batch	60	-		1.6	-	(Levin <i>et al.</i> 2006)
<i>Thermococcus kodakaraensis</i> KOD1	Starch	Continuous	85	-		3.33	0.21	(Fukui <i>et al.</i> 2005)
<i>Thermotoga maritima</i>	Glucose	Batch	80	-		4	-	(Schröder <i>et al.</i> 1994)
<i>Thermotoga elfi</i>	Glucose	Batch	65	-		3.33	-	(van Niel <i>et al.</i> 2002)

Microorganism	Substrate/ Carbon source	Culture operation	T °C	pH	Light intensity W/m ²	Hydrogen yield mol H ₂ /mol glucose	Hydrogen production rate L H ₂ /L/h	Reference
<i>Caldicellulosiruptor saccharolyticus</i>	Glucose	Batch	70	-		3.4	-	(Mars <i>et al.</i> 2010)
	Hydrolyzed potato steam peels	Batch	70	-		3.4	-	
<i>Thermotoga neapolitana</i>	Glucose	Batch	80	-		2.9	-	(Mars <i>et al.</i> 2010)
	Hydrolyzed potato steam peels	Batch	80	-		3.3	-	
Photosynthetic bacteria								
<i>Rhodospseudomonas capsulata</i>	Acetate, Propionate, Butyrate	Continuous	32	6.8	50	-	0.02	(Shi and Yu 2006)
<i>R. faecalis</i> RLD-53	Ethanol, Acetate	Batch	35	7	4k lux	3.49	0.28	(Liu <i>et al.</i> 2009a)
<i>R. palustris</i> P4	Glucose	Batch	37	7	-	2.76	0.37	(Oh <i>et al.</i> 2002)
<i>R. palustris</i>	Acetate, Lactate	Batch	30-33	6.8-7.6	150-200	5.52	0.03	(Özgür <i>et al.</i> 2010)
<i>R. sphaeroides</i> RV	Lactate	Continuous	30	7.2	10 k lux	-	0.06	(Fascetti and Todini 1995)
<i>R. sulfidophilum</i> W-1S	Succinate	Batch	30	-	40-45 μmol/m ² /s	-	0.03	(Maeda <i>et al.</i> 2003)

Microorganism	Substrate/ Carbon source	Culture operation	T	pH	Light intensity	Hydrogen yield	Hydrogen production rate	Reference
			°C		W/m ²	mol H ₂ /mol glucose	L H ₂ /L/h	
<i>Rhodobacter capsulatus</i>	Acetate, Lactate	Batch	30-33	6.8-7.6	150-200	4.68	0.02	(Özgür <i>et al.</i> 2010)
<i>R. capsulatus hup⁻</i> mutant	Acetate, Lactate	Batch	30-33	6.8-7.6	150-200	6.85	0.03	(Özgür <i>et al.</i> 2010)
<i>R. sphaeroides</i> O.U. 001	Malate	Batch	30	7	2.4k lux	-	0.01	(Sasikala <i>et al.</i> 1992)
<i>R. sulfidophilus</i> LMG 5202	Lactate	Batch	30	6.75	6k lux	2.2	-	(Peng <i>et al.</i> 1987)
<i>Rhodospirillum rubrum</i> S-1	Lactate	Continuous	30	7	400	-	0.18	(Zürter and Bachofen 1982)
<i>Chromatium</i> sp. miami PSB 1071	Succinate and Thiosulphate	Batch	34	7.4-8.1	140 μE/m ² /s	-	0.03	(Ohta <i>et al.</i> 1981)
Green algae								
<i>Chlamydomonas reinhardtii</i>	Acetate	Batch	27-29	7	100 μE/m ² /s	-	0.002	(Laurinavichene <i>et al.</i> 2006)
<i>Chlamydomonas</i> MGA 161	5% CO ₂	Batch	30	8	25	-	0.004	(Ohta <i>et al.</i> 1987)
<i>Chlorella sorokiniana</i> Strain Ce	Acetate	Batch	30	-	120 μE/m ² /s	-	0.001	(Chader <i>et al.</i> 2009)
Cyanobacteria								
<i>Anabaena variabilis</i>	1% CO ₂	Continuous	30	-	32	-	0.007	(Berberoglu <i>et al.</i> 2008)

Microorganism	Substrate/ Carbon source	Culture operation	T °C	pH	Light intensity W/m ²	Hydrogen yield mol H ₂ /mol glucose	Hydrogen production rate L H ₂ /L/h	Reference
<i>Aphanocapsa montana</i>	Air	Batch	25-30	7.1	20 μmol/m ² /s	-	0.009	(Howarth and Codd 1985)
<i>Gleobacter</i> PCC 7421	CO	Batch	20	-	20 μE/m ² /s	-	0.043	(Moezelaar <i>et al.</i> 1996)
<i>Chroococcidiopsis thermalis</i> CALU 758	1% CO ₂	Batch	26	-	70 μE/m ² /s	-	0.009	(Serebryakova <i>et al.</i> 2000)
<i>Oscillatoria miami</i> BG7	CO ₂	Batch	35	7.75	100 μE/m ² /s	-	0.006	(Philips and Mitsui 1983)

2.2.3 Photo fermentative hydrogen production

In photo fermentation, photosynthetic non-sulfur bacteria (PNSB) are catalyzed by nitrogenase to produce hydrogen under nitrogen-deficient and anaerobic conditions from reduced compounds (organic acids) with light energy input (Figure 2.7).

Figure 2.7 Schematic diagram of photo fermentation from organic acids.

Eq. (2.13) presents H_2 formation from acetic acid by photo fermentation, where external energy input is required because of the positive free energy of the reaction. The overall reaction of hydrogen production from glucose can also be given as Eq. (2.14).

Although photo fermentation has the advantage to well stoichiometrically convert carbohydrates to hydrogen, several drawbacks exist in this process: (1) high energy demand of nitrogenase enzyme for efficient H_2 production is required (Brentner *et al.* 2010); (2) limited H_2 production rate due to low nitrogenase activity ($\sim 5 \text{ s}^{-1}$) (Hallenbeck and Benemann 2002); (3) the low solar energy conversion efficiencies; and (4) re-oxidation of the produced H_2 by uptake hydrogenase (Koku *et al.* 2002).

2.2.3.1 PNSB bacteria

The most widely known bacteria used in photo fermentative H_2 production are *Rhodospseudomonas* sp (Barbosa *et al.* 2001, Shi and Yu 2006, Su *et al.* 2009, Lo *et al.* 2010), *Rhodobacter* sp (Peng *et al.* 1987, Kim *et al.* 2006b, Özgür *et al.* 2010, Kim *et al.* 2012a), and *Rhodospirillum* sp (Zurrer and Bachofen 1979, Melis and Melnicki 2006, Basak and Das 2007, Argun and Kargi 2011).

Rhodopseudomonas species are Gram-negative, rod-shaped, and motile by means of polar or sub-polar flagella. They belong to the family of *Bradyrhizobiaceae*. *Rhodopseudomonas* show polar growth with budding and asymmetric cell division. Lamellar internal photosynthetic membranes underlie and parallel to the cytoplasmic membrane. Optimal growth conditions are in mesophilic freshwater and neutral pH. Several growth modes present in different conditions: preferred photoheterotrophic growth is under anoxic conditions in the light; photoautotrophic growth is possible under anoxic conditions with hydrogen, thiosulfate, or sulfide as electron donor; and chemotrophic growth occurs under microoxic to oxic conditions (Brenner *et al.* 2005, Bueno *et al.* 2012). *Rhodopseudomonas faecalis* RLD-53 was isolated from indigenous freshwater pond sludge by Ren *et al.* (2009). Hydrogen productivity was study using different carbon and nitrogen sources under different light intensities. H₂ yields of 2.64 mol H₂/mol acetate, 2.34 mol H₂/mol propionate, 1.75 mol H₂/mol lactate and 3.55 mol H₂/mol malate, respectively were achieved at pH 7.0 and 35 °C under light intensity of 4000 lux. H₂ production yield and rate decreased with increasing light intensity and reached highest under light intensity of 3000–5000 lux.

Rhodobacter species are Gram negative, ovoid or rod-shaped, and belong to the family of *Rhodobacteraceae*. Some cells are motile by polar flagella and may form chains. They multiply by binary fission or budding, and may produce capsules or slime. The color of phototrophic cultures is yellow-green to yellow-brown, while aerobic cultures are pink to red. *Rhodobacter* species grow in mesophilic freshwater with neutral pH and in the absence of NaCl. They are usually found in the sewage ponds or eutrophic lakes. Photoheterotrophic and photoautotrophic growth occur under the similar conditions of *Rhodopseudomonas*. However, chemotrophic growth is by aerobic respiration under anaerobic dark conditions, also by denitrification, fermentation, or oxidant-dependent metabolism. Sasikala *et al.* (1992) reported the hydrogen production by isolated *Rhodobacter sphaeroides* O.U. 001 from the wastewater of a distillery. Wastewater with low concentrations (5% and 10%) could be utilized to produce hydrogen, but the hydrogen production rate

was low. Calcium alginate immobilization could increase the hydrogen productivity by 2-3 folds.

Rhodospirillum species are Gram negative, vibrioid to spiral shaped, motile by bipolar flagella, and belong to the family of *Rhodospirillaceae*. They divide by binary fission. Similar growth molds like *Rhodopseudomonas* species occur under special conditions. Some species are very sensitive to oxygen, in opposite others can also grow aerobically in the dark. They are found in mesophilic freshwater and prefer neutral pH. Zurrer *et al.* (1982) studied the hydrogen production by *Rhodospirillum rubrum* strain S-1 in a 2 L fermenter with four 100 W spotlight tungsten lamps. The hydrogen production rate was constant in the process, with the maximum rate of around 180 mL/h/L (cell suspension). The composition of the evolved gas mixture (CO₂ and H₂) varied and determined by pH and pressure.

2.2.3.2 Metabolism pathway

The metabolism of photo fermentation has been studied extensively in purple non-sulfur bacteria (Figure 2.8). Nitrogenase and hydrogenase are two important enzymes in the H₂ evolution metabolism, where nitrogenase is used to produce H₂ in the absence of nitrogen but hydrogenase oxidizes H₂ to recycle electrons, protons and ATP for energy metabolism (Akkerman *et al.* 2002, Hallenbeck and Benemann 2002, Koku *et al.* 2002, Mathews and Wang 2009, Brentner *et al.* 2010, McKinlay and Harwood 2010a, Hadicke *et al.* 2011).

Figure 2.8 H₂ production metabolic pathway in PNSB (McKinlay and Harwood 2010a, Oh *et al.* 2011).

Under anaerobic conditions, the electrons scavenged from organic acids are transferred to oxidize ferredoxin (Fd_{ox}) through a series of membrane-bound, electron transport carrier molecules. These electrons are then transported to nitrogenase by ferredoxin. When nitrogen is not present, nitrogenase catalyzes the reduction of protons to produce H_2 by using energy from ATP (Eq. 2.15). In opposite, the electrons are used to reduce molecular nitrogen to ammonia (NH_3) by nitrogenase (Eq. 2.16). A large amount of energy is required for the reduction and electron transfer in this whole process (Oh *et al.* 2011).

2.2.4 Integrated processes

Integration of different biological hydrogen processes can both enhance the hydrogen production and utilize the substrate and energy more sustainably (Melis and Melnicki 2006, Liu *et al.* 2010a, Wang *et al.* 2011). Multi-stage biohydrogen production processes involving three or even four stages has been proposed including photolysis, photo fermentation, dark fermentation and microbial electrolysis (Show *et al.* 2011).

Melis *et al.* (2006) reported a two-dimensional integration of photo-biological H_2 production by an unicellular green algae of *Chlamydomonas reinhardtii* and purple photosynthetic bacteria *Rhodospirillum rubrum*, which resulted in better solar irradiance (visible and infrared) utilization and integration of nutrient utilization for the cost-effective production of substantial amounts of hydrogen gas. Another high-yield, sustainable, and commercially viable H_2 production integrated process was proposed and discussed. Oxygenic and anoxygenic photosynthesis were employed in tandem to convert solar energy into H_2 , which could be collected using a light–dark cycle. By use of industrial enzymes, biomass extracted from the photosynthetic system (green algae and PNSB) was converted into cellulolytic material composed of hydrolysates of polyglucose and protein, which was directly fed into dark fermentation system by using cellulosome-containing fermentative bacteria. A

variety of small organic acids in the effluents from dark fermentation were then fed back into the photosynthetic system.

Lo *et al.* (2010) designed a high-yield and CO₂-free biohydrogen process by a sequential dark-photo fermentation and autotrophic microalgal system. Sucrose was proved to be the most effective carbon source for the sequential dark (with *Clostridium butyricum* CGS5) and photo (with *Rhodospseudomonas palustris* WP3-5) fermentation process. A maximum H₂ yield of 11.61 mol H₂/mol sucrose and a H₂ production rate of 673.93 mL/h/L were obtained. The CO₂ produced from the sequential dark-photo fermentation process was completely consumed by a microalga culture (*Chlorella vulgaris* C-C) for its autotrophic growth at 30 °C under 60 mmol/m²/s illumination.

Among various integration biohydrogen production processes, integrated dark and photo fermentations in the form of sequential or combined fermentations are much attractive (Yokoi *et al.* 1998a, Kim *et al.* 2006b, Su *et al.* 2009, Argun *et al.* 2009b, Liu *et al.* 2010b, Sagnak and Kargi 2011). The overall reaction of sequential or combined dark and photo-fermentation is in Eq. (2.14).

2.2.4.1 Sequential dark and photo fermentation

The scheme of sequential dark and photo fermentation from biomass is described in Figure 2.9. In this process, pretreatment of dark fermentation effluent (DFE) before photo fermentation and neutralization after acid hydrolysis are important steps to consider (Chen *et al.* 2008a, Xie *et al.* 2010). Take into account the cost of pretreatments and reactors setups, an overall yield of 8 mol H₂/mol glucose is expected as an economic target of this whole process (Argun and Kargi 2011). In general, sequential dark and photo fermentations improved H₂ yields to more than 5 mol H₂/mol glucose, while H₂ production rates in sequential fermentation are lower (<700 mL H₂/L/h) than those of dark fermentation alone (Shi and Yu 2006, Kim *et al.* 2006b, Liu *et al.* 2009b, Su *et al.* 2009, Lo *et al.* 2010, Özgür *et al.* 2010, Ozmihci and Kargi 2010a, Liu *et al.* 2010b, Özkan *et al.* 2012).

Figure 2.9 Bio-hydrogen production from biomass by sequential dark and photo fermentation.

Dr Kargi's group studied and concluded certain conditions for the effective sequential dark and photo fermentation (Argun *et al.* 2008a, Ozmihci and Kargi 2010a). Since residual glucose in the DFE resulted a shift from glucose to VFA fermentation by the PNS bacteria, hydrogen yields and production rates of the whole system would decrease. Besides the requirement of glucose deficient in DFE, TVFA and NH_4^+ concentrations from DFE must be below 2500 mg/L and 40 mg/L, respectively. Therefore, DFE pretreatments such as dilution, ammonium stripping, centrifugation and sterilization were required.

Yokoi *et al.* (2002) reported a high hydrogen yield of 7.2 mol H_2 /mol glucose by using a sequential dark and photo fermentation process from starch with *Enterobacter aerogenes* HO-39 and *Clostridium butyricum* in dark fermentation and *Rhodobacter* sp. M-19 in photo fermentation. Individual yields of dark and photo-fermentations were 2.7 mol H_2 /mol glucose and 4.5 mol H_2 /mol glucose, respectively. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ and EDTA addition, and pH control at 7.5 were stated to enhance photo fermentation yield.

Liu *et al.* (2009b) investigated the sequential process by *Ethanoligenens harbinense* B49 and immobilized *Rhodopseudomonas faecalis* RLD-53 with a total hydrogen yield of 6.32 mol H_2 /mol glucose by using 9 g/L glucose and 30 mmol/L phosphate buffers. Acetate and ethanol were the main soluble metabolites from dark-fermentation. It was found that the ratio of acetate to ethanol increased with increasing phosphate buffer concentration that is favorable to further photo hydrogen production.

2.2.4.2 Combined dark and photo fermentation

Combined dark and photo fermentation is realized in a single reactor to convert biomass to hydrogen and the other metabolites by dark fermentative bacteria and photo fermentative bacteria, which is considered a cost-effective process that can both save the bioreactor and time (Miyake *et al.* 1984, Yokoi *et al.* 1998a, Asada *et al.* 2006). The total fermentation time is reduced by simultaneous formation and utilization of VFAs; in addition, accumulation of VFAs in the medium is also eliminated. Hydrogen yields in combined fermentation are considerably higher than dark and photo-fermentations alone. However, H₂ production rates are still a bit lower than single-stage dark fermentation (Fang *et al.* 2006, Melis and Melnicki 2006, Ding *et al.* 2009, Argun *et al.* 2009a, Argun *et al.* 2009b, Kargi and Ozmihci 2010, Sun *et al.* 2010, Xie *et al.* 2010, Argun and Kargi 2010a, Liu *et al.* 2010a, Argun and Kargi 2010b, Ozmihci and Kargi 2010b, Ozmihci and Kargi 2010c, Sagnak and Kargi 2011, Jiao *et al.* 2012).

Yokoi *et al.* (1998a) compared the hydrogen production from a sequential process and a combined process by *Clostridium butyricum* and *Rhodobacter* sp. M-19 from starch. For the sequential process, the total hydrogen yield was 5.5 mol H₂/mol glucose with 1.9 mol H₂/mol glucose from dark fermentation and 3.6 mol H₂/mol glucose from photo fermentation. The hydrogen yield of the mixed cultures was 6.6 mol H₂/mol glucose.

Due to the great difference on growth rate and acid-resistant capacity between dark-fermentative bacteria and photo-fermentative bacteria, the ratio of the two kinds of bacteria in the combined system was proved crucial to the hydrogen production (Liu *et al.* 2010a). Fang *et al.* (2006) investigated the combined dark and photo fermentation system from glucose by *Clostridium butyricum* and *Rhodobacter sphaeroides*. Results showed that *R. sphaeroides* with cell populations 5.9 times higher could not compete with *C. butyricum* for glucose but used the acetate and butyrate to produce hydrogen. When the pH was lowered to 6.5 resulting from the accumulation of fatty acids, hydrogen production in the co-culture system was ceased. Kargi *et al.* (2010) investigated a combined dark and photo fermentation system from ground wheat using fed-batch operation. Different initial dark/light

biomass ratios (D/L) of anaerobic sludge and the mixture of *Rhodobacter* sp. were investigated. When the D/L ratio was 1/2, the highest cumulative hydrogen volume (1.5 L), hydrogen yield (65.2 mL/g starch), and specific hydrogen production rate (5.18 mL/g/h) were obtained.

2.2.5 Factors affecting bio-hydrogen production

Bio-hydrogen production is such a complex biochemical process that many factors have effects on hydrogen production including pH, temperature, nutrients, light, hydrogen partial pressure, HRT and metabolic engineering methods.

2.2.5.1 pH

pH is an important impacting factor for biological hydrogen production because it takes effect on both the enzymes activities and metabolic pathways of the fermentative bacteria. The optimum initial pH reported in a wide range from 4.5 to 9.0 dependent on inoculated organisms per se, substrate type and concentrations, medium buffering capability, and various operation conditions. Some researchers used the mathematical models narrowed the range to 5.5-5.7. In continuous experiments, to achieve the maximum hydrogen yield or hydrogen production rate, pH was suggested to control between 5.2 and 6.0 (Fang and Liu 2002b, Oh *et al.* 2003a, Hwang *et al.* 2004, Li and Fang 2007b, Lin *et al.* 2007b, Liu *et al.* 2011). In addition, pH 4.0 was regarded as the operational limit for the anaerobic biohydrogen production process since the activity of all the microorganisms was inhibited below pH 4.0. Ginkel *et al.* (2001) used fractional factorial design batch experiments to test the effect of varying pH (4.5-7.5) on hydrogen production from a sucrose rich synthetic wastewater. Heating pretreatment was applied to the mixed bacterial cultures obtained from a compost pile, a potato field, and a soybean field to inhibit hydrogen-consuming methanogens. It was determined that the highest hydrogen production rate of 74.7 mL H₂/L/h occurred at a pH of 5.5 and a substrate concentration of 7.5 g COD/L with a conversion efficiency of 38.9 mL-H₂/g-COD/L.

VFAs including acetate, butyrate, propionate, and ethanol that are the major products by anaerobic fermentation from carbohydrates, are varied as pH. An ethanol-acetate fermentation at pH of 5.0 ± 0.2 and HRT of 3 days was studied (Hwang *et al.* 2004). The change in the operational pH induced a change in the main fermentation pathway, where butyrate, ethanol and propionate were the main products in different pH ranges of 4.0-4.5, 4.5-5.0 and 5.0-6.0, respectively. Fang *et al.* (2002b) also studied the distribution of the key VFA and alcohols in the effluents at various pH. Butyrate and acetate were the two most abundant species in the effluent. Increase of pH from 4.0 to 7.0 resulted in the decrease of butyrate but in the increase of acetate. At pH 4.0 to 6.0, the effluent contained more butyrate than acetate. At pH 6.5 and 7.0, acetate and butyrate became about equally abundant.

2.2.5.2 Temperature

Fermentative reactions can be operated at mesophilic (25-40 °C), thermophilic (40-65 °C) or hyperthermophilic (>80 °C) temperatures by various bacteria with different characteristics. Mesophilic conditions are more favorably in most of studies (Li and Fang 2007b, Wang and Wan 2009, Sinha and Pandey 2011). Effect of temperature on H₂ production is explained thermodynamically by considering the changes in Gibb's free energy and in standard enthalpy. The Van't Hoff equation can be used to explain the effect of the temperature on equilibrium constant. In the selected glucose glycolysis reaction, acetate, carbon dioxide and hydrogen are converted from glucose. With the positive enthalpy in the reaction, the equilibrium kinetic constant will increase with the increase in temperature (Eq. 2.17-2.20), which demonstrates the hydrogen production enhancement.

$$K = \frac{[H_2]^4 [CO_2]^2 [CH_3COOH]^2}{[C_6H_{12}O_6]}
 \tag{2.18}$$

$$\ln \frac{K_1}{K_2} = -\frac{\Delta H^0}{R} \left[\frac{1}{T_1} - \frac{1}{T_2} \right] \quad (2.19)$$

$$\frac{K_1}{K_2} = \frac{[H_2]_1^4 [CO_2]_1^2 [CH_3COOH]_1^2}{[H_2]_2^4 [CO_2]_2^2 [CH_3COOH]_2^2} \quad (2.20)$$

Zhang *et al.* (2006) investigated the effects of culture temperature on biological hydrogen production from sucrose by anaerobic mixed culture. In the range of 25-40 °C, hydrogen production increased with culture temperature increase, however, the hydrogen production was almost inhibited when the temperature was higher than 45 °C. Temperature effects on H₂ production of a novel carrier-induced granular sludge bed reactor fed with sucrose-based synthetic wastewater by mixed culture from the sludge were investigated (Lee *et al.* 2006). At 40 °C the overall maximum hydrogen production rate and yield were observed of 7.66 L/h/L and 3.88 mol H₂/mol sucrose, respectively. Specific H₂ production rate increased with increasing temperature, while the biomass content tended to decrease.

2.2.5.3 Metal ions

Essential micronutrients such as trace metals Fe, Ni, Mg or Zn are important for bacterial growth and fermentation metabolism for hydrogen production. Iron and nickel are important nutrient elements to form the enzymes of [Fe-Fe] hydrogenases and [Ni-Fe] hydrogenases (Dabrock *et al.* 1992, Lee *et al.* 2001, Lacey *et al.* 2005, Wang *et al.* 2007, Alshiyab *et al.* 2008, Kim *et al.* 2008b, Wang and Wan 2008, Zhao *et al.* 2012). Magnesium also functions as cofactor of enzymes and is one of the constituents of cell walls and membranes, which plays a very crucial role of activator of many kinases and synthetases (Mandal and Mandal 1998, Wang *et al.* 2007). Other heavy metals such as Cr, Cu, Cd, Pb and Zn also influence the biological processes most because of their toxicity (Lin 1993, Li and Fang 2007a, Lin and Shei 2008).

Zhao *et al.* (2012) investigated the effects of Fe²⁺, Mg²⁺ ions on hydrogen production from glucose and cell growth of *C. beijerinckii* RZF-1108 in dark fermentation. The hydrogen yield slightly increased from 1.28 to 1.50 mol H₂/mol glucose when the Fe²⁺ concentration increased from 0 to 0.2 g/L, and then decreased

with Fe^{2+} concentration further increasing. On the other side, hydrogen yield decreased significantly when the Mg^{2+} concentration increased from 0.1 to 1.0 g/L. A maximum hydrogen yield of 1.96 mol H_2 /mol glucose and production rate of 106.0 mL- H_2 /L-medium/h were obtained using optimized culture medium by adding 0.2 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$.

Li *et al.* (2007a) studied the toxicity effects of six metal ions on the hydrogen productivity of mixed cultures in granular sludge. The relative toxicity was found in the decreasing sequence of Cu (most toxic) $\gg$ Ni $\sim$ Zn > Cr > Cd > Pb (least toxic). It was also concluded that H_2 -producing sludge exhibited higher resistance to metal toxicity than methanogenic granular sludge.

2.2.5.4 Light

Light is an important factor that affects hydrogen production in photo fermentation and integrated dark and photo fermentation systems from various aspects such as light source, intensity, and illumination protocols (Zurrer and Bachofen 1979, Tao 2008, Ozmihci and Kargi 2010b). Uyar *et al.* (2007) reported that light intensity and light-dark cycles were two key parameters. Hydrogen productivity of *R. sphaeroides* O.U. 001 was investigated with 2 mM sodium glutamate and 15 mM malate using 150 W tungsten lamps. Light conversion efficiency decreased from 1.11% to 0.25% as light intensity increased from 88 to 405 W/m^2 . The hydrogen production by photosynthetic bacteria ceased in dark. It was also concluded that the infrared region (750-950 nm) of light spectrum where the bacteriochlorophyll *a* absorption maxima exist was very important for hydrogen production, whereas the left part of spectrum was not significantly effective where the carotenoid absorption maxima exist.

Argun *et al.* (2010b) studied the effects of the light source, intensity and lighting regime on bio-hydrogen production in combined dark and photo fermentations. A mixture of heating treated anaerobic sludge and *R. sphaeroides*-RV were used to converted ground wheat starch to hydrogen. Tungsten, fluorescent, infrared (IR), tungsten + infrared, halogen lamps were selected as light sources with a light intensity of 270 W/m^2 along with sunlight. Halogen lamp was found to be the most

suitable light source with the highest cumulative hydrogen production of 178 mL and yield of 218 mL/g starch. Light intensities of 1-10 klux of halogen were tested to conclude that hydrogen production was limited at low light intensities below 10 klux. In addition, hydrogen production increased with increasing dark/light cycle time and continuous lighting resulted in the highest cumulative hydrogen production volume and hydrogen yield.

2.2.5.5 Hydrogen partial pressure

Since hydrogen evolution pathways are sensitive to H₂ concentrations and are subject to end-product inhibition, hydrogen partial pressure is an important factor to be considered. As hydrogen concentration increases, H₂ synthesis decreases and metabolic pathways shift towards production of more reduced substrates, such as lactate, ethanol, acetone, butanol or alanine (Yerushalmi *et al.* 1985, Hawkes *et al.* 2002, Levin 2004). The acetate conversion fermentation is also strongly determined by the hydrogen partial pressure (Nath and Das 2004a). Continuous hydrogen synthesis requires a hydrogen partial pressure of 50 kPa, 20 kPa and 2 kPa, at different temperatures of 60 °C, 70 °C, and 98 °C respectively (van Niel *et al.* 2003).

Mandal *et al.* (2006) investigated the effect of hydrogen partial pressure on hydrogen production by *E. cloacae* from glucose. As the partial pressure of H₂ was decreased by lowering the total pressure in the headspace of the reactor from 760 mm Hg to 380 mm Hg, the hydrogen yield increased from 1.9 to 3.9 mol H₂/mol glucose. Besides, the maximum production rate of 0.017 mmol H₂/L/h was at 380 mm Hg.

Gas sparging has been found to be a useful technique to reduce hydrogen partial pressure in the liquid phase for enhancement of hydrogen yield (Mizuno *et al.* 2000, Kim *et al.* 2006a). Kim *et al.* (2006a) investigated the effects of gas sparging on continuous fermentative H₂ production in completely stirred-tank reactors using internal biogas, and external gasses of N₂ and CO₂. The sparging with external gases of N₂ and CO₂ showed higher H₂ yield than the control of no sparging or internal biogas sparging. CO₂ sparging was especially more effective in the reactor

performance than N₂ sparging, accompanied by higher production of H₂ and butyrate. In addition, high CO₂ partial pressure had inhibitory effect on other microorganisms such as acetogens and lactic acid bacteria that were competitive with H₂-producing bacteria.

2.2.5.6 Hydraulic retention time (HRT)

Hydraulic retention time (HRT) has a pronounced effect on metabolic balance, which can also be used to select microbial populations due to the different growth rates. Typically, the specific growth rate of hydrogen-producing bacteria is about 0.172/h (Chen *et al.* 2001). Lay *et al.* (1999) studied the hydrogen productivity from organic municipal solid waste by two seed microorganisms, namely heat-pretreated digested sludge and hydrogen-producing bacteria enriched from soybean-meal silo. The results showed hydrogen production began immediately after a lag-period of 2.2 days and reached a maximum yield of 230 mL on day 3.2. Butyrate production began on day 1.8 along with hydrogen production, and continued until day 3.2 with a yield of 2.81 g/L when hydrogen production stopped. At that moment, the formation of acetate and propionate were 0.97 and 0.61 g/L, respectively. Propanol and butanol formation began on day 2.8 and 3.2, while maximum concentrations of 3.75 and 0.46 g/L were obtained on day 4, respectively.

Moreover, HRT is a key parameter considered in bioreactor design and optimization study for hydrogen production (Ueno *et al.* 1996, Yu *et al.* 2002, Oh *et al.* 2004a, Aguilar *et al.* 2013). Wu *et al.* (2008) operated a CSTR to study the effect of HRT on fermentative hydrogen production. The hydrogen production rate increased with a decrease of HRT from 12 to 6 h, giving an optimal rate of 0.6 L/h/L at HRT 6 h. However, when the HRT was further shortened to 4 h, both hydrogen production rate and hydrogen yield decreased significantly, which was mainly attributed to the marked biomass decrease in the reactor because of severe cell washout. Therefore a high substrate-feeding rate was applied to improve cell retention.

2.2.5.7 Metabolic engineering application

Although the above operation parameters could be adjusted and optimized, metabolic pathways involving the bacteria are diverse except the hydrogen production. Therefore, metabolic engineering which can modify the microorganisms in order to change and improve the existing metabolic pathway could help increase hydrogen production. As well, genomic study will provide great potential for biological hydrogen production breakthrough. The application of

Molecular biological methods in environment engineering area have facilitated the microbes characterizing in engineered and natural environments, production of biofuels enhancement, and also optimizing biological processes to improve the performance of biological treatment.

Hellenbeck (2012) reviewed the latest metabolic engineering strategies applied in dark fermentative bacteria: 1) eliminating up-taken hydrogenase; for example, in one case elimination of *hyd1* and *hyd2* gave a 37% increase in H_2 yield compared to the wild type strain (Bisaillon *et al.* 2006). 2) inactivating competing pathways. The most obvious mutation to introduce is to inactivate *ldhA*, lactate dehydrogenase, since normally this potentially could drain the pyruvate pool which otherwise could be used to make formate, and in turn H_2 . 3) Introducing new enzymes and/or pathways to overcome thermodynamic/metabolic barriers to increasing hydrogen yields. Rollin *et al.* (2015) presented a model of hydrogen production from corn stover via in vitro metabolic engineering. There were 11 enzymes utilized in glucose 6-phosphate (G6P) generation and hydrogen production pathway. Hydrogenase for hydrogen production was isolated from *Pyrococcus furiosus*. Results showed hydrogen yield of 12 mol H_2 /mol glucose and 10 mol H_2 /mol xylose, which were the maximum possible yields. Hydrogen production rate was enhanced to 54 mmol H_2 /L/H after increasing reaction temperature, substrate (100 mM G6P) and enzyme concentrations.

Gene expression that is linked to microbial functions for incorporation into predictive fate and transport models is important to be studied. High throughput sequencing of the transcriptome (RNAseq) has the potential to transform gene

structure and expression study. RNAseq could easily investigate mRNA transcripts, identify novel exons and splice sites and quantify gene expression differences among samples. RNAseq is sensitive and has highly reproducible sequencing data, while increasing the number of biological replicates could improve the precision and generalizability. Wei *et al.* (2014) compare the transcriptional profiles of *Clostridium thermocellum* grown on cellobiose and pretreated yellow poplar by using RNAseq technology. Results showed that *C. thermocellum* fermentation of pretreated yellow poplar (PYP) produced 30 and 39% of ethanol and hydrogen product concentrations, respectively, compared to fermentation of cellobiose. From the transcriptional profiles investigation, the PYP-grown cells showed 1211 genes up-regulated and 314 down-regulated by more than two-fold compared to the cellobiose-grown cells. Finally, the up-regulated genes for cells growing on PYP compared to cellobiose contained 47 cellulosomal protein-encoding genes, genes for 4 pairs of SigI-RsgI for polysaccharide sensing, 7 cellodextrin ABC transporter genes, and a set of NAD(P)H hydrogenase and alcohol dehydrogenase genes, which were targeted for further regulatory mechanism study. Qin *et al.* (2015) studied the different transcriptome profile of *Bacillus coagulans* 2-6 response to sodium lactate and calcium lactate during lactic acid production. The enhancement of lactate production varied when adding calcium carbonate or sodium hydroxide during fermentation. By investigating gene expression changes in *B. coagulans* 2-6 cultured in non-stress, sodium lactate stress and calcium lactate stress condition, it was found that lactate fermentation was less affected by calcium lactate stress than by sodium lactate stress. Respectively, 712 and 1213 significantly regulated genes in response to calcium lactate stress and sodium lactate stress were identified. ATP-binding cassette transporters and amino sugar and nucleotide sugar metabolism were significantly affected by calcium lactate stress and sodium lactate stress, separately. Sodium lactate stress had negative effect on the expression of ‘glycolysis/gluconeogenesis’ genes but positive effect on the expression of ‘citrate cycle (TCA cycle)’ genes. However, calcium lactate stress had the opposed effects.

2.2.6 Reactor types

Various bioreactors are studied and investigated for biohydrogen production. The requirements for dark fermentation and photo fermentation systems are different. Combination systems are also a challenge to cultivate dark and photo fermentative bacteria together. From the practical engineering standpoint, only continuous hydrogen production bioreactors are discussed.

2.2.6.1 Bioreactors for dark fermentation

Reactors for continuous or fed-batch dark fermentative hydrogen production include continuous stirred tank reactor (CSTR) (Hussy *et al.* 2005), anaerobic sequencing batch reactor (ASBR) (Vijaya Bhaskar *et al.* 2008, Buitrón and Carvajal 2010), membrane bioreactor (MBR) (Oh *et al.* 2004a), fixed-bed bioreactor or packed-bed reactor (Chang *et al.* 2002, Oh *et al.* 2004b), fluidized-bed bioreactor, and upflow anaerobic sludge blanket (UASB) bioreactor (Wang *et al.* 2010b) have been developed with high production yields and output.

Continuous stirred tank reactors (CSTR)

Continuous stirred tank reactors (CSTR) are commonly used for continuous hydrogen production. In a CSTR, hydrogen-producing microbes are completely mixed and suspended in the reactor liquor from the mixing pattern, which results in a good substrate-microbes contact and mass transfer. However, CSTR has the drawback that is unable to maintain high levels of fermentative biomass because of the rapidly mixed operating pattern. Hydrogen production rates are considerably restricted because of biomass washout at short HRTs. The highest reported CSTR hydrogen production rate was 1.12 L/h/L in fermentation of sucrose with a mixed hydrogen-producing culture (Mizuno *et al.* 2000, Show *et al.* 2007, Wu *et al.* 2008, Aguilar *et al.* 2013).

Anaerobic sequencing batch reactor (ASBR)

Buitron *et al.* (2010) studied the biohydrogen production from Tequila vinasses in an anaerobic sequencing batch reactor. Hydrogen was produced at longer HRT of 24 h but neglectable at HRT of 12 h. A maximum volumetric hydrogen production

rate of 50.5 mL H₂/h/L was obtained when the reactor was fed with 3 g COD/L at 35 °C and 12 h HRT.

Membrane bioreactor (MBR)

MBRs were reported with hydrogen production rates in a range of 0.25-0.69 L/h/L. Increasing the sludge retention time could further enhance biomass retention that favored substrate utilization, but resulted in a decrease in hydrogen production rate. Membrane fouling and high operating cost also limited the use of MBR process in bio-hydrogen fermentation (Li and Fang 2007b, Show *et al.* 2011).

Fixed-bed bioreactor

A fixed-bed bioreactor is operated with support carriers packed within the tank. The hydraulic mixing regime is less turbulent comparing with the CSTR, which results in higher mass transfer resistance along with lower rates of substrate conversion and hydrogen production (Lo *et al.* 2009). High hydrogen yield could not be maintained consistently in a fixed-bed reactor, because pH gradient distribution along the reactor column caused a heterogeneous distribution of microbial activity. Recirculation flow was recommended to overcome the mass transfer resistance and pH heterogeneous distribution. In addition, support carriers play an important role in biomass retention and hydrogen production in fixed-bed reactors. Activated carbon was considered as a better choice for support carriers (Lee *et al.* 2006).

Fluidized-bed bioreactor

An efficient fluidized-bed bioreactor reported by (Zhang *et al.* 2007b) presented a hydrogen production rate of 2.36 L/L/h and a specific hydrogen production rate of 4.34 mmol H₂/g-VSS/h, which were linearly correlated to the effective loading rate. It was also observed that retained biomass helped the reactor run at a high organic loading rate and enhance hydrogen production.

Upflow anaerobic sludge blanket reactor (UASBr)

UASB bioreactors were investigated with good treatment efficiency and capability in retaining high biomass concentration (Chang and Lin 2004, Lo *et al.* 2009). A

continuous UASB reactor applied to produce hydrogen from coffee drink manufacturing wastewater was reported (Jung *et al.* 2010). The maximum H₂ yield of was 1.29 mol H₂/mol hexose achieved at HRT of 6 h in UASBr operation with the dominant acids of butyric and caproic acids.

2.2.6.2 Photo-bioreactors for photo fermentation

Bioreactors used in photo fermentation have more restrict requirements than those in dark fermentation because of the light dependent characteristics. For efficient biohydrogen production, it is critical to dilute the light and distribute it over the reactor volume, but also mix the culture at high rates so that cells are exposed to the light only for a short time. Trapping and removal of hydrogen gas in the system are also important design considerations for photo-bioreactors (Evens *et al.* 2000, Akkerman *et al.* 2002). Photo-bioreactors of different geometries have been reported for biohydrogen production: tubular, flat plate, fermentor types etc., while airlift, helical tubular and flat plate reactors are found most suitable with respect to biomass production (Kaidi *et al.* 2012, Adessi and De Philippis 2014, Zhang *et al.* 2014b). These bioreactors may be employed for hydrogen production with necessary modifications to overcome the existing bottlenecks like gas hold up, oxygen toxicity and poor agitation (Uyar *et al.* 2007).

A flat panel reactor and a tubular reactor were setup under outdoor conditions and evaluated the hydrogen productivity of *Rhodococcus capsulatus* using a nitrogen source of sodium glutamate and carbon sources of acetate and lactate (Gebicki *et al.* 2009). Both the panel and tubular reactor showed comparable productivity per illuminated surface area of 1250 and 1100 mL H₂/m²/d. It suggested the design of a bioreactor should aim to a maximum ratio of illuminated surface area to land space covered by the reactors, which in the panel reactor was 8.5 times higher than in the tubular system.

Fermentor-type reactors are widely used in the optimization studies, but their scaling up may be limited due to low surface area to volume ratio (Adessi and De Philippis 2014). Agitation is provided with the help of impellers or magnetic stirrers. Some photo-bioreactors can be internally illuminated with fluorescent

lamps, which have the advantages: heat-sterilizing under pressure to minimize contamination, maintaining supply of light to the photo-bioreactor continuously (both day and night) by integrating artificial and solar light devices (Ugwu *et al.* 2008, Eroglu and Melis 2011, Prachanurak *et al.* 2014)

2.2.6.3 Hybrid bioreactors for integrated dark and photo fermentations

As the basic principle of the sequential two-stage bioreactors is that dark-fermentation of substrate conversion to hydrogen and organic acids takes place in a conventional reactor in the first stage of process, and additional gaseous energy from organic acids is extracted in the second stage. In optimizing gas production, a different reactor for the second stage operated under different conditions (such as higher pH and longer HRT) is applied (Hwang *et al.* 2011). Bioreactors for sequential systems seem much easier than combined system, where dark fermentation and photo fermentation take place in separate reactors. Ozgur *et al.* (2010) studied hydrogen productivity by a sequential dark and photo fermentation system using sugar beet molasses as a feedstock. The sequential bioreactor was consisted of a pH-controlled jacketed 2 L bioreactor with a working volume of 1 L for dark fermentation, followed by glass bottles with 55 mL liquid volume for photo fermentation. Dark fermentation effluents were centrifuged and sterilized by autoclaving prior to use, as well as applied several adjustments to optimize the conditions for following photo fermentative hydrogen production. The overall hydrogen yield from sucrose increased from the maximum of 4.2 mol H₂/mol sucrose in dark fermentation to 13.7 mol H₂/mol sucrose by sequential dark and photo fermentation.

Limited studies have been investigated the hybrid reactors for combined dark and photo fermentation system. Argun *et al.* (2010a) used a 7.63 L annular hybrid bioreactor (AHB) for hydrogen production from ground wheat starch by *C. beijerinckii* and *R. sphaeroides* RV. This annular hybrid bioreactor was a novel design consisting of two coaxial cylindrical reactors with internal circulation between them. Effects of hydraulic residence time on hydrogen production rate and yield were investigated. Hydrolysis of starch and fermentation of glucose to organic acids were HRT-independent, while conversion of VFAs to H₂ and CO₂ by photo-

fermentation was slow. Besides, specific and volumetric hydrogen production rates decreased with increasing HRT. The highest hydrogen yield was 90 mL H₂/g starch at HRT of 6 days. HRT longer than 5 days was required for effective hydrogen production in AHB.

2.3 Food Waste to Energy

Simple sugars such as glucose, sucrose and lactose are preferred substrates for hydrogen production. However, pure carbohydrate sources are expensive raw materials for hydrogen production. H₂ production from organic wastes is more desirable as it is feasible process for industrialization to realize the goal of waste reduction and energy production. Food waste (FW) with its high content of organic matter and carbohydrates, and its easily hydrolysable and biodegradable nature has a high hydrogen production potential (Kim *et al.* 2004, Kapdan and Kargi 2006).

Food waste, as an important municipal and agricultural waste, can be an economical source for fermentative hydrogen production. Food waste includes uneaten food and food preparation leftovers from residences, commercial establishments such as restaurants, institutions like school cafeterias, and industrial sources like factory lunch-rooms. Although food waste can be highly variable depending on their resources, generally they consist mainly of starch, protein, and fat, with a small amount of cellulose and hemi-cellulose. Food wastes have the characteristics of moisture contents of 74-90%, carbon to nitrogen ratios (C/N) of 14.7-36.4, and volatile solids to total solids ratios (VS/TS) of 80-97%. These volatile solids are very biodegradable, of which about 80-90% can be converted to biogas under mesophilic conditions (Han and Shin 2004a, Zhang *et al.* 2007a, Zhu *et al.* 2008).

On the other hand, currently, recycled efficiency for food waste is quite low, while most of food wastes are disposal directly to landfills or incineration plants. The disposal of biodegradable waste to landfill leads to the formation of landfill gas and leachate that can result in adverse environmental impacts (Cantrell *et al.* 2008, Guo *et al.* 2010, Iacovidou *et al.* 2012). The operation efficiency of incineration plants decreases because of the moisture from collected food wastes. Biohydrogen

production from food waste can both recycle the energy but also relieve the burden of waste treatments.

A variety of substrates can be used for bio-hydrogen production not only simple sugars (glucose or sucrose, etc.) but also the complex polysaccharides and organic acids (cellulose or VFAs, etc.) (Kumar and Das 2000, Barbosa *et al.* 2001, Tao 2008, Sun *et al.* 2010). Besides the pure substrate, organic wastes like wastewater or food waste are being investigated for hydrogen production in recent years (Kim *et al.* 2006b, Hsiao *et al.* 2009, Argun and Kargi 2010a).

Bacteria have the substrate selectivity and perform differently for hydrogen production. Glucose and sucrose are most widely used and easily assimilable substrates for fermentative hydrogen production, while some compounds such as cellulose are required to hydrolyze to monosaccharides first. Lo *et al.* (2008) evaluated the hydrogen production by seven bacteria from xylose and sucrose. All the seven pure bacteria (*C. butyricum* CGS2, *C. butyricum* CGS5, *C. pasteurianum* CH1, *C. pasteurianum* CH4, *C. pasteurianum* CH5, *C. pasteurianum* CH7, and *Klebsiella* sp.) were able to utilize sucrose for cell growth and H₂ production. However, xylose could be only used by *C. butyricum* CGS2, *C. butyricum* CGS5, and *Klebsiella* sp. Among the seven strains, *C. pasteurianum* CH4 exhibited the best H₂-producing performance with H₂ production rate and yield of 512 mL/h/L and 1.58 mol H₂/mol hexose from sucrose. Ueno *et al.* (1995) obtained the hydrogen yield of 2.4 mol H₂/mol hexose from cellulose although a relatively longer fermentation time was required.

Furthermore, substrate concentration is also important to the hydrogen production performance that in an appropriate range increasing substrate concentration can increase the ability of hydrogen-producing bacteria to produce more hydrogen, but at much higher concentration levels substrate toxicity occurs and results in the inhibition of bacteria growth and hydrogen production (Zhang *et al.* 2006, Lo *et al.* 2008). Lo *et al.* (2008) concluded the best sucrose concentration was 40 g COD/L for *C. pasteurianum* CH4 to obtain the highest H₂ production rate (569 mL/h/L) and yield (4.14 mol H₂/mol sucrose or 2.07 mol H₂/mol hexose). However the optimal concentration for *C. butyricum* CGS5 was 20 g COD/L xylose. Oh *et al.* (2003a)

observed the increase of glucose concentration (1-20 g/L) in the culture medium resulted in higher H₂ production, but glucose utilization efficacy (mol H₂/mol glucose) gradually decreased as glucose concentration increased.

2.3.1 Pretreatment of food waste

Since hydrolysis of food waste is considered as the rate-limiting step in the overall anaerobic fermentation process, to enhance the hydrogen production, various pretreatment methods of substrate have been used to release the organics to liquid as well as to suppress hydrogen-consuming microbial activity. The studied pretreatment methods mainly include thermal pretreatment, alkaline pretreatment, acidification, ultrasonic pretreatment, freezing and thawing, chloroform, etc. (Penaud *et al.* 1999, Cai *et al.* 2004, Baghchehsaraee *et al.* 2008, Elbeshbishy *et al.* 2011, Elbeshbishy *et al.* 2012). Different pretreatment processes have different property, and their impacts on biohydrogen production from various substrates and with different cultures also vary much. Therefore many researchers did the study to get a better pretreatment for the specific conditions.

Elbeshbishy *et al.* (2011) evaluated the effects of seven single and combined pretreatment methods applied on food wastes for hydrogen production. Four individual pretreatment methods were ultrasonication, heat, acid, and base; while three combined pretreatment methods were comprised ultrasonication with heat (UH), ultrasonication with acid (UA), and ultrasonication with base (UB). Food wastes used in this study had the average characteristics were (mg/L): total chemical oxygen demand (TCOD): 91900, soluble chemical oxygen demand (SCOD): 49900, total solids (TS): 65500, volatile solids (VS): 46100, particulate carbohydrate: 26500, soluble carbohydrate: 20000, particulate protein: 6250, and soluble protein: 8710. The volatile fatty acids (VFAs) concentration was 1990 mg COD/L. In the results, a hydrogen yield of 42 mL/g VS_{initial} was observed for the untreated food waste. The seven pretreatment methods enhanced the hydrogen productivity in different degrees. Among the four individual pretreatment methods, ultrasonic pretreatment produced the highest hydrogen yield of 97 mL/g VS_{initial}, while base pretreatment had the lowest hydrogen yield of 46 mL/g VS_{initial}. Ultrasonication had a positive enhancement with acid pretreatment, but a negative

effect on base and heat pretreatments on hydrogen production. The solubility of chemical oxygen demand and protein was enhanced most by UB pretreatment, while the highest increase in soluble carbohydrate was observed for UA pretreatment. Meanwhile, the VFAs concentration in the effluents with UA pretreatment was most, and that with base pretreatment was least of 9700 mg COD/L.

2.3.2 Biohydrogen production from food waste

Biohydrogen production from food waste has been proved to be feasible and promising although some limitations exist (Han and Shin 2004a, Han and Shin 2004b, Shin and Youn 2005). Therefore, further studies and efforts are needed in this area. Kim *et al.* (2006c) tested the possibility and the feasibility of hydrogen production from food waste by fermentation. Heat-treated sludge was fed in a reactor with 5 L working volume. The experimental results showed that hydrogen production could be possible from food waste but with a relatively low hydrogen production rate. Besides, COD concentrations were very high due to slowly biodegradable matters. Further study to maximize hydrogen production by optimizing operational parameters such as HRT, temperature, pH and agitation speed and a two-stage process were suggested.

Redondas *et al.* (2012) evaluated the dark fermentation from food wastes using a completely stirred tank reactor at different hydraulic retention times. The results obtained demonstrate that the H₂ yields from the fermentation of food wastes were affected by HRTs due to the incomplete hydrolysis. The decrease in the duration of fermentation had a negative effect on the conversion of the substrate into soluble products. This resulted in a lower amount of soluble substrate being available for metabolism by H₂ producing bacteria leading to a reduction in specific H₂ production. The highest hydrogen yield and specific hydrogen production were 0.6 L H₂/L/d and 20.58 L H₂/kg VSS_{added}, respectively.

Due to the lack of nitrogen in food wastes, the concept of co-digestion of FW and sewage sludge (primary sludge and waste activated sludge) has been investigated to improve biohydrogen production, since the addition of sludge to FW supplied a

more balanced carbon to nitrogen (C/N) ratio (Zhu *et al.* 2008, Sreela-or *et al.* 2011, Tawfik and El-Qelish 2012, Kim *et al.* 2012b). Zhou *et al.* (2013) investigated hydrogen production in batch anaerobic co-digestion system using mixtures of food waste (FW), primary sludge (PS), and waste activated sludge (WAS) in different volumetric ratios at 37 °C and an initial pH of 5.5 ± 0.2 . The results showed that co-digestion of FW and sludge had a positive impact on the hydrogen production. The maximum hydrogen yields by co-digestion of FW + PS, FW + WAS, and FW + PS + WAS were achieved at volumetric ratios of 75:25, 75:25, and 80:15:5, respectively. The highest cumulative hydrogen production rate of 11 mL/h was achieved by M2 (90% FW+10% PS) and M3 (75% FW + 25% PS), 13.5 mL/h was achieved by M7 (90% FW + 10% WAS), and 12.7 mL/h was achieved by M13 (80% FW + 15% PS + 5% WAS). Besides, the mixtures with low concentration of food waste produced minimal hydrogen. The synergistic effect of co-digestion was proven that the measured hydrogen productions were higher than the sums of the hydrogen productions calculated from each fraction.

2.5 Summary

Biohydrogen production has been proved a promising method for the future energy. Various bacteria are found to be capable of hydrogen production from water and many carbohydrates. Integrated dark and photo fermentation systems can maximize the substrate utilization and enhance the hydrogen production by individual process. However, the hydrogen production rate needs to be improved. The optimal pairs or mixtures of inoculum and the efficient hybrid bioreactors are two challenges for the integration systems. Optimal conditions such as pH, temperature or HRT need to be investigated more.

To make the biohydrogen technologies more feasible and development, food waste is a good choice as substrate. Pretreatment of food waste is required to reduce the hydrogen-consuming microorganisms and increase the hydrolysis of the solid wastes. In practice operation, co-digestion is considered and studied for the supplement of nutrients to food waste fermentation. In this respect, hydrogen production from food waste using the integration systems is a promising method but requires further and more detailed studies. In addition, molecular biological

methods application in environment engineering area have promising positive effect on characterizing the functional capacity of microbial communities, and also optimizing biological processes to improve the bioreactor performance.

Chapter 3 Comparative Study of Dark Fermentative

Bacteria

3.1 Introduction

Dark fermentation is an efficient biological process for hydrogen production with its obvious advantages: a) hydrogen is produced at higher rate in the absence of light source and b) it can use various organic waste and wastewater enriched with carbohydrates as the substrate. Among the dark fermentative hydrogen producers, *Clostridium* sp. and *Enterobacter* sp. have attracted more attention due to their high growth rate (Lee *et al.* 2011, Zhang *et al.* 2011).

In this study, hydrogen production potential of four dark fermentative bacteria (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii*, and *E. aerogenes*) using glucose as substrate was investigated under anaerobic conditions. Batch experiments were conducted to study the effects of initial glucose concentration on glucose utilization efficiency, cumulative hydrogen production, hydrogen yield, hydrogen production rate, specific hydrogen production rate, concentration and composition of volatile fatty acids (VFAs) in the effluents.

3.2 Materials and Methods

3.2.1 Bacteria strains and medium

C. butyricum DSM 10702, *C. pasteurianum* DSM 525, *C. beijerinckii* DSM 791 and *E. aerogenes* DSM 30053 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in lyophilized form. The medium (RCM) used to cultivate the four species consisted of 3 g/L yeast extract, 10 g/L 'Lab-Lemco' powder (beef extract), 10 g/L peptone, 5 g/L glucose, 1 g/L soluble starch, 5 g/L sodium chloride, 3 g/L sodium acetate, 0.5 g/L cysteine hydrochloride. The medium was adjusted at pH 6.8 ± 0.2 . *Clostridium* species and *E. aerogenes* were cultivated in incubators at 37 °C and 30 °C, respectively.

3.2.2 Batch fermentation experiments

The dark fermentation batch experiments were performed in 60 mL serum bottles that were purged with nitrogen and then filled with 30 mL medium. The bottles were sealed with both butyl rubber stoppers and aluminum crimp caps. The medium was heated until boiling, purged with nitrogen to maintain anaerobic conditions and sterilized at 121 °C for 15 min. When the cells entered mid-exponential growth phase, the inoculants were injected into serum bottles by 3 mL syringes (10% v/v). Afterward, the bottles were placed in two shaking incubators operating at 150 rpm at 37 °C for *Clostridium* species and 30 °C for *E. aerogenes*, respectively. The initial glucose concentrations were ranged from 5 to 20 g/L. The batch experiments were conducted in triplicate and lasted for 72 h.

3.2.3 Analytical methods

Gas products (H₂ and CO₂) were analyzed by gas chromatography equipped with a thermal conductivity detector, GC-TCD-TCD (Agilent, USA). Argon and helium were used as carrier gases. Columns used in the analysis were molecular sieve 13X and molecular sieve 5A. Oven and detector temperatures were 115 °C and 150 °C, respectively. Gas production was measured periodically by using a 100 mL glass syringe (SAMCO, UK). The cumulative H₂ volume was calculated from the H₂ percentage and total volume of the biogas produced.

Liquid samples (0.1 mL) were taken with 1 mL conventional plastic syringe to measure glucose concentration, which was quantified by the phenol-sulfuric acid method and detected by UV-spectrophotometer (Agilent Cary 50, USA). The volatile fatty acids (VFAs) in the supernatant of the culture broth were determined using gas chromatography with flame ionization detector, GC-FID (Agilent 7890A, USA). The liquid samples were filtered through a 0.45 µm nylon membrane before free acids were analyzed. Nitrogen was used as carrier gas. Biomass concentrations in the experimental bottles were measured as cell dry weight (CDW) according to Standard Methods by filtering sample through a 0.45 µm Millipore filter paper, drying overnight at 105 °C, cool to room temperature in a desiccator jar, and determining the constant dry weight (Eaton and Franson 2005) .

3.3 Results and Discussion

3.3.1 Glucose consumption and utilization efficiency

Figure 3.1 shows glucose consumption and utilization efficiencies by the four dark fermentative bacteria at different initial glucose concentration levels (5, 10, 15, 20 g/L). Glucose was the sole carbon source during the dark fermentation process. The glucose utilization efficiency (amount of substrate utilized / total amount of substrate supplied) was calculated according to the remaining amount of glucose in the solution. All the bacteria consumed more glucose as initial glucose concentration increased, but glucose utilization efficiency varied among the strains. It was observed that glucose utilization efficiency increased with the increase of initial glucose concentration by *E. aerogenes* and *C. beijerinckii*. On the contrary, *C. butyricum* and *C. pasteurianum* presented different trends. Among the fermentative bacteria, *E. aerogenes* had the higher glucose utilization efficiency (more than 85%). The difference on substrate utilization among these four bacteria was mainly because: 1) Nutrient requirements varied with respect to the diverse metabolic conditions of different bacteria. 2) Some components of the nutrients might be used up and became the limiting factors of multiplication, although the substrate (glucose) was not yet completely exhausted (Málek and Fenel 2013). There was a trace amount of increase in glucose consumption for *C. butyricum* and *C. pasteurianum* at the initial glucose concentration above 10 g/L, as a result their glucose utilization efficiency decreased sharply. 3) The metabolic pathway for hydrogen production might change within the same bacterium dependent on initial glucose concentrations, therefore, the maximum substrate consumption was affected correspondingly (Oh *et al.* 2003a, Cai *et al.* 2010). Similar results have been reported regarding the initial glucose concentration about the consumption (Liu *et al.* 2009b).

Figure 3.1 Glucose consumption and utilization efficiencies (A) 5 g/L glucose; (B) 10 g/L glucose; (C) 15 g/L glucose; (D) 20 g/L glucose.

3.3.2 Effect of initial glucose concentration on cumulative hydrogen production and hydrogen yield

Effect of initial glucose concentration (5, 10, 15, 20 g/L) on biohydrogen production was also studied (Figure 3.2). During the early stage, the initial glucose concentrations had less impact on hydrogen production (adaptation period). After that, the hydrogen production increased rapidly during the first 20 h. It was observed that at low glucose concentration (5 g/L), the cumulative hydrogen production was around 30 mL for *Clostridium* sp., while *E. aerogenes* produced less than 5 mL. After 30 h, the production practically ceased. At higher glucose concentration, the fermentation process could continue even after 45 h.

Figure 3.2 Cumulative hydrogen production at the initial glucose concentration of: (A) 5 g/L glucose; (B) 10 g/L glucose; (C) 15 g/L glucose; (D) 20 g/L glucose.

By increasing the initial glucose concentration, the hydrogen production was increased for *E. aerogenes* and *C. beijerinckii*. The maximum value of hydrogen production was recorded when the initial glucose concentration was 20 g/L, i.e. 47.07 mL for *C. beijerinckii*, 12.94 mL for *E. aerogenes*. On the contrary, the hydrogen production of *C. pasteurianum* was higher at low glucose concentration (10 g/L), while with the increase of the glucose concentration less hydrogen was produced. Cumulative hydrogen produced by *C. butyricum* was stable with initial glucose more than 10 g/L. These results indicated that high glucose concentration inhibited the fermentation process; in other words, other nutrients might limit the fermentation process instead of glucose (Argun *et al.* 2008b). Several studies have reported similar results regarding the relationship of initial substrate concentration and hydrogen production (Kotay and Das 2007, Shin *et al.* 2007). Furthermore, it was also found the cumulative biogas volume (data not shown) produced by *E. aerogenes* was nearly the same with *C. butyricum*, i.e. 102 mL and 109 mL, respectively, at 15 g/L glucose. However, the composition of produced biogas (H_2 to CO_2 ratio) was quite different since *Clostridium* sp. favored more hydrogen production.

Hydrogen yield (mol/mol) is calculated by dividing mol of glucose consumed into mol of hydrogen produced (Sen *et al.* 2008). Figure 3.3 presents the effect of initial glucose concentration on hydrogen yield. *E. aerogenes* and *C. butyricum* had more stable hydrogen yields at the initial glucose concentration ranges. As well, *C. butyricum* and *C. pasteurianum* appeared higher in hydrogen yield than the other two species. In addition, hydrogen yields of all the strains dropped in various degrees as the initial glucose concentration increased, which indicated the portion of the total consumed glucose used for hydrogen production was reduced. For comparison with the literature, Table 3.1 lists hydrogen yields from other studies that used glucose as substrate by pure cultures. It seemed that the hydrogen yields found in this study were varied with different initial glucose concentrations but quite comparable to the yields of pure cultures in other studies. Many studies have shown that besides substrate concentration, factors like pH, temperature, and nutrients content could affect hydrogen productivity (Jo *et al.* 2008, Sinha and

Pandey 2011). The optimal levels of key factors and the interactions should be further investigated.

Figure 3.3 The effect of initial glucose concentration on hydrogen yield.

Table 3.1 Hydrogen yield using glucose as substrate by pure cultures.

Microorganism	Substrate	Culture operation	Hydrogen yield mol-H ₂ /mol-glucose	Reference
<i>Enterobacter aerogenes</i> HU-101 wt	glucose	batch	0.6	(Rachman <i>et al.</i> 1997)
<i>Enterobacter aerogenes</i> IAM1183	glucose	batch	0.9	(Lu <i>et al.</i> 2011)
<i>Enterobacter aerogenes</i> DSM 30053	glucose	batch	0.1-0.3	This study
<i>Clostridium beijerinckii</i> L9	glucose	batch	2.8	(Lin <i>et al.</i> 2007b)
<i>Clostridium beijerinckii</i> DSM1820	glucose	batch	1.5	(Masset <i>et al.</i> 2012)
<i>Clostridium beijerinckii</i> RZF-1108	glucose	batch	2.0	(Zhao <i>et al.</i> 2011)
<i>Clostridium beijerinckii</i> DSM 791	glucose	batch	0.6-1.6	This study
<i>Clostridium butyricum</i> CWB11009	glucose	batch	1.7	(Masset <i>et al.</i> 2010)
<i>Clostridium butyricum</i> ATCC19398	glucose	batch	2.3	(Lin <i>et al.</i> 2007b)
<i>Clostridium butyricum</i> SC-E1	glucose	continuous	1.4-2.3	(Kataoka <i>et al.</i> 1997)
<i>Clostridium butyricum</i> DSM 10702	glucose	batch	2.4-3.1	This study
<i>Clostridium pasteurianum</i> DSM525	glucose	batch	0.7	(Masset <i>et al.</i> 2012)
<i>Clostridium pasteurianum</i> DSM 525	glucose	batch	1.8-3.0	This study
<i>Clostridium saccharoperbutylacetonicum</i> ATCC 27021	glucose	batch	1.4	(Ferchichi <i>et al.</i> 2005)
<i>Citrobacter</i> sp. Y19	glucose	batch	2.5	(Oh <i>et al.</i> 2003a)
<i>Clostridium paraputrificum</i> M-21	glucose	batch	1.1	(Evvyernie <i>et al.</i> 2001)
<i>Clostridium acetobutylicum</i>	glucose	batch	1.8	(Lin <i>et al.</i> 2007b)

3.3.3 Hydrogen production rates

3.3.3.1 Effect of initial glucose concentration on hydrogen production rate and specific hydrogen production rate

Variations of volumetric hydrogen production rate and specific hydrogen production rate (HPR and SHPR) at different initial glucose concentrations are presented in Figure 3.4. Hydrogen production rate (R_v , mL- H_2 /L-medium/h) and specific hydrogen production rate (R_x , mL- H_2 /g-biomass/h) are defined as follows:

$$R_v = \frac{V_{H_2}}{V} \quad \text{and} \quad R_x = \frac{V_{H_2}}{VX}$$

where V_{H_2} is the volume of hydrogen gas produced per hour (mL H_2 /h); V is the volume of fermentation broth (30 mL); X is the biomass concentration in the serum bottles (g/L); the initial biomass was ranged from 0.75 to 0.79 g/L.

Figure 3.4 The effect of initial glucose concentration on volumetric hydrogen production rate and specific hydrogen production rate.

In the batch experiments, Figure 3.4 presents that *Clostridium* species had higher HPR and SHPR compared to *E. aerogenes*. In general, hydrogen production rates had the same trends with the cumulative hydrogen production. For *E. aerogenes* and *C. beijerinckii*, the hydrogen production rates increased as initial glucose concentration increased having highest hydrogen production rates of 9.21 and 21.79 mL-H₂/L-medium/h, respectively. At initial glucose concentration of 10 g/L, *C. pasteurianum* achieved its highest hydrogen production rate of 17.88 mL-H₂/L-medium/h. The HPR of *C. butyricum* remained stable within a range of 18 to 21 mL-H₂/L-medium/h at the glucose concentration of 10 to 20 g/L. Regarding the

SHPR, *C. butyricum* presented the highest specific hydrogen production rate of 3.90 mL-H₂/g-biomass/h at 10 g/L of glucose. The SHPRs of *E. aerogenes* and *C. beijerinckii* slightly increased but *C. pasteurianum*'s SHPR decreased with the increase of the initial glucose concentration that was in consistence with the HPR results. Generally, biomass increased with the increase of the initial glucose concentration for all the strains in this study, only a slight decrease for *C. pasteurianum* and *C. butyricum* at the initial glucose concentration above 15 g/L glucose. Kotay (2007) reported the similar result regarding the initial glucose concentration about the cell biomass. The relationship between cell growth and hydrogen production was complex that they had a competing for substrate but a synergy for production. Several researchers have discussed the phenomenon with a various results (Xie *et al.* 2012) (nitrogenase activity more at the stationary phase). For example, both HPR and SHPR of *C. pasteurianum* dropped at glucose of 20 g/L, however, more glucose was consumed (Figure 3.1). This was probably due to the metabolic change with respect to the substrate pressure, as well other reduced compounds other than hydrogen were produced (Cai *et al.* 2010, Qin *et al.* 2015).

Overall, *C. butyricum* was proved as the most efficient dark fermentative bacteria because of its stably higher hydrogen production rates and hydrogen yield both in the low and high glucose concentrations.

3.3.3.2 Maximum hydrogen production rate of different cultures

Dynamics study on hydrogen production could help to find the maximum hydrogen production rate in order to optimize the reactor development. An AMPTS test was applied to monitor hydrogen production among the four bacteria by using 5g/L glucose. Results in Figure 3.5 revealed that hydrogen production process was very fast after cells adaption. From the observation of *C. butyricum* and *C. pasteurianum*, after 12 hours lag phase, around 65% of the total hydrogen was produced within 3 hours. It took double time for *E. aerogenes* adaption to produce hydrogen compared to *C. butyricum* and *C. pasteurianum*. *C. beijerinckii* started to produce hydrogen rapidly when incubation started, however, because of the butyrate metabolism preference the hydrogen yield was lower. Maximum hydrogen production rate was 357.4 mL/L/h observed at 14 h from *C. butyricum*.

Figure 3.5 Hydrogen production rate study from AMPTS test.

3.3.4 Effect of initial glucose concentration on volatile fatty acids production and final pH

In order to identify the constraints of the dark fermentation process on the hydrogen production, further analyses were done on the liquid samples. Soluble metabolites like volatile fatty acids (VFAs) were formed with hydrogen simultaneously in the fermentation process. To some extent, VFAs production represented hydrogen evolution efficiency. Figure 3.6 presents the VFAs concentration as well as the ratio of acetic and butyric acids. For *E. aerogenes*, only acetic acid was the main VFAs. It was observed that acetate was produced less as initial glucose concentration increased, but in opposite hydrogen was produce more (Figure 3.2). Because *E. aerogenase* produced hydrogen via formic fermentation pathway (Lee *et al.* 2011), and acetate is one of complete substances with formate (Figure 2.5). For *Clostridia*, among soluble metabolites in the dark-fermentation effluent, acetic and butyric acid were the two main VFAs accompanied by hydrogen production. But the VFAs compositions for each strain were different. *C. butyricum* and *C. pasteurianum* produced more acetate but less butyrate compared to *C. beijerinckii*. This agreed with hydrogen content in biogas that *C. butyricum* and *C. pasteurianum* had a higher hydrogen content range of 34% ~ 41%, but it was only 18% ~ 23% for *C. beijerinckii*. The VFAs profile highly affected hydrogen production.

Theoretically, 4 mol H₂/mol glucose can be achieved when acetic acid is the only byproduct., 2 mol H₂/mol glucose is obtained If butyric acid is the only VFAs, and 1 mole H₂ will be consumed as 1 mole propionic acid forms (Espinoza-Escalante *et al.* 2008, Vardar-Schara *et al.* 2008). Furthermore, to obtain the maximum total hydrogen yield, two-stage systems have been investigated widely, such as the combination system of dark and photo fermentation. Photo fermentation can further convert the organic acids to hydrogen, which are obtained from the effluents of dark fermentation. VFAs as the substrate of photo fermentative bacteria play an important role on the hydrogen production of photo fermentation. In addition, the A/B ratio changes also indicated the metabolic shift as the initial glucose concentration increased (Hawkes *et al.* 2002). The final pH was also presented in Table 3.2. Due to the higher VFAs concentrations of *C. butyricum* and *C. pasteurianum* produced in the effluent, the lower the final pH-values were occurred. It was important to relief the pH stress by the accumulation of VFAs that affected hydrogen production.

Table 3.2 The effect of initial glucose concentration on final pH.

Glucose Concentration	<i>E. aerogenes</i>	<i>C. butyricum</i>	<i>C. pasteurianum</i>	<i>C. beijerinckii</i>
5 g/L	6.2	4.86	4.88	5.43
10 g/L	6.4	4.74	4.76	5.22
15 g/L	6.32	4.71	4.84	5.13
20 g/L	6.34	4.7	4.84	5.04

Figure 3.6 VFAs concentrations in dark fermentation effluents by the four bacteria species at the initial glucose concentration of: (A) 5 g/L glucose; (B) 10 g/L glucose; (C) 15 g/L glucose; (D) 20 g/L glucose.

3.3.5 Evaluation on hydrogen production potential of artificial mixed cultures

Pure bacterial strains present better hydrogen yields than mixed, natural microbial communities (Masset *et al.* 2012). However the main drawback with the pure cultures is the system stability. Therefore, artificial co-cultures composed of well-characterized strains were proposed for H₂ production study. It was expected to investigate whether there was any positive interaction between the species for hydrogen production. According to the four selected dark fermentative bacteria, six artificial consortia (calculated as C_4^2) were constructed: *C. butyricum* (abbreviated as C1) and *C. pasteurianum* (abbreviated as C5), *C. butyricum* and *C. beijerinckii* (abbreviated as C7), *C. butyricum* and *E. aerogenes* (abbreviated as E), *C. pasteurianum* and *C. beijerinckii*, *C. pasteurianum* and *E. aerogenes*, *C. beijerinckii* and *E. aerogenes*. An equal volume (mL) of the two respective strains was added to initiate a co-culture system. As well, a more complex consortium with all the four bacteria was observed as MIX group, where each strain took up one quarter of the total inoculum volume (10% v/v). In total, there were 7 mixed groups used in this study.

Figure 3.7 shows the hydrogen content in biogas produced by different culture consortia in the glucose fermentation. Co-culture systems had the consistent results with the pure culture performance. C1 and C5 had more hydrogen content than C7 and E. In the co-culture studies, there was not significant improvement on the hydrogen content in biogas. The most hydrogen purity of 39.6% was achieved by C1+C5 and C5+C7. Since every culture took effect in the MIX group, it only had a hydrogen content of 30.4% at the end.

Figure 3.7 Hydrogen content in the fermentative biogas production.

Cumulative hydrogen production result (Figure 3.8) showed obvious improvement in the co-culture systems. For instance, C1+C5 and C1+C7 increased hydrogen production by 21.2% and 16.2 % compared with C1, which was the pure culture producing the maximum hydrogen. This phenomenon proved the existing of the cooperation to enhance the target product production. Meanwhile, the combination with E has no improvement on hydrogen production, but the negative effect by E on C1 and C7 seems much weaker than on C5. The co-culture of C5+E only produced 55% of cumulative hydrogen production by pure C5. C1 and C7 almost had no harm impact on the others. While the mixed group with all the four culture did not have the best result, it revealed that the co-operation existing in the bacteria have the preference, which also demonstrated the mixed enrichment sometimes had a lowed yield. Therefore, the cooperation study could help optimize a consortium for the target substances production.

Figure 3.8 Cumulative hydrogen production by different bacteria consortia.

Figure 3.9 illustrates final pH in dark fermentation effluent. Final pH of C1, C1+C5, and C1+C7 group were stable around 4.9. With the existing of E, group final pH was a bit higher.

Figure 3.9 Final pH of the fermentation effluent.

Ammonium is an important factor affecting photo fermentative hydrogen production (McKinlay and Harwood 2010a). At 5g/L glucose concentration, ammonium concentration in dark fermentation effluent was in the decreasing sequence of E, C7, C1 and C5. The maximum ammonium concentration was 1.6 mM by E. Similar with the final pH results, ammonium concentration in the group of C1+E and mixed was higher than the other co-culture groups. Although ammonium concentration decreased slightly in the co-culture systems, there was no significant effect on ammonium production by the cultures interaction (Figure 3.10).

Figure 3.10 NH₃-N production in dark fermentation.

The interaction effect on VFAs production among C1, C5 and C7 was obvious in Figure 3.11. The co-cultures C1+C7 and C1+C5 produced more VFAs than pure strains. The acetate production of the co-culture with C1 and C5 was 50% and 60% higher than pure C1 and C5. Especially, the acetate to butyrate ratio of co-culture with C1 and C5 was higher than C1 and C7, which was consistent with higher hydrogen production by the co-culture of C1+C5.

Figure 3.11 VFAs production in dark fermentation.

As a result, the co-operation among C1, C5, and C7 had a positive effect on the hydrogen and VFAs production. However, the effect on final pH, ammonium production and hydrogen content was not significant.

3.4 Conclusions

The characteristics of hydrogen production by four dark fermentation bacteria (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii* and *E. aerogenes*) was compared. Bacteria could consume more glucose by increasing initial glucose concentration, but the glucose utilization efficiencies were varied. *C. butyricum* had higher hydrogen yield and hydrogen production rate compared to the other bacteria. Hydrogen was evolved very fast in dark fermentation after cells adaption. More than 60% hydrogen was produced within an average period of 3 hours. The high acetate to butyrate molar ratio showed that more hydrogen could be produced by the bacteria. Interactions among *Clostridium* species had a positive effect on hydrogen production and system stability.

Chapter 4 Hydrogen production from food waste

4.1. Introduction

Simple carbohydrates such as glucose, sucrose and lactose are ideal substrates for biological hydrogen production. However, pure carbohydrate sources are expensive raw materials. Organic waste is more economic substrate as it can both reduce global waste and produce renewable energy. Food waste (FW) is a good candidate for hydrogen production because of its biodegradable characteristics. In recent years, it has been reported that a variety of anaerobic and photosynthetic microbes could produce hydrogen from food waste in different metabolic pathways (Hallenbeck and Benemann 2002, Wang *et al.* 2005). Hydrolysis of food waste is considered as the rate-limiting step in the overall anaerobic fermentation process. In order to enhance hydrogen production, different pretreatment methods have been proposed such as thermal, alkaline, freeze and thaw, etc. (Penaud *et al.* 1999, Cai *et al.* 2004, Baghchehsaraee *et al.* 2008, Elbeshbishy *et al.* 2011). Recently, autoclaving was introduced for municipal solid waste treatment, which involves steam processing under the action of pressure. The high temperature and pressure enhance the hydrolysis of the solid wastes. In Europe, it is considered as an alternative treatment method for diverting solid wastes from landfilling (Ibrahim *et al.* 2011).

pH is an important factor that significantly influences the hydrogenase activity and biological metabolic pathways, in order to affect hydrogen production potential from food waste (Guo *et al.* 2010). In batch studies, initial pH is rather important and can't be ignored. A pH range from 4 to 8 has been investigated, which showed that at neutral pH conditions hydrogen production was enhanced. Although higher initial pH heightened hydrogen production rate, however the rate would not last for long time because of the fast pH decrease by acids generation and accumulation. Lower pH values resulted in longer lag periods for bacteria to produce hydrogen due to unfavorable initial condition for fermentative bacteria (Sinha and Pandey 2011).

To our knowledge, there are limited fermentation studies with pure cultures and food waste. The purpose of this study was to evaluate hydrogen production potential from food waste by four fermentative pure cultures (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii* and *E. aerogenes*). In addition, the effect of autoclaving and initial pH adjustment as pretreatment methods of food waste were investigated to further enhance hydrogen production potential. The fermentation process was monitored in terms of hydrogen yield, volatile fatty acids, pH and ammonia production.

4.2. Materials and Method

4.2.1 Feedstock and bacteria cultures

Food waste (FW) was collected from a canteen at Nanyang Technological University (Singapore). FW was mainly food remaining in plates after lunch consisted of peelings of fruits or vegetables like salad, water melon, cooked potatoes, garden peas, fried potatoes, noodles, rice, cooked meat and bread. After bones and all non-food materials were removed, FW was thoroughly mixed to promote homogeneity of the particulate organics. Then, it was diluted with DI water (the ratio was 100 g FW to 1000 mL DI water) for the subsequent experiments. The characteristics of FW solution are presented in Table 4.1.

Table 4.1 Characteristics of raw food waste.

Parameter	Value	Unit	Parameter	Value	Unit
pH	4.8 ± 0.2	-	NH ₃ -N	16.0	mg/L
C	47.2	%	Carbohydrates	15.48	g/L
N	2.7	%	Na	112.6	mg/L
H	7.4	%	K	97.63	mg/L
C/N	17.5	-	Ca	16.32	mg/L
TS	28.05	g/L	Mg	9.61	mg/L
VS	27.22	g/L	Zn	724	ug/L
VS/TS	97	%	Fe	170	ug/L
Acetate	1.22	mM	Mn	32	ug/L
Propionate	0.15	mM	Cu	21	ug/L
Butyrate	0.05	mM	Mo	8	ug/L
TVFAs	1.98	mM	Co	6	ug/L
TCOD	33.55	g/L	Ni	5	ug/L
SCOD	8.95	g/L	Cd	2	ug/L

C. butyricum, *C. pasteurianum*, *C. beijerinckii* and *E. aerogenes* were used in the study. The medium used to cultivate the four species consisted of 3 g/L yeast extract, 10 g/L ‘Lab-Lemco’ powder (beef extract), 10 g/L peptone, 5 g/L glucose, 1 g/L soluble starch, 5 g/L sodium chloride, 3 g/L sodium acetate, and 5 g/L cysteine hydrochloride. The medium was adjusted at pH 6.8 ± 0.2. Bacteria were cultivated in an incubator at 37 °C and 150 rpm.

4.2.2 Experimental design

The dark fermentation batch experiments were performed in 60 mL serum bottles that were purged with argon and then filled with 30 mL food waste solution. The bottles were sealed with both butyl rubber stoppers and aluminum crimp caps. Four different kinds of medium were used: raw food waste (RFW), pH adjustment food waste (PFW), autoclaving food waste (AFW), and autoclaving and pH adjustment food waste (APFW). For pH adjustment, small amounts of 1 M NaOH were added to ensure minimum increase of the food waste solution. For autoclaving, the sealed bottles were sterilized at 121 °C for 15 min. When cells reached mid-exponential growth phase, the inoculants were injected into serum bottles by 3 mL syringes (cell dry weight was 0.75-0.79 g/L). Afterwards, the bottles were placed in the shaking incubator operating at 200 rpm, at constant temperature 35 °C. The batch

experiments were conducted in duplicate and lasted for 120 h. To avoid the influence of the microorganisms in food waste, substrate control experiments were run in order to exclude any hydrogen or other biogas production.

4.2.3 Analytical methods

Samples were analyzed for TS and VS according to the standard methods for the examination of water and wastewater (Eaton and Franson 2005). Carbohydrate concentrations were determined adapting the colorimetric method (Dubois *et al.* 1956) with UV visible wavelength at 490 nm using glucose as standard (Agilent Cary 50, USA). The C/N ratio was determined by measuring the total carbon and nitrogen using an elemental analyzer (Vario EL cube CHNOS, Germany). Trace metals concentration was measured by an Inductively Coupled Plasma - Optical Emission Spectrometer (Perkin Elmer Optima 8300, USA). COD and NH_4^+ concentrations were measured colorimetrically using a DR 2800 spectrophotometer (Hach, USA). VFAs concentration and hydrogen production measurements were the same as described in Chapter 3.

4.3. Results and discussion

4.3.1 Bio-hydrogen production from raw food waste by four pure cultures

Figure 4.1A shows the hydrogen yield and hydrogen production rate of the four pure cultures using raw food waste (RFW). Results indicate that *C. beijerinckii* could produce sufficient hydrogen from RFW. On the contrary, *E. aerogenes* produced very limited hydrogen, while *C. pasteurianum* and *C. butyricum* were unable to directly produce hydrogen. It seems that *C. beijerinckii* was capable to utilize the dissolved carbohydrates as carbon source to produce hydrogen easily and quickly. In case of *C. butyricum* and *C. pasteurianum*, it was unable to directly use carbohydrates in the RFW, although they are capable hydrogen producers using other substrates like glucose. In previous study (Kim *et al.* 2008a), it was investigated the hydrogen production from food waste by *C. beijerinckii*. They addressed that initial pH was rather important for hydrogen and VFA production. In addition, acetate to butyrate ratio was much higher when initial pH was adjusted to

7.0.

The cumulative hydrogen production by *C. butyricum*, *C. pasteurianum*, *C. beijerinckii*, and *E. aerogenes* using RFW is presented in Figure 4.1B. During the early stage (less than one day), there was no hydrogen production (adaptation period). After that, *C. beijerinckii* rapidly started producing hydrogen around 8 mL, while *E. aerogenes* produced less than 2 mL. After 50 h, the production practically ceased. On the contrary, *C. butyricum* and *C. pasteurianum* were not able to produce hydrogen from RFW. It has previously reported that one day lag time could be essential for the hydrolytic/acidogenic phase (Ramos *et al.* 2012). For this reason, pretreatment of RFW is required to enhance solubilization of the organic matter.

Figure 4.1 (A) Hydrogen yield and hydrogen production rate; (B) cumulative hydrogen production, by *C. butyricum*, *C. pasteurianum*, *C. beijerinckii*, and *E. aerogenes* using raw food waste (RFW).

4.3.2 Effect of food waste pretreatments on hydrogen production

To avoid hydrogen consuming bacteria and other bacteria competing for the product and substrates, as well to provide a better condition for dark fermentative growth and producing hydrogen, autoclaving and pH adjustment pretreatments were investigated in this study in order to maximize hydrogen production. The effect of initial pH adjustment and autoclaving on solubilization of food waste is presented in Figure 4.2. After pH adjustment, TVFAs and soluble carbohydrates were increased by 21.7% and 10.5%, respectively, while SCOD was slightly increased by 4%. When autoclaving occurred, SCOD, TVFAs, and soluble carbohydrates were increased by 27.4%, 27.8%, and 19.4%, respectively. By combination of pH adjustment and autoclaving, maximum solubilization achieved since SCOD, TVFAs, and soluble carbohydrates were increased by 55.3%, 67.2%, and 19.3%, respectively. These findings were consistent with previous studies that pretreatment could enhance the solubility of organic wastes such as food waste and sludge (Elbeshbishy *et al.* 2011).

Figure 4.2 Solubilization of raw food waste after pretreatment techniques.

Figure 4.3 presents the impact of food waste pretreatment techniques on biohydrogen yield. The results showed that hydrogen production was enhanced after initial pH adjustment. More specifically, *C. butyricum* and *C. pasteurianum* became adequate to produce limited amount of hydrogen, while *C. beijerinckii* and *E. aerogenes* increased hydrogen yield by 5.8 and 6.8 mL-H₂/g-VS_{added}, respectively. By autoclaving pretreatment, hydrogen yield of *C. butyricum* was significantly increased to 38.6 mL-H₂/g-VS_{added}. Others, *C. beijerinckii* and *E. aerogenes* produced sufficient amount of hydrogen. However, autoclaving had negligible effect on *C. pasteurianum*. The maximum hydrogen yield was achieved after pH adjustment and autoclaving pretreatment by all cultures. This implies that the solubilization of food waste intensified hydrogen production. Moreover, *C. butyricum* produced a hydrogen yield of 39.2 mL-H₂/g-VS_{added}, which was 92-fold higher than with RFW. When considering pH adjustment on the autoclaving process, *C. pasteurianum* seems to have the greater correspondence. In other words, without any pH initial adjustment, *C. pasteurianum* couldn't utilize the soluble carbohydrates in food waste.

Figure 4.3 Effect of pretreatment technique on hydrogen yield by pure cultures from food waste.

Overall, autoclaving with or without pH adjustment had the higher influence for hydrogen production. After autoclaving pretreatment, most species could efficiently utilize food waste to produce hydrogen. That was because autoclaving could preserve the actual nutrient substances in FW and break down biodegradable

compounds into simpler organic compounds through physical and chemical reactions, therefore, the hydrolysis of carbohydrates was enhanced (Glass and Bozzi 2000). Similarly, other types of organic compounds presented in food waste could be employed for hydrogen production by different species. The composition of food waste, especially the carbohydrates, should be further studied.

Moreover, to investigate the cost-benefit of autoclaving pretreatment on hydrogen production from food waste in practical application, an energy analysis was conducted by using the hydrogen production results of the strain *C. butyricum*. Net energy gain analysis was conducted by calculating the energy input utilized for autoclaving and potential energy output generated from hydrogen gas produced (Jung *et al.* 2011, Perera *et al.* 2012). It was obtained that net energy gain of the increased hydrogen production after applying autoclaving pretreatment (121 °C, 15 min) was 0.13 KJ/g raw food waste compared with hydrogen production without pretreatments.

4.3.3 Effect of food waste pretreatments on VFAs production

The concentrations of TVFAs and their main components after 5 days of dark fermentation are shown in Figure 4.4. It was presented in Figure 4.2 that food waste substrates contained some amount but low concentration of TVFAs, therefore, the accessional concentration was derived through the metabolic pathways of the fermentation process. The phenomenon was observed in glucose fermentation (Chapter 3) that acetic and butyric acids were the two dominant compounds in the metabolic pathways for hydrogen production of *Clostridium* sp., acetic acid was the main metabolic volatile fatty acid of *E. aerogenes*. The food waste fermentation showed the same result as glucose fermentation for all the strains. The production of TVFAs in relation to pretreatment techniques showed the positive effect, especially autoclaving had the greater influence on *C. butyricum* with a 150% increase of TVFAs concentration. Because of the positive correlation between hydrogen and VFAs production as mentioned in 3.3.4, it was further proved the pretreatment conditions favored hydrogen production. Another phenomenon was observed that VFAs were produced in RFW fermentation although little hydrogen was detected (Figure 4.3), which indicated the hydrogen-consuming

microorganisms such as homoacetogens might exist in the system (Elbeshbishy *et al.* 2011). Besides, TVFAs concentration in AFW fermentation was lower than that in PFW, more hydrogen was produced from AFW by *C. beijerinckii*. This phenomenon was also observed in *E. aerogenes* for RFW and AFW fermentation. Further microorganism community analysis of food waste would help explain these observations. Moreover, little amounts of VFAs were produced in RFW and AFW fermentation in consistence with the hydrogen production results by *C. pasteurianum*, as it did not grow well in low pH condition (Dabrock *et al.* 1992).

Figure 4.4 Effect of pretreatment technique on VFAs concentration after fermentation experiments cultures: (A) *C. butyricum*; (B) *C. pasteurianum*; (C) *C. beijerinckii*; and (D) *E. aerogenes*.

The ratio acetic to butyric acid (HAc/HBu) has great impact on hydrogen production. Generally, higher HAc/HBu ratio means more hydrogen could be yielded when the same volume of biogas is produced. Considering the hydrogen yields of *C. pasteurianum* in APFW and *C. beijerinckii* in RFW (Figure 4.3), it seems that *C. pasteurianum* produced more hydrogen in APFW, although the amount of produced biogas and TVFAs were almost the same. That was consistent with the HAc/HBu ratio between the two experiments. Moreover, Figure 4.4 also reveals that HAc/HBu ratios of *Clostridium* sp. using food waste with autoclaving pretreatment were lower than without autoclaving pretreatment. This suggests that

butyrate metabolic pathway was preferred in the fermentation process, because different substrates and pH changes could have impact on the metabolic activities (Ren *et al.* 2008, Guo *et al.* 2010). Overall, VFAs results further supported the pretreatment effect on hydrogen production.

4.3.4 Effect of food waste pretreatments on pH variation and NH_4^+ production

It has been observed that pH significantly influences the hydrogen production (Kim *et al.* 2008a). The initial pH of the food waste used in this study was 4.8, while at the pH adjustment experiments, it was fixed at 7.0. Table 4.2 presents the final pH after the fermentation experiments. It seems that experiments without pretreatment had the lower pH range 3.3 ± 0.15 . The initial adjustment at pH 7 slightly increased final pH, which had a minor influence on the hydrogen production. When autoclaving was applied for food waste pretreatment, it had significant influence on pH variation, since pH was ranged between 4-4.5. In practice, the pH adjustment after autoclaving had no impact on the final pH. Such results are in accordance with hydrogen production efficiency, because higher pH favors the activity of hydrogenases (Guo *et al.* 2010).

Ammonia is one of the hydrolysis products during the degradation of proteins (ammonification). It has previously been addressed that in an appropriate concentration range, ammonia nitrogen was beneficial to fermentative hydrogen production. However, the high ammonia concentration could inhibit the fermentation process because (1) it changes the intracellular pH of hydrogen-producing bacteria; and (2) it increases the maintenance energy requirement for hydrogen-producing bacteria (Sung and Liu 2003, Tada *et al.* 2005).

Ammonia concentration was monitored after dark fermentation of food waste (Table 4.2). It seems that ammonia concentration increased from 16 mg/L $\text{NH}_3\text{-N}$ to more than 50 mg/L $\text{NH}_3\text{-N}$ after the fermentation process. The effluents from autoclaving pretreatment contained relatively less ammonia than effluents without autoclaving. In addition, the highest ammonia concentration was observed in the effluents from PFW fermentation. Among the bacteria cultures, less ammonia was produced by *C. butyricum* followed by *C. pasteurianum*.

Table 4.2 Final pH and ammonia in the end of biohydrogen fermentation.

	<i>C. butyricum</i>		<i>C.pasteurianum</i>		<i>C.beijerinckii</i>		<i>E.aerogenes</i>	
	pH	NH ₃ -N mg/L	pH	NH ₃ -N mg/L	pH	NH ₃ -N mg/L	pH	NH ₃ -N mg/L
RFW	3.2	46.7	3.3	40.0	3.4	53.6	3.2	52.4
PFW	3.3	48.1	3.4	44.4	3.6	55.8	3.4	57.1
AFW	4.0	18.3	4.8	29.2	4.5	33.6	4.5	42.3
APFW	4.2	18.2	4.7	22.5	4.5	36.0	4.5	44.9

In order to increase hydrogen production or substrate utilization efficiency, subsequent photo fermentation or anaerobic digestion is necessary. Attention should be given to high NH₄⁺ concentration in the effluent since it could be toxic to methanogens and photo fermentative bacteria. It has been reported that 2709 mg/L of NH₄⁺ could completely prevent the growth of *Methanobacterium formicicum*. Other photo fermentative bacteria, like *Rhodobacter sphaeroids*, were not able to produce hydrogen when ammonia was more than 2 mM, but they used ammonia only for cell growth. Further studies showed that hydrogen production activity could be resumed only once ammonia was consumed (Kapdan and Kargi 2006, Wang *et al.* 2009).

4.3.5 Mixed-cultures study on hydrogen production from food waste

To investigate the co-operation between cultures effect on hydrogen production from food waste, seven bacteria consortia were studied. Unlike the results from glucose fermentation (Figure 3.8), mixed cultures had limited effect on hydrogen production enhancement, while only the co-cultures of *C. butyricum* + *C. beijerinckii*, *C. pasteurianum*. + *C. beijerinckii*, and MIX Group with all the four bacteria successfully produced hydrogen from food waste (Figure 4.5). *C. butyricum* and *C. beijerinckii* were still good at converting food waste to hydrogen, where *C. butyricum* produced most hydrogen from food waste and *C. beijerinckii* digested food waste easily. Hydrogen productions from the co-cultures of *C. butyricum* + *C. beijerinckii*, *C. pasteurianum*. + *C. beijerinckii* and MIX Group were 60%, 74% and 59% of the hydrogen production 1474 mL/L by C1, respectively. Although the co-culture of *C. butyricum* + *C. beijerinckii* produced 40% less hydrogen than pure *C. butyricum*, the system stability was enhanced in view of

the less difference from the replicates. Mixed group could produce hydrogen for some amount but not stable. *E.aerogenes* had a negative effect on the co-operation for hydrogen production. It was noticed that consortia could make the system more stable.

Figure 4.5 Cumulative hydrogen production by different bacteria consortia.

Figure 4.6 presents the final pH decrease as the sequence of *E.aerogenes*, *C. beijerinckii*, *C. pasteurianum* and *C. butyricum*, which was the same as the result from glucose fermentation, but all the final pH slightly decreased in food waste fermentation. Final pH of co-cultures was still within the range less than 5.29 of E and higher than 4.62 of *C. butyricum*. The lowest co-culture final pH was found in *C. butyricum* + *C. beijerinckii*, which might be one of the reasons for its lower hydrogen production compared with *C. butyricum*. + *C. beijerinckii*.

Figure 4.6 Final pH of the fermentation effluent.

Compared with glucose fermentation study by mixed cultures (Figure 3.7-3.11), it was clearly shown that mixed consortia had different trend on the hydrogen production improvement in food waste fermentation. The maximum hydrogen production of the mixed consortia was less than that of the pure cultures (*C. butyricum*). Besides, the consortia that had good performance in glucose fermentation such as the co-culture of *C. pasteurianum* and *C. butyricum* (C1+C5) only produced limited hydrogen from food waste. C1+C7, C5+ C7 and MIX group with all the four species were stable for hydrogen production in both glucose and food waste fermentation. In addition, the consortia with a higher hydrogen production resulted in a lower final pH in food waste fermentation. Moreover, acetate/butyrate ratio was much lower in food waste fermentation that indicated butyrate production was favored compared to glucose fermentation. Overall, the co-culture of *C. butyricum* and *C. beijerinckii* could be considered to apply in food waste fermentation for the system stability enhancement, however, the pure culture *C. butyricum* produced maximum hydrogen.

4.4. Conclusions

The present study investigated the biological hydrogen production from food waste

by selected pure cultures (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii* and *E. aerogenes*). It revealed the positive effects of pH adjustment and autoclaving pretreatment on raw food waste. *C. beijerinckii* and *E. aerogenes* were able to produce hydrogen from food waste without any pretreatments. The fermentation tests showed that further pH adjustment had minor effect in autoclaving pretreatment. In addition, the lower ammonia production had positive effect on the hydrogen production. It is believed that an integrated or combined dark-photo fermentation system could further benefit bio-hydrogen production.

In order to enhance bio-hydrogen production from food waste, pretreatment methods are widely used. The influence of the initial pH and autoclaving were investigated in batch experiments. Fermentative studies showed that pure cultures like *C. beijerinckii* could directly utilize raw food waste to produce hydrogen, while other cultures (*C. butyricum* and *C. pasteurianum*) could produce hydrogen only after pH adjustment. In this case, the optimal starting pH of the culture was found to be 7. Autoclaving could further enhance hydrogen yields due to increased hydrolysis of food waste. The maximum hydrogen yield was achieved by *C. butyricum* (38.9 mL-H₂/g-VS_{added}) after autoclaving food waste with pH adjustment at 7. In addition, the ratio acetic to butyric acid was decreased by autoclaving pretreatment, because butyrate metabolic pathway was favored in the fermentation process. However, suitable pH for bacteria growth and the low ammonia production could be achieved from autoclaving food waste. The co-culture of *C. butyricum* and *C. beijerinckii* could be used in food waste fermentation to enhance the system stability.

Chapter 5 Photo fermentative hydrogen production

5.1 Introduction

Purple non-sulfur photosynthetic bacteria (PNSB) have been reported to convert a wide variety of organic compounds to hydrogen and carbon dioxide under anaerobic conditions in the presence of light (Keskin *et al.* 2011). These bacteria species mainly include *Rhodopseudomonas* sp., *Rhodobacter* sp., and *Rhodospirillum* sp. (Zurrer and Bachofen 1979, Barbosa *et al.* 2001, Kim *et al.* 2012a). They prefer to grow in mesophilic freshwater and neutral pH, while *Rhodobacter* sp. could be also found in the sewage ponds or eutrophic lakes (Brenner *et al.* 2005). Ren (Ren *et al.* 2009) isolated *Rhodopseudomonas faecalis* RLD-53 from indigenous freshwater pond sludge, and it has been well used for hydrogen production. Sasikala (Sasikala *et al.* 1992) reported hydrogen production by isolated *R. sphaeroides* O.U. 001 using distillery wastewater as substrate. PNSB convert light energy into chemical bond energy (stored in ATP) for growth. The use of photosynthetically derived energy permits the conversion of substrate (organic acids) into hydrogen, where the reaction would not be thermodynamically effective without energy input (Keskin *et al.* 2011). PNSB are capable to use broad spectra of light wavelengths (Zhang *et al.* 2015b). As well, different light sources such as tungsten, fluorescent, infrared, halogen lamps have been reported for hydrogen production, but the performances varied among different conditions (Argun and Kargi 2010b, Ozmihci and Kargi 2010b). Light energy strongly affect hydrogen production rate and yield in photo fermentation process, which is a key factor that could not be neglected for hydrogen evolution (Zürer and Bachofen 1982, Boran *et al.* 2009, Carlozzi 2009). A wide variety of substrates have been utilized by photosynthetic bacteria including carbohydrates such as glucose, sucrose and organic acids like short chain organic acids (Keskin *et al.* 2011). Thus, industrial and agricultural waste effluents containing residual carbohydrates, and organic acids produced from anaerobic digestion could be treated as the feedstock for hydrogen production via the photo fermentation process. Especially, acetic, butyric and lactic acids are the main dark fermentation products from food waste, malic

acid from wine distillery waste are common substrates for PNSB utilization (Barbosa *et al.* 2001, Carlozzi 2009). However, reported substrate conversion efficiencies are quite varied according to different carbon sources and different strains (Koku *et al.* 2002). Few studies have been carried out on substrate selection for hydrogen production, which could maximize the hydrogen production efficiency by culture selection and in order to benefit the energy recovery from complex waste/wastewater. Therefore, it is a necessity to verify the effects of carbon sources and concentrations on photo fermentative hydrogen production before any industrial application.

Biomass yield and hydrogen production are kind of competing for substrate, as any increase in H₂ yield must come at the expense of biomass yield. To avoid the competition between cell growth and hydrogen production, a starvation strategy was reported to enhance hydrogen production by *R. palustris* (McKinlay *et al.* 2014). When starved for nitrogen, non-growing cells continued to metabolize acetate source and produce H₂. Metabolism shift was observed from hydrogen production by non-growing cells. H₂ yields approaching 80% of the theoretical maximum is supposed to be achieved (Akkerman *et al.* 2002).

In this chapter, two types of lighting system were investigated for biohydrogen production using four PNSB with different carbon sources and concentrations. The selection was made to simulate dark fermentation effluents. Kinetics study on cell growth and hydrogen production was further performed to improve hydrogen evolution. The effects of yeast extract and non-growing cells on hydrogen production were discussed and evaluated.

5.2 Materials and Methods

5.2.1 Bacteria strains and medium

Rhodopseudomonas palustris DSM 127, *Rhodobacter sphaeroides* DSM 158 and *Rhodobacter capsulatus* DSM 1710 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in lyophilized form. *Rhodospirillum rubrum* WT was provided by Prof. Nordlund (Stockholm

University, Sweden). Growth medium (per liter) consisted of 0.3 g yeast extract, 1 g sodium succinate, 0.5 mL ethanol, 0.4 g NH_4Cl , 0.5 g ammonium acetate, 5 mL ferric citrate (0.1%), 0.5 g KH_2PO_4 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g NaCl , 0.05 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.4 mL Vitamin B_{12} solution (0.1 g/L), 1 mL trace element solution SL-6. Trace element solution SL-6 (per liter) contained: 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.3 g H_3BO_3 , 0.2 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.03 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. In hydrogen production medium, carbon source was replaced by acetate, butyrate, lactate or malate, and 4.2 g/L glutamate was used as the sole nitrogen source. Initial pH value was adjusted to 6.8 ± 0.2 by using 1 M HCl or NaOH solution. Both growth and hydrogen production medium was packed into 120 or 60 mL serum bottles, purged with argon gas to create anaerobic conditions, and then sealed with butyl rubber stoppers and aluminum crimp caps. After that, sealed serum bottles with fresh medium were autoclaved at 121°C for 15 minutes.

R. palustris strain CGA676 NifA* obtained from Harwood Lab was used in this experiment. The strain CGA676 was an engineered mutant strain by deleting 48 bp of the Q-linker that was part of master transcriptional regulator of nitrogenase genes (McKinlay and Harwood 2010b). It was defective in uptake hydrogenase activity and having constitutive nitrogenase activity allowing it to produce H_2 in the presence of NH_4^+ . Nitrogen-fixing medium (NFM) and defined mineral medium (PM) were used for different studies. NFM (per L) contained 1 mL of 0.1 M $\text{Na}_2\text{S}_2\text{O}_3$, 12.5 mL of 0.5 M KH_2PO_4 , 12.5 mL of 0.5 M Na_2HPO_4 , 1 mL of 10% PABA, and 1 mL of concentrated base (Kim and Harwood 1991). PM contained all the same components as in NFM but with additional 10% $(\text{NH}_4)_2\text{SO}_4$. *R. palustris* was incubated anaerobically in front of a 60 W incandescent light bulb at 30°C in sealed 16 mL anaerobic culture tubes containing 10 mL PM with nitrogen headspace.

5.2.2 Batch fermentation experiments

For lighting and carbon source study

Bacteria were first cultivated at 30°C in 120 mL serum bottles containing 60 mL

growth medium. Cells in the mid-exponential growth phase were then injected into 60 mL serum bottles with 30 mL hydrogen production medium for hydrogen production study. The incubator was operated at 180 rpm and 30 °C, illuminated by fluorescent or incandescent light. Fluorescent lighting system consisted of 6 fluorescent lamps (Phillips Essential TL5 14W/830) with an average intensity of 2400 lux. Incandescent lighting system was composed of 6 incandescent bulbs (Phillips A55 25W) with an average intensity of 2000 lux.

Experimental conditions were described in Table 5.1. In Run 1, four PNSB were cultivated in growth medium illuminated by fluorescent and incandescent light. Growth kinetics of *R. palustris* was investigated under fluorescent and incandescent lighting systems. In Run 2, four carbon sources (acetate, butyrate, malate and lactate) were used to compare the photo fermentative hydrogen production by *R. palustris* under fluorescent and incandescent lighting systems. Initial concentration ranges of different organic acids (Table 5.1) were taken from previous food waste fermentation study (Hu *et al.* 2014). The batch experiments lasted for 32 days (50 days for butyrate study). In Run 3, the initial concentration of each carbon source was selected according to Run 2 results and utilized to compare hydrogen production potential by these four photo fermentative bacteria.

Table 5.1 Experiment conditions.

Runs	Cultures	Lighting system	Carbon source	Concentration (g/L)
Run 1	<i>R. palustris</i>	Fluorescent	Acetate	2
	<i>R.sphaeroides</i>	Incandescent		
	<i>R.capsulatus</i>			
	<i>R.rubrum</i>			
Run 2	<i>R. palustris</i>	Fluorescent	Acetate	2, 3, 4
		Incandescent	Butyrate	1, 2, 3
			Lactate	2.5, 4.5, 6.5
			Malate	2, 4, 6
Run 3	<i>R. palustris</i>	Incandescent	Acetate	2
	<i>R.sphaeroides</i>		Butyrate	2
	<i>R.capsulatus</i>		Lactate	2.5
	<i>R.rubrum</i>		Malate	6

For photo fermentative hydrogen production enhancement study

In Run 1, acetate, butyrate and lactate were compared for photo fermentative hydrogen production study. The carbon source concentrations were selected according to the previous dark fermentation studies with the range of 10 to 60 mM acetate, 5 to 40 mM butyrate, and 10 to 70 mM lactate. Cells were incubated in sealed 27-mL anaerobic culture tubes containing 10 mL NFM with N₂ gas as the sole nitrogen source. Tubes were illuminated using a 60 W incandescent light bulb in a 30 °C incubator. In Run 2, 0.1% yeast extract was added to NFM and PM tubes. 20 mM acetate was used as the sole carbon source. In Run 3, cells were grown anaerobically in 120 mL serum bottles containing 50 mL NFM. Cells were flushed with Ar for 20 min to remove N₂ in the headspace in the mid-exponential phase (0.4~0.5 of OD₆₆₀). Different carbon sources, 20 mM acetate, 10 mM butyrate and 20 mM lactate were compared for the non-growing cells study. Concentration effect for non-growing cells on hydrogen production was investigated by using 5, 10, 20, and 40 mM of butyrate.

5.2.3 Analytical methods

Methods to determine biogas production, hydrogen contents, VFAs, biomass concentration were the same as described in Chapter 3 & 4. Besides, lactate and malate concentrations were analyzed at 210 nm by high performance liquid

chromatography, HPLC (UltiMate 3000, Thermo Scientific Dionex, USA), with a diode-array detector and a 300 x 7.8 mm Aminex HPX-87H Column (Bio-Rad, USA). The mobile phase was an aqueous solution of 0.005 M H₂SO₄ with a flow rate of 0.8 mL/min. The liquor samples were filtered through a 0.45 µm nylon membrane before free acids analysis. Optical density (OD) was quantitated as cell concentration and measured by a spectrophotometer at 660 nm (Agilent Cary 50, USA). Light intensity was measured by using a light meter (LT Lutron LX-1108, Taiwan).

H₂ was quantified with a gas chromatograph equipped with a thermal conductivity detector (GC-2014, Shimadzu, Japan). Sample lock syringes were used to inject gas samples into the equipment. Organic acids were analyzed by HPLC (Huang *et al.* 2010). Cell growth was quantified by optical density at 660 nm using a spectrophotometer (Genesys 20, Thermo Scientific, USA). The intensity of the light reaching the front of the culture tubes was 35 µmol/m²/s, measured using a quantum meter (Spectrum Technologies, Plainfield, IL, USA).

5.2.4 Kinetic study and data analysis

Modified Gompertz equation is used to simulate cell growth and hydrogen production. a) For cell growth modeling, the equation (Gardini *et al.* 2001) is described as follows:

$$y = A \cdot \exp \left\{ -\exp \left[\left(\frac{\mu_{\max} e}{A} \right) \cdot (\lambda - t) + 1 \right] \right\} \quad (5.1)$$

where y is the OD at time t, A represents the maximum OD reached, $\mu_{\max}$ is the maximum OD increase rate (as $\Delta OD/h$) and λ is the lag time (h) for OD increase.

b) For hydrogen production modeling, the equation expression (Xie *et al.* 2012) is

$$H = H_{\max} \cdot \exp \left\{ -\exp \left[\left(\frac{R_{\max}}{H_{\max}} \right) \cdot (\lambda - t) + 1 \right] \right\} \quad (5.2)$$

where H represents the cumulative hydrogen production (mL-H₂/L-medium) at time t (h); H_{max} denotes maximum cumulative H₂ production (mL-H₂/L-medium); R_{max} is the maximum hydrogen production rate (mL-H₂/L-medium/h); and λ is the lag-phase duration (h) for hydrogen production. Statistical significance was evaluated by one-way ANOVA.

5.3 Results and Discussion

5.3.1 Effect of lighting and carbon source on hydrogen production

5.3.1.1 Effect of light sources on the kinetics of PNSB cell growth

To investigate the effect of light energy on PNSB growth, fluorescent and incandescent lighting systems were applied and compared in photo fermentation study. Results showed that all the four PNSB (*R. palustris*, *R. sphaeroides*, *R. capsulatus* and *R. rubrum*) could grow under both fluorescent and incandescent lighting systems. They grew in different colors: *R. palustris* in red, *R. rubrum* in purple/red, both *R. capsulatus* and *R. sphaeroides* in yellow-brown. Color is an indicator of PNSB growth status, for instance, phototrophic cultures of *Rhodobacter* species are yellow-green to yellow-brown, while aerobic cultures are pink to red (Brenner *et al.* 2005). However, the cell growth characteristics varied under different lighting conditions. It took longer time for PNSB to start growth under the fluorescent system compared to the incandescent system. For example, the lag phase for *R. capsulatus* and *R. sphaeroides* to grow was around 4 d under the incandescent system, but more than 10 d under the fluorescent system. Among these four strains, *R. palustris* grew faster than the other three strains, which was the only bacteria producing hydrogen within 21 d study under the fluorescent system. Therefore, it was selected to further investigate the effect of light on cell growth and hydrogen production.

Figure 5.1 presents cell growth of *R. palustris* under different lighting systems. Under incandescent system, *R. palustris* began to grow at early stage and reached

its maximum cell density of 1.78 at 78 h. Cells grew slow under fluorescent system, and maximum cells density OD₆₆₀ of 1.69 was obtained at 162 h. To further investigate the effect of light on cell growth, simulated cell growth kinetics of *R. palustris* according to Eq. (1) was illustrated in Figure.1. The model well fitted the experimental results with a correlation coefficient (R^2) of 0.965 and 0.977 for fluorescent and incandescent lighting, respectively. The estimated maximum cell growth rates ($\mu_{\max}$) for fluorescent and incandescent lighting system were 0.01344/h and 0.03675/h, respectively. Cells growth rate in incandescent lighting system was three-folds higher that in fluorescent lighting system. The lag phase time (λ) under fluorescent system was around 9.2 h, while λ was 0.1h under incandescent system. The simulated OD_{max} was 1.75 and 1.77 under fluorescent and incandescent system, respectively. Cell growth rate and lag phase were quite different ($p < 0.005$) under two lighting systems, but maximum cell density has no significant difference ($p = 0.142$). Compared with fluorescent light, cell growth began to accelerate under incandescent light.

Figure 5.1 Growth study of *R. palustris* under fluorescent and incandescent lighting systems.

5.3.1.2 Effect of carbon source and substrate concentration on hydrogen evolution by *R. palustris*

Four carbon sources were selected for hydrogen production by *R. palustris* under these two lighting systems. Acetate, butyrate, and lactate are dominant products of dark fermentation process from food waste. Malate was chosen as a reference since it is one of the most widely used carbon sources for photo fermentative hydrogen production. Substrate conversion efficiency (SCE) is used to evaluate the hydrogen production performance of a specific substrate (Koku *et al.* 2002, Lazaro *et al.* 2015). It is the ratio of the actual hydrogen produced to the theoretical hydrogen production amount.

$$SCE(\%) = \frac{\text{moles of } H_2 \text{ produced}}{\text{theroretical maximum hydrogen yield}} \times 100\% \quad (5.3)$$

Theoretical hydrogen production is calculated according to the following hypothetical reaction, which denotes all of the substrate is used for hydrogen and carbon dioxide production.

Table 5.2 lists the final fermentation characteristics under fluorescent system by *R. palustris*. *R. palustris* could utilize acetate for hydrogen production, but hardly convert butyrate, malate and lactate to hydrogen. In addition, *R. palustris* did not grow well using butyrate and lactate. Hydrogen yield at different initial acetate concentrations had no obvious difference with a ranged of 1.46 to 1.69 mol/mol, while at 3g/L of acetate *R. palustris* produced the most hydrogen of 1735.4 mL/L medium. Most of acetate (70.5 ~ 100%) was consumed for hydrogen production and cell growth. Only small amount of butyrate and lactate (less than 20%) was consumed, which resulted in the limited cell growth and no hydrogen evolution. *R. palustris* could use malate for cell growth, and cell density decreased as initial malate concentration increased. Meanwhile, it was observed that the final pH of malate fermentation was above 9, which was neutral in acetate fermentation. The effect of pH was more significant on hydrogen production than on cell growth.

Table 5.2 Final characteristics of photo fermentation by *R. palustris* under fluorescent lighting system.

Carbon source	Concentration g/L	Final pH	OD _{max}	Substrate Consumption	Hydrogen yield mol H ₂ /mol acetate
Acetate	2	7.37	1.90	100.0%	1.57
	3	7.32	1.70	97.0%	1.69
	4	7.40	1.89	70.5%	1.46
Butyrate	1	6.47	0.17	13.6%	-
	2	6.50	0.23	12.1%	
	3	6.54	0.24	7.1%	
Lactate	2.5	6.40	0.11	16.2%	-
	4.5	5.56	0.13	12.2%	
	6.5	5.44	0.11	9.7%	
Malate	2	9.14	1.88	84.0%	-
	4	9.18	0.99	31.0%	
	6	9.11	0.46	16.9%	

Photo fermentation results under incandescent lighting system are illustrated in Figure 5.2. It shows butyrate, lactate and malate were well utilized by *R. palustris* for hydrogen production. As well, limited hydrogen was produced from acetate, although *R. palustris* grew well with acetate (Figure 5.1). Final effluents test showed that system pH in acetate fermentation was out of self-control and sharply increased above 9.5, which was unfavorable for hydrogen production by PNSB (Lazaro *et al.* 2015). *R. palustris* started to produce hydrogen using 1 g/L butyrate after 220 h, while it took longer time for hydrogen evolution using higher concentrations of butyrate (Figure 5.2b). In opposite, *R. palustris* could easily convert malate and lactate to hydrogen at the fermentation commencement. It was observed from Figure 5.2c that no further hydrogen was produced as initial lactate concentration increased. Conversely, cumulative hydrogen production increased as the initial malate concentration increased (Figure 5.2d). The optimal initial carbon concentrations for cumulative hydrogen production were 2g/L butyrate, 2.5 g/L lactate, and 6 g/L malate. Interestingly, slight decrease of cumulative hydrogen production was observed in Figure 5.2 after maximum production was obtained. It was due to the small amount of leakage during biogas sampling; another reason might be hydrogen consumption by the possible existing up-taken hydrogenase in PNSB (Rey *et al.* 2006, Kars *et al.* 2008).

Figure 5.2 Effect of carbon sources (acetate (a), butyrate (b), lactate (c), and malate (d)) and initial concentrations on hydrogen evolution by *R. palustris* under incandescent lighting system.

To better understand the effects of carbon source and initial concentration on hydrogen production, four conditions were further studied on the kinetics modeling, including acetate fermentation under fluorescent system, butyrate, lactate and malate fermentation under incandescent system. The simulated results according to Eq (5.2) from the four carbon sources by *R. palustris* are summarized in Table 5.3. As the carbon concentration increased, the estimated maximum hydrogen production rate ($R_{\max}$) decreased for acetate, lactate and malate. $R_{\max}$ of butyrate did not present a general trend and varied between 13.9 to 19.9 mL/L/h. The highest $R_{\max}$ was achieved at 2 g/L malate of 27.6 mL/L/h. Lag phase duration (λ) for hydrogen production increased as the initial carbon concentration increased. λ was much longer in butyrate than the other carbon sources, which demonstrated butyrate was relatively more difficult to adapt for hydrogen production by *R. palustris*. Maximum potential of hydrogen evolution ($H_{\max}$) varied according to the carbon sources in the sequence of 2170.9 mL/L/h at 6 g/L malate, 2153.7 mL/L/h at 2 g/L butyrate, 1866.1 mL/L/h at 3 g/L acetate, and 1670.7 mL/L/h at 2.5 g/L lactate.

Table 5.3 Hydrogen evolution by *R. palustris* under different carbon sources and concentrations.

Carbon source	Concentration	R _{max}	H _{max}	λ	R ²	SCE	Final pH
	g/L	ml/l/h	ml/l	h			
Acetate*	2	3.9	1228.7	70.2	0.977	38.6%	7.37
	3	2.9	1866.1	116.4	0.997	42.3%	7.32
	4	2.6	1609.6	131.7	0.989	36.7%	7.40
Butyrate	1	13.9	1039.9	395.6	0.999	42.4%	7.37
	2	19.9	2153.7	725.0	0.993	49.2%	6.80
	3	14.3	1358.5	722.3	0.865	47.2%	6.64
Lactate	2.5	8.4	1670.7	39.4	0.999	42.9%	6.82
	4.5	7.2	1654.7	42.8	0.982	23.3%	6.73
	6.5	7.0	1528.9	73.2	1.000	16.0%	7.01
Malate	2	27.6	801.2	16.2	1.000	39.7%	8.02
	4	10.3	1644.9	45.9	0.997	39.7%	7.22
	6	8.9	2170.9	50.9	0.989	38.8%	7.16

*Acetate results under flurescent lighting system.

The highest substrate conversion efficiency was consistent with $H_{\max}$ of each carbon source except malate. SCE of 6 g/L malate was slightly lower than the other concentrations. Compared to the other carbon sources, substrate conversion efficiency from butyrate was generally higher, while the highest SCE of 49.2 % was obtained at initial concentration of 2 g/L. A stable neutral pH range of 6.64 to 8.02 for hydrogen production by *R. palustris* was found according to the final pH results. The final pH of butyrate and lactate was slightly lower than acetate and malate, but no significant difference ($p = 0.109$) existed. Considered all the results including the failed hydrogen production samples, pH control was proved important for *R. palustris* to form hydrogen from carbon sources. There was no further pH control during the investigation period, except the initial pH adjustment (7.0 ± 0.2). Light and substrate conversion efficiency highly affected *R. palustris* to adjust environmental pH in order to affect hydrogen production. The relation between hydrogen production and pH self-adjustment by *R. sphaeroides* ZX-5 was previously reported by Tao (2008). However, unlike their results of a wide pH range between 5.5 to 9.5 for hydrogen production, *R. palustris* in our study required neutral system to evolve hydrogen, which was consistent with the results that most PNSB was quite sensitive to pH and had their own preferred pH range especially for hydrogen production (Lazaro *et al.* 2015). pH self adjustment was quite useful but its mechanism still required further study.

In sum, the lighting system had significant effect on hydrogen production by *R. palustris*. While under fluorescent system only acetate could be converted to hydrogen, most carbon sources (butyrate, malate and lactate) could be utilized with incandescent light. Seldom report was related to light source effect on carbon source selection for hydrogen production by PNSB. Moreover, the effects of carbon sources on hydrogen production mainly focused on lag phase, hydrogen production rate, and substrate conversion efficiency. Butyrate had the highest SCE for hydrogen evolution, in consistence with the previous studies that more reduced carbon source had higher conversion efficiency for PNSB to produce hydrogen (Koku *et al.* 2002, McKinlay and Harwood 2011). Acetate and malate were much easier to be utilized by *R. palustris* for growth, but without pH control no hydrogen was converted from them. In general, higher initial concentration resulted in longer

lag phase, so that it was suggested to use lower concentration at the beginning of biological fermentation for culture adjustment to the environment and hydrogen production. The optimal concentration for cumulative hydrogen production and SCE varied upon carbon sources. As a result, 2 g/L acetate, 2 g/L butyrate, 2.5 g/L lactate, 6 g/L malate were selected for hydrogen production evaluation under incandescent lighting system by several PNSB strains in Run 3.

5.3.1.3 Hydrogen production evaluation and carbon sources selection by several PNSB

All the four PNSB (*R. palustris*, *R. sphaeroides*, *R. capsulatus* and *R. rubrum*) could grow under both lighting systems. However, cell growth rate was much slower under florescent system, especially for *R. sphaeroides*, *R. capsulatus* and *R. rubrum*. Therefore, hydrogen production evaluation by these four PNSB was conducted using incandescent light. Figure 5.3 illustrated these four cultures could produce hydrogen with selected carbon sources. Similar to the results of *R. palustris*, limited hydrogen was produced from acetate. The consumed acetate was only used for cells growth but not for hydrogen production. Butyrate was still difficult for the other three bacteria to utilize for hydrogen production. *R. capsulatus* only produced hydrogen well from lactate, while *R. sphaeroides* preferred to utilize malate for hydrogen production. Both lactate and malate could be easily converted to hydrogen by *R. rubrum* with a relatively high hydrogen yield of 2.44 and 5.07 mol/mol, respectively. *R. palustris* had a wide range of carbon sources utilization, although hydrogen yield from malate was a bit lower than *R. sphaeroides* and *R. rubrum*. In general, hydrogen production rate had a positive correlation with hydrogen yield. The maximum hydrogen production rate in this study was 19.85 mL/L/h, achieved by *R. palustris* using butyrate as carbon source. The results showed a significant preference on substrate selection for photo fermentative hydrogen production by different PNSB, which would benefit to optimize the practical treatment and recycling on complex waste and wastewater.

Figure 5.3 Comparison on hydrogen production by *R. sphaeroides*, *R. capsulatus*, *R. rubrum* and *R. palustris* from acetate, butyrate, lactate and malate. (Columns present hydrogen yield, symbol lines indicate hydrogen production rate.)

Table 5.4 presents a further analysis of hydrogen production characteristics by these four PNSB. Higher final pH (>9.0) was observed from the limited hydrogen production condition, such as acetate fermentation, lactate fermentation by *R. capsulatus* and malate fermentation by *R. sphaeroides*. pH control would be an important strategy to maintain and improve photo fermentation. *R. palustris* had higher SCE on butyrate and lactate than the other bacteria. However, the highest SCE (84.6%) was obtained by *R. rubrum* from malate fermentation, which was more than 2-fold SCE of *R. palustris*. The carbon source efficiency comparison by different PNSB was vital for the optimization on waste treatment/recycling. According to characteristics of food waste fermentation effluents (rich in acetate, butyrate and lactate), *R. palustris* will be selected for further integrated dark and photo fermentation study.

Table 5.4 Evaluation on hydrogen production from oraginic acids by PNSB.

Carbon Source	<i>R. palustris</i>		<i>R. sphaeroides</i>		<i>R. capsulatus</i>		<i>R. rubrum</i>	
	SCE	Final pH	SCE	Final pH	SCE	Final pH	SCE	Final pH
Acetate	2.5%	9.79	3.5%	9.325	2.6%	9.94	4.4%	8.62
Butyrate	49.2%	6.80	3.9%	8.23	10.4%	8.12	8.7%	7.65
Lactate	42.9%	6.82	31.0%	7.47	5.4%	9.35	40.7%	7.14
Malate	38.8%	7.16	5.4%	9.19	77.2%	7.88	84.6%	7.38

5.3.2 Strategies to enhance photo fermentative hydrogen production

5.3.2.1 Hydrogen production by different carbon sources (acetate, butyrate, lactate)

Nongrowing PNSB (usually nitrogen-starved) are attractive as H_2 yields approaching 80% of the theoretical maximum can be achieved (Akkerman *et al.* 2002). Hydrogen production from acetate, butyrate and lactate by *R. palustris* NifA* were illustrated in Figure 5.4. Initial organic acids concentrations were determined by the composition of food waste fermentation effluents. Each carbon source had four initial concentration levels: 10, 20, 40, 60 mM acetate; 5, 10, 20, 40 mM butyrate; and 10, 20, 50, 70 mM lactate. In general, lag phase for hydrogen production from butyrate and lactate was shorter than lactate. For each carbon source, maximum cell density increased as the initial concentration increased. Within a concentration range, cell growth was slightly affected, while lag phase increased as the initial concentration increased. However, beyond the range of each carbon source, lag phase increased a lot, for instance, 70 mM lactate. As well, lower concentration limited cell growth in order to hydrogen production. Maximum cell density was less than 0.5 at initial acetate concentration of 10 mM. From the above results, it was suggested to dilute the substrate to lower concentration at the start-up of operation.

Figure 5.4 Effect of carbon sources on *R. palustris* cells growth.

Within one-week experiment period, hydrogen production from different carbon sources was summarized in Table 5.5. For acetate and lactate, within a concentration range, hydrogen production was increased as initial concentration increased. After normalization with final OD, hydrogen production efficiency from acetate was higher than lactate. For butyrate, the most hydrogen was produced at 10 mM, while hydrogen production efficiency had no significant trend according to the different concentrations. Higher concentration of butyrate had some negative effect on hydrogen production.

Table 5.5 Hydrogen production from different carbon sources by *R. palustris* NifA*.

Acetate mM	H ₂ Production mL/L	H ₂ /OD _{final} *	Butyrate mM	H ₂ Production mL/L	H ₂ /OD _{final} *	Lactate mM	H ₂ Production mL/L	H ₂ /OD _{final} *
10	260	572.7	5	160	222.8	10	340	400.5
20	568	555.3	10	792	714.8	20	570	426.3
40	890	562.2	20	628	449.7	50	660	440.0
60	393	426.6	40	100	192.7	70	280	517.6

* Hydrogen production was normalized by the final OD₆₆₀ to account for differences in cell density between cell suspensions.

5.3.2.2 Effect of yeast extract on hydrogen production

Yeast extract was a complex organic nutrient, which was favored by a lot of cultures (Alvarado-Cuevas *et al.* 2013). Trace amount of yeast extract might increase reaction rate and product production (Zhang *et al.* 2014a). *R. palustris* NifA* grew faster in PM compared to NFM (Figure 5.5), which revealed that compared to nitrogen, ammonium was preferred for cell growth. Yeast extract highly increased cell growth rate, as well lag phase was shorted to one third (~ 20h).

Figure 5.5 Effect of yeast extract on cells growth.

R. palustris NifA* could produce hydrogen when ammonium existing. However, hydrogen produced in PM was 20 % less than NFM. It might be because cell grew better in PM, so less carbon source was used for hydrogen production. Besides, yeast extract had a positive effect on hydrogen production. 52.9% and 46.5% hydrogen was increased separately in PM and NFM after adding 0.1% yeast extract.

Table 5.6 Effect of yeast extract on hydrogen production.

Medium	H ₂ production (mL/L)	Increase
PM	445.1	
PM-Y	680.6	52.9%
NFM	556.1	24.9% (compared with PM)
NFM-Y	814.8	46.5%

5.3.2.3 Non-growing study for hydrogen production enhancement

R. palustris non-growing cells shifted their metabolism from the tricarboxylic acid (TCA) cycle to glyoxylate cycle when converting acetate to hydrogen, which enabled cells to more fully oxidize acetate, providing the necessary reducing power for more H₂ yield (McKinlay *et al.* 2014). The effect of carbon source on hydrogen production by non-growing cells was studied. Acetate, butyrate and lactate were used for this study. Results in Table 5.7 show that in the same condition, non-growing cells highly increased 40 to 60% hydrogen production than growing cells. The non-growing system from lactate was illustrated in Figure 5.6 to present the relationships among substrate consumption, cell growth and hydrogen production. When cells grew to some extent, nitrogen was removed from the system, and cells began to starvation. Biomass yield consumed around 70% of acetate. After calculating the substrate conversion efficiency to hydrogen after starvation, it was high to 78.3%. From optical density result, non-growing cells grew a little bit after starvation because of the stored nutrients in the system, and then it was quite stable with slightly vibration.

Table 5.7 Hydrogen production by non-growing cells from different carbon sources.

Carbon source	Conc (mM)	H ₂ production (mL/L)		Increase
		Growing	Non-growing	
Acetate	20	567.5	806.4	42.1%
Butyrate	10	792.0	1125.0	42.0%
Lactate	20	570.0	910.7	59.8%

Figure 5.6 Hydrogen production by non-growing cells from lactate.

The concentration effect on hydrogen production by non-growing cells was investigated in Figure 5.7. Three butyrate concentration levels were selected according to the previous study to reduce lag phase. Hydrogen production was normalized for the OD₆₆₀ at the onset of nitrogen starvation to account for differences in cell density between cell suspensions. After the starvation, hydrogen production rate was only decided by the carbon concentration and cells density. Hydrogen production was more as the concentration increased. Hydrogen production rate was kept at high level until carbon was consumed. However, as it was known that nitrogenase was the gene for hydrogen production in *R. palustris* and most PNSB, cells concentration at the onset of the starvation could affect the production rate. Previous studies (Xie *et al.* 2012) reported that most nitrogenase

activities were observed in the exponential log phase of cell growth. However, there was no report about the relationship between nitrogenase activity and carbon consumption.

Figure 5.7 Hydrogen production by non-growing cells from different concentration of butyrate.

Starvation and non-growing cells could help solve the lighting penetration problem in photo fermentation because of the low cell density. As well, instead of biomass growth, more carbon electrons were converted to hydrogen, which highly increased substrate conversation efficiency. A near-theoretic maximum H_2 yield could be achieved if the system was well operated. Therefore, substrate N/C ration control is necessary for the non-growing bacteria photo-fermentation system. Zhang *et al.* (2015a) modeled the N/C ratio effect on hydrogen production by non-growing PNSB. It was reported that high N/C ratios resulted in cell growth instead of hydrogen production when bacteria using the electrons from carbon sources consumption. In addition, nitrogenase was repressed by fixed nitrogen, indicating high C/N ratio substrate was preferred (Androga *et al.* 2011, Adessi and De Philippis 2014). In conclusion, using low N/C ratio or sole carbon waste in non-growing PNSB system is a promising strategy in photo fermentative hydrogen production.

5.4 Conclusions

Two lighting systems (fluorescent and incandescent) were compared for hydrogen

production by PNSB. Incandescent lighting system was more efficient for PNSB growth and photo fermentative hydrogen production. From carbon source study, substrate selection on hydrogen production was observed by four different PNSB (*R. palustris*, *R. capsulatus*, *R. sphaeroides* and *R. rubrum*). Lactate was the preferable carbon source by *R. capsulatus*, while *R. sphaeroides* produced more hydrogen from malate. *R. rubrum* and *R. palustris* could converted several kinds of carbon sources to hydrogen but with different efficiencies. Although pH self-adjustment was observed in some conditions, pH control was very important for photo fermentative hydrogen production. Initial substrate concentration had the significant effect on lag phase duration of both cell growth and hydrogen production. Compared to the other three bacteria, *R. palustris* grew faster and showed a high capacity to use wide range of light and carbon sources. Some strategies to enhance hydrogen production by *R. palustris* NifA* were discussed. Yeast extract had a positive effect on hydrogen production, while 52.9% and 46.5% hydrogen was increased in PM and NFM after adding 0.1% yeast extract, respectively. Non-growing cells were promising to utilize for hydrogen production since it increased both the substrate conversion efficiency and lighting efficiency.

Chapter 6 Integrated dark and photo fermentation for hydrogen production from food waste

6.1 Introduction

To maximize hydrogen production, the dark and photo fermentation integration attracted more attention, because the characteristics of dark and photo fermentative bacteria. In general, dark fermentative converted carbohydrates to hydrogen and organic acids, which could be further utilized by photo fermentative bacteria. Therefore, sequential and combined dark and photo fermentation were proposed to enhance hydrogen yield (Yokoi *et al.* 1998a, Xie *et al.* 2010, Ozmihci and Kargi 2010c).

In sequential (two-step) dark and photo fermentation system (Figure 6.1a), the pretreatment of dark fermentation effluent and photo fermentation start-up were two considerable issues, due to the sensitivity of photo fermentative bacteria to light. On the other hand, combined dark and photo fermentation seemed more complex, which was a co-culture operation system (Figure 6.1b). The ratio of the initial ratio of different culture for startup, and the balance between cultures for continues hydrogen production were important for the system development. As well, limited insights study has been carried out to investigate the interspecies relationship in the co-culture systems.

The objective of this study was to develop and evaluate the integrated dark and photo fermentations system for hydrogen production. For the sequential system in Section 6.3.1, an overall performance was presented. To optimize the sequential system for hydrogen production, the pH, dilution ratio of effluent, and inoculum concentration effects were investigated and discussed. For combined system in Section 6.3.2, a sustainable co-culture system was expected to obtain for hydrogen production by investigating different D/P inoculum ratios, and then a comprehensive genomic study was conducted by using RNAseq technology. The insights study into the co-culture system could help on the further optimization of

combined dark and photo fermentation for hydrogen production, as well as an efficient and stable engineered consortium.

Figure 6.1 Schematic diagram of the integrated dark and photo fermentations system.

6.2 Materials and Methods

6.2.1 Bacteria strains and medium

C. butyricum was selected as the dark fermentative bacteria based on the previous studies. Seed cells were cultivated in RCM and incubated at 35 °C, 120 RPM. The RCM composition was described in Chapter 3.

R. palustris CGA676 NifA* and *R. palustris* CGA009 were the two photo fermentative bacteria used in this study. *R. palustris* CGA009 was a wild type strain naturally defective in uptake hydrogenase activity with a frameshift in the hupV gene. They were incubated anaerobically in front of a 60 W incandescent light bulb at 30 °C in sealed 16-mL anaerobic culture tubes containing 10 mL PM (see Chapter 5) with nitrogen headspace.

6.2.2 Experiment procedures

For sequential dark and photo fermentations system study

In this sequential system study, *C. butyricum* and *R. palustris* CGA676 NifA* were the two cultures inoculated in the dark and photo fermentations. A semi-continuous CSTR system (Figure 6.2) was applied as the 1st stage dark fermentation reactor. The dry-heating fermentor (Winpact, USA) was consist of a 3.8 L vessel with an effective working volume of 3 L, a control unit monitoring pH, DO, ORP, T and RPM, and four built-in peristaltic pumps. To study the pH effect on the sequential system for hydrogen production, two fermentors with base heating or jacket heating were used (no difference for the fermentation result between the two heating methods, data not shown). 1.5 L RCM with 5g/L glucose was filled in the fermentors. The pH was controlled at 7.0 in fermentor R1 by automatically adding 2 M NaOH, The fermentor R2 was set the beginning pH at 7.0 and then did not control pH during the fermentation process. Glucose was fed into the system to a concentration of 5g/L when it was consumed.

The 2nd stage photo fermentation study was carried out in 60 mL serum bottles to investigate the diluted ratio of effluent and photo fermentative bacteria inoculum concentration effects on hydrogen production. Collected dark fermentation effluent

(DFE) was diluted with DI water according to the proportion of 1:0 (no dilution), 1:1 and 1:4, and then packed into serum bottles (30 mL each) for autoclaving (121 °C, 15 min). The autoclaved bottles were adjusted pH to 7.0 by injecting phosphate buffer solution. The phosphate buffer was filtered by 0.2 µm membrane for sterilization. Initial inoculum concentrations were determined by OD₆₆₀ value of 0.01, 0.05, 0.1 and 0.2 in photo fermentation. After inoculation, the bottles were incubated at 35 °C and 110 rpm under 100 w/m² illumination by incandescent lamps.

Figure 6.2 Schematic diagram of the CSTR fermentor.

For combined dark and photo fermentations system study

R. palustris NifA* and CGA009 were cultivated with *C. butyricum* together for the combined system study. The photo to dark fermentative bacteria ratios according to OD were listed in Table 6.1.

Table 6.1 Compositions of different co-culture consortia (OD ratio).

Group	<i>R. palustris</i> NifA* : <i>C. butyricum</i>	Group	<i>R. palustris</i> CGA009 : <i>C. butyricum</i>
CR1	2:1	CR3	2:1
CR2	1:1	CR4	1:1

The hydrogen production potential of the co-culture groups was investigated in 27-mL sealed Hungate tubes containing 10 mL NFM by adding 5g/L glucose with

argon headspace. Each co-culture group was continuously transferred 1mL to the next generation when both bacteria grew well.

A RNAseq study was conducted to investigate the co-culture stress to the monoculture of *C. butyricum* and *R. palustris* NifA*, and substrate stress on the co-culture.

6.2.3 Analytical methods

Hydrogen, organic acids, as well as the cell growth were test according to the same methods listed in Chapter 3~5. Glucose concentration was analyzed by high performance liquid chromatography, HPLC (UltiMate 3000, Thermo Scientific Dionex, USA), with a RI detector and a 300 x 7.8 mm Aminex HPX-87H Column (Bio-Rad, USA). The mobile phase was DI water with a flow rate of 0.9 mL/min.

6.2.4 RNAseq experimental procedures

RNA extraction and purification

5 mL of the cell suspensions was used for RNA extraction in mid-exponential phase. The cell culture was centrifuged at 4000 rpm for 10 min to pellet the bacterial cells. The cell pellets were then resuspended in 1 mL QIAzol Lysis Reagent and placed into 2 ml screw-capped tubes containing approximately 1 mL of 0.1 mm zirconia/silica beads. The suspension was then treated in a BeadBeater for 1 min, cooling on ice after each period, for a total of 4 times. The broken cells and beads were added with 200 μ L chloroform and sedimented by centrifugation. 520 μ L colorless upper aqueous phase was transferred to 1.5 mL tube. 780 μ L ethanol (100%) was added to the combined cell lysate and mixed thoroughly. After that, 700 μ L sample was applied to a RNeasy Mini-column to isolate 30 μ L RNA.

DNase digestion was performed with the TURBO DNase set. The eluted RNA samples were then applied to RNeasy MinElute columns to purify the RNA. After RNA concentration was determined by Nanodrop, 1 μ g RNA was pipetted in 0.2 mL tubes and keep them at -80°C for further application.

The quality of RNA (integrity and DNA contamination) was determined with an Agilent 2200 TapeStation (Agilent, Palo Alto, CA), and by reverse transcription (RT)-PCR using the 16S rRNA-targeted primer set of 27F (AGA GTT TGA TCC TGG CTC AG) and 519R (GTA TTA CCG CGG CTG CTG). PCR samples were loaded to run agarose gel electrophoresis. Figure 6.2 shows that there was no DNA contamination with the harvested RNAs.

Figure 6.3 Agarose gel electrophoresis image of RNA samples.

cDNA library construction and sequencing

cDNA libraries were prepared using Encore Complete Prokaryotic RNA-Seq DR Multiplex System (NuGEN, San Carlos, CA) following the manufacturer's instruction. For first strand cDNA synthesis, 100 ng of total RNA was mixed with 2 μ L of primer mix v10 in a 5 μ L reaction volume, and incubated at 65 °C for 5 min. After snap chilling on ice, 2 μ L of 5x First-Strand Buffer, 2 μ L of 10 mM dNTP mix, 0.5 μ L of 0.1 M DTT, 0.5 μ L of SuperScript III Reverse Transcriptase were added, and the mixture was further incubated at 40°C for 90 min. To generate the second strand, 63 μ L of Second Strand Buffer Mix, 2 μ L of Second Strand Enzyme Mix were mixed with 10 μ L of first strand samples, and then incubated at 16 °C for 2 h. The reaction was stopped by adding 45 μ L of Second Strand Stop Buffer.

Double-stranded cDNA samples were then purified by using the MinElute PCR Purification Kit (Qiagen, Valencia, CA) and eluted with 12 μ L of Buffer EB. 10 μ L of the purified double-stranded cDNA sample was mixed with 5 μ L of ER Mix and incubated for end repair. The most important step was to add 3 μ L of appropriate Ligation Adaptor Mix to 15 μ L of the end repaired sample, mixed with 12 μ L of Ligation Mix and incubated for 30 min. After two times strand selection, adaptor cleavage and library amplification were performed. Finally, the cDNA library was purified by RNAClean XP beads and quantified with KAPA SYBR FAST qPCR kit for sequencing.

All cDNA libraries were sequenced with an Illumina HiSeq at Fred Hutch (WA, USA), and raw sequencing data were processed with CLRNASeq provided by ChunLab (Korea). Solexa reads were aligned to the reference genome sequences of *C. butyricum* (GenBank Accession number: AQQF000000000) and *R. palustris* (GenBank Accession number: BX571963). The uniquely mapped reads aligned with less than 2 mismatches to a single genomic locus were subjected to further analysis, while the reads that can not be unambiguously mapped were excluded. The above alignment approach and criteria were similar to those used in literature (McKinlay *et al.* 2014, Wei *et al.* 2014).

Data Analysis

The software CLRNASeq was used for data analysis. Three normalized methods – RPKM (reads per kilobase per million mapped reads), RLE (Relative Log Expression) and TMM (Trimmed Mean of M-values) - were provided to facilitate comparison of gene expression levels (Dillies *et al.* 2013, Maza *et al.* 2013). DESeq analysis was used to evaluate whether, for a given region, an observed difference in read counts between tx and t0 was significant. Genes were considered to be differentially expressed from levels observed immediately if they had a p value $\leq$ 0.05 and with fold change (FC) ratios $\geq$ 2.

The identified, differentially expressed genes were interpreted and discussed in the context of biological processes and functions, using eggNOG database, hierarchical clustering and KEGG. eggNOG is a database that classifies all conserved genes

according to their homologous relationships (Tseng *et al.* 2015). Hierarchical clustering uses the distance matrix of data between samples, and progressively forms clusters by merging the closest elements first (Murtagh and Contreras 2012). KEGG (Kyoto Encyclopedia of Genes and Genomes) is a database that deals with genomes, biological pathways, disease, drugs, and chemical substances.

6.3 Results and discussions

6.3.1 Sequential dark and photo fermentation

6.3.1.1 Effect of pH on semi-continuous dark fermentation

Table 6.2 presents the hydrogen production results from fed-batch dark fermentation reactors. Reactor R1 with pH control showed a stable hydrogen production and 100% substrate consumption in each cycle. An average substrate conversion efficiency (SCE) of 29.5% was achieved. Besides, in the semi-continuous run, butyrate was accumulated gradually, while acetate did not increase but a bit decrease. Acetate could be converted to other organic acids, which was reported in previous studies (De Beer *et al.* 1992). Fermentor R2 without pH control produced hydrogen in the first two cycles with an average SCE of 32.6%. In the last cycle, glucose could not be converted to hydrogen, and only a small portion (12.7%) was utilized for cells growth. VFAs had no accumulation from cycle #2 to cycle #3 in consistence with no hydrogen production result.

Table 6.2 Hydrogen production from fed-batch fermentors.

Reactor	Hydrogen production (L/L)	OD	NH ₃ -N (mg/L)	Acetate (mM)	Butyrate (mM)	Substrate consumption	Hydrogen yield (mol/mol)	SCE
1 st cycle (0~16 h)								
R1	0.97	5.9	10.3	58.57	23.03	100%	1.45	36.1%
R2	0.88	5.7	3.2	52.13	22.23	100%	1.31	32.6%
2 nd cycle (16~58h)								
R1	0.66	2.7	12.6	47.71	42.10	100%	0.98	24.5%
R2	0.88	4.8	10.7	47.06	39.75	100%	1.31	32.7%
3 rd cycle (58~85h)								
R1	0.75	2.6	19.3	46.17	62.95	100%	1.12	27.9%
R2	0	5.0	8.6	48.26	39.74	12.7%	-	-

The oxidation-reduction potential (ORP) measures the activity of electrons involved in oxidation-reduction reactions within an aqueous environment. In most complex biological systems, ORP was used to monitor oxidative conditions and biological activity (Peddie *et al.* 1990, Kishida *et al.* 2003). Figure 6.4 illustrated the monitoring profile of fed-batch hydrogen production in terms of pH and ORP. It was observed that ORP decreased when hydrogen started to produce. After hydrogen production, ORP started to increase in the biosystem. Especially, ORP in fermentor R2 continued to increase. At the beginning of the 3rd cycle, ORP had increased to around 300 and bacteria stopped to produce hydrogen in this cycle. pH in fermentor R2 was stably around 4.9 with a neglectable decrease after hydrogen production in the first cycle.

Figure 6.4 Semi-continuous hydrogen production in fermentors.

pH control in continuous dark fermentation was important that highly affected hydrogen production. ORP could monitor the system stability and hydrogen production process. Some strategies should be applied for hydrogen yield enhancement.

6.3.1.2 Effect of initial inoculum on 2nd step photo fermentation

In the second step study of the sequential dark and photo fermentation, results showed that DFE dilution was important for sequential photo fermentation process. Only the substrate diluted by 5 times could be utilized for hydrogen production. Small red dots were observed in the other two diluted solutions, that meant limited cells grew but no hydrogen produced. Meanwhile, from the result of diluted solution in Figure 6.5, initial inoculum concentration almost had no effect on hydrogen production. At the beginning, high inoculum concentration started to produce hydrogen faster. Later on they all had similar hydrogen production potential. So according to these results, OD of 0.05 was suggested as the initial inoculum concentration.

Figure 6.5 Hydrogen production by *R. palustris* from DFE.

Sequential study also revealed that initial inoculum concentration had less effect on hydrogen production compared to pH and substrate concentration. The optical density range of 0.05 to 0.1 was optimal inoculum concentration for photo fermentative hydrogen production.

6.3.1.3 Perspective in the hydrogen production from glucose and food waste

From the previous results, a system was calculated and proposed for hydrogen production using glucose and food waste. A sequential dark and photo fermentation system was investigated by *C. butyricum* and *R. palustris* NifA*. An overall sequential system hydrogen yield of 7.01 mol/mol glucose was achieved, which was 58.5% of the maximum theoretical hydrogen yield. From the stage analysis, it was observed the hydrogen yield was highly affected by the dark fermentation due to its lower substrate conversion efficiency. For food waste fermentation, 9.5 L hydrogen could be converted from 100 g-food wastes by using the sequential dark and photo fermentation system.

6.3.2 Combined dark and photo fermentations

6.3.2.1 Hydrogen production combined dark and photo fermentations

Combined dark and photo fermentation was a complex system since two different cultures were employed in the same condition (Fang *et al.* 2006, Cheng *et al.* 2015). Because of the different hydrogen production rates that dark fermentation was much faster than photo fermentation, the co-culture inoculum ratio was selected as 2:1 and 1:1 of photo and dark fermentative bacteria according to the CFU. To explain the system composition, we also used the agar plate to cultivate *C. butyricum* and checked it from the microscope. We tried to explain this phenomenon and expected a more stable co-culture system for the long term running.

At the beginning, all of the four co-culture system produced hydrogen and more than the single dark and photo fermentation. When cells grew ready (seen from the color because of *R. palustris*), the next generation was transferred and glucose was added. From physical color observation, the second generation grew slower compared to the first generation for CR2, CR3, CR4. The third generation of CR2, CR3 and CR4 stopped to produce hydrogen. From the observation, cells did not grow well except CR1. We continued to transfer CR1, cells growth image was illustrated in Figure 6.6. The fourth generation grew well in red color, while both *C. butyricum* (bigger one) and *R. palustris* NifA* (smaller one) were observed from the microscope.

Figure 6.6 Co-culture CR1 growth after 4th generation cultivation.

Table 6.3 presents the hydrogen production result by the four pairs of co-culture. For all the co-culture samples, CR4 of 1st generation produced the most hydrogen of 16.8 mL, which was 22.5% of the theoretical maximum hydrogen production from glucose. However, CR4 ceased to produce hydrogen from the 3rd generation. Among the four co-culture consortia, CR1 with *R. palustris* NifA* and *C. butyricum* (OD ratio 2:1) presented a stable hydrogen production potential. The last (6th) generation co-culture sample was used for the following RNAseq study.

Hydrogen yield from the co-culture in this study was lower than the so-far reported maximum hydrogen yield of 7.1 mol H₂/mol glucose (Asada *et al.* 2006). However, the hydrogen yields achieved by the co-culture consortia were higher than the sum hydrogen production of single dark and photo fermentation. For instance, the 1st generation of CR1 produced 15.6 mL hydrogen, which increased 92.9% than the sum hydrogen produced by *C. butyricum* in 5g/L glucose fermentation and *R. palustris* NifA* in 20 mM acetate fermentation. The overall hydrogen production could be enhanced by the future optimization study. Most importantly, the main objective of this combination system study to get a sustainable co-culture consortium had been achieved. The results also revealed that the co-operation between the two bacteria existed in the co-culture system for the converting

efficiency enhancement from glucose to hydrogen. Next, it was expected to use RNA sequencing technology to investigate the insight into the co-culture consortium.

Table 6.3 Hydrogen production by co-cultures from NFM and glucose.

Generation	CR1		CR2		CR3		CR4	
	HP (mL)*	**	HP (mL)		HP (mL)		HP (mL)	
1st	15.6	92.9%	13.8	70.6%	10.2	28.1%	16.8	111.0%
2nd	9.9	31.4%	10.8	43.3%	12.6	67.2%	10.2	35.4%
3rd	11.2	48.7%	0		0		0	
4th	12.5	65.9%						
5th	10.9	44.7%						
6th	11.6	54.0%						

* Hydrogen production per tube (mL); **Hydrogen production increase compared to the sum of hydrogen produced by single culture.

6.3.2.2 RNAseq analysis on the co-culture *C. butyricum* and *R. palustris* (CR1)

The RNA sequencing of four samples were compared and analysed in three groups: co-cultures of *C. butyricum* and *R. palustris* compared with single 1) *C. butyricum* or 2) *R. palustris* in NFM, as well as the co-cultures in NFM and PM.

- Co-culture of *C. butyricum* and *R. palustris* vs single *C. butyricum*

In this set comparison, RLE nomination was recommended for the changes analysis in global gene expression. To compare the differential expression of genes between co-culture and sole *C. butyricum* cells, fold change (FC) was computed as the ratio of the RLE value obtained for individual gene in co-culture against single culture cells. The analysis of changes in global gene expression identified 977 genes that showed a two-fold or greater increase in expression (i.e., $FC \geq 2.0$), and 922 genes with a two-fold or greater decrease (i.e., $FC \leq 0.5$) in transcript abundance for co-culture relative to sole *C. butyricum*. The eggNOG distribution for these up- and down-regulated genes in the transcriptome was determined and shown in Figure 6.7.

INFORMATION STORAGE AND PROCESSING

- [A] RNA processing and modification
 - [B] Chromatin structure and dynamics
 - [J] Translation, ribosomal structure and biogenesis
 - [K] Transcription
 - [L] Replication, recombination and repair
-

CELLULAR PROCESSES AND SIGNALING

- [D] Cell cycle control, cell division, chromosome partitioning
 - [M] Cell wall/membrane/envelope biogenesis
 - [N] Cell motility
 - [O] Posttranslational modification, protein turnover, chaperones
 - [T] Signal transduction mechanisms
 - [U] Intracellular trafficking, secretion, and vesicular transport
 - [V] Defense mechanisms
 - [Z] Cytoskeleton
-

METABOLISM

- [C] Energy production and conversion
 - [E] Amino acid transport and metabolism
 - [F] Nucleotide transport and metabolism
 - [G] Carbohydrate transport and metabolism
 - [H] Coenzyme transport and metabolism
 - [I] Lipid transport and metabolism
 - [P] Inorganic ion transport and metabolism
 - [Q] Secondary metabolites biosynthesis, transport and catabolism
-

POORLY CHARACTERIZED

- [R] General function prediction only
 - [S] Function unknown
-

Figure 6.7 eggNOG functional categories analysis on the significant regulated genes in co-culture relative to *C. butyricum* (FC>2).

After removing the two unknown categories [R] General function prediction only (equivalent to unclassified) and [S] Function unknown, the top categories for up- and down-regulated genes belong to the categories [K] Transcription for information storage and processing, and [E] Amino acid transport and metabolism, [G] Carbohydrate transport and metabolism related. Besides, categories [C] Energy production and conversion and [I] Lipid transport and metabolism that are important for the energy consuming process of biofuel end-product production, were also well represented for the up-regulated genes in the transcriptome. Figure 6.8 clearly illustrates the different expression among these categories [C, E, K, G, I] in DEG map (FC>0).

Figure 6.8 The distribution of different expression genes between co-culture (Y-axis) and *C. butyricum* (X-axis) in DEG map (FC>0).

The key enzymes employed in hydrogen production are hydrogenase and nitrogenase (McKinlay and Harwood 2010a). Especially, hydrogenase was the main enzyme *C. butyricum*, and nitrogenase was the functional gene for hydrogen production in *R. palustris*. Figure 6.9 presents the differential expression profiles between co-culture and sole *C. butyricum*. The lower profile belongs to the co-culture, while the upper one presents the expression in *C. butyricum*. Table 6.4 listed the fold changes between co-culture and *C. butyricum*. The three genes for hydrogenase (K670_RS03965 and K670_RS15110) and nitrogenase

(K670_RS16100) enhanced expression in co-culture by 3.2, 5.5, and 1.6, respectively.

Figure 6.9 Hydrogenase and nitrogenase genes expression profiles between co-culture and *C. butyricum*.

Enriched pathways analysis finds eggNOG categories with meaningful changes calculated using the Fisher Exact Test. In Enrichment Analysis, the p-value is used to designate DEG-selected categories that are significantly important. If the p-value is lower than 0.05, there is a greater probability that the selected category is of more importance, and if the p-value is higher than 0.05 the reverse would be true.

After the enrichment analysis, the following three eggNOG categories and expression levels of significantly changed genes are listed in Table 6.4. Genes for ABC transporter were also expressed more. Cell motility gene expressed well in *C. butyricum*.

Table 6.4 Significant differential gene between co-culture and *C. butyricum* (p<0.05).

Gene name	Co-culture	<i>C. butyricum</i>	FC	Annotation
<u>[C] Energy production and conversion</u>				
K670_RS03965	220.3	69.4	3.2	Iron hydrogenase
K670_RS15110	217.7	39.5	5.5	Periplasmic [Fe] hydrogenase large subunit
K670_RS00815	2.6	226.7	0.0	Ferredoxin-NADP ⁺ reductase subunit alpha
K670_RS00795	7.1	1547.5	0.0	Butanol dehydrogenase
K670_RS05840	33.5	415.4	0.1	NAD-dependent 4-hydroxybutyrate dehydrogenase
K670_RS20640	56.4	2747.4	0.0	Reverse rubrerythrin
<u>[N] Cell motility</u>				
K670_RS05235	2.6	62.5	0.0	Flagellar hook protein FlgL
<u>[P] Inorganic ion transport and metabolism</u>				
K670_RS12985	150.7	1.2	124.6	Operon proteins affect spore coat composition
K670_RS14605	15.9	0.0	-	ABC transporter permease
K670_RS07265	396.6	18.1	21.9	Sulfonate ABC transporter permease
K670_RS16100	345.5	215.8	1.6	nitrogenase iron protein
K670_RS08565	3.5	64.1	0.1	iron transporter FeoA

- Co-culture of *C. butyricum* and *R. palustris* vs single *R. palustris* NifA*

Figure 6.10 shows the up- and down- regulated genes between co-culture and *R. palustris* with significantly changes distributed in eggNOG categories. RLE values were recommended for expression comparison of individual genes in co-culture against sole culture cells. After the FC analysis in global gene expression, 1028 genes were identified with a two-fold or greater increase in expression, and 2143 genes with a two-fold or greater decrease in transcript abundance for co-culture against sole *R. palustris*. The distribution of genes in Inorganic ion transport and metabolism, Energy production and conversion, Amino acid transport and metabolism, Transcription and Lipid transport and metabolism differ significantly between co-culture and *R. palustris*. These are all the most important metabolisms for hydrogen production since photoproduction of hydrogen in PNS bacteria is primarily related to nitrogen metabolism, together with electron transport and photosynthesis (Gürkan *et al.* 2015).

Figure 6.10 eggNOG functional categories analysis on the significant regulated genes in co-culture relative to *R. palustris* (FC>2).

Two unknown categories [R] General function prediction only (equivalent to unclassified) and [S] Function unknown were removed as previous study, after that the distribution of the other top up- and down- regulated categories is illustrated in Figure 6.11. In total, 78 genes for energy production and conversion, 108 genes for amino acid transport and metabolism, 58 genes for carbohydrate transport and metabolism, 61 genes for lipid transport and metabolism, 119 genes for transcription, 102 genes for inorganic iron transport and metabolism were more expressed in co-culture relative to *R. palustris*. To point out, the genes for biosynthesis of fatty acids were up regulated.

Figure 6.11 The distribution of different expression genes between co-culture (Y-axis) and *R. palustris* (X-axis) in DEG map (FC>0).

Hydrogen consumption was a phenomenon existing in most of PNSB, which was typically mediated by uptake hydrogenases catalyzing the oxidation of hydrogen (Koku *et al.* 2002, Sinha and Pandey 2011, Das *et al.* 2014). Electrons derived from this process were then transferred to ferredoxins and cytochromes. It was reported that *R. palustris* had a cluster of 22 genes encoding a membrane-bound nickel-iron uptake hydrogenase and many accessory proteins for the synthesis and assembly of this enzyme (Rey *et al.* 2006, McKinlay *et al.* 2014).

The sequenced strain of *R. palustris* (strain CGA009) behaved as if it was a hydrogenase uptake mutant with a frameshift mutation in its *hupV* gene, which was involved in predicted hydrogen sensor protein encoding. *R. palustris* NifA* was an engineered mutant from *R. palustris* strain CGA009, which should have the same sequencing as the wild type if no other mutation happened. To understand how *R. palustris* regulated its uptake hydrogenase could avail hydrogen production enhancement. From the sequencing transcriptome, it was found both in co-culture and *R. palustris* NifA* the expressions of *hupV* (RPA0960) genes were zero. Figure 6.12 presents the expression profile of RPA0960.

Figure 6.12 Expression profile related to uptake hydrogenase in co-culture and *R. palustris* NifA*.

After the enrichment analysis, Energy production and conversion, Posttranslational modification, protein turnover, chaperones and Inorganic ion transport and metabolism these three were found with significant changes ($p \leq 0.05$). The important changed genes are listed in Table 6.5. Genes for ABC transporter were also expressed more. Nitrogenase was highly expressed in co-culture cells with a FC more than 2 for RPA1379 and RPA1437. The encoding components of hydrogenase RPA1911, RPA1501, RPA 2315 and RPA 2306 were all up-regulated in co-culture. The more genes expression of these two important enzymes was in consistence with the result that more hydrogen was produced by the co-culture. Inorganic ion transport is also very important in the electron transportation for hydrogen production, while the expression of RPA 0694 in co-culture was 28-fold increase in *R. palustris* NifA*.

Table 6.5 Significant differential gene between co-culture and *R. palustris* NifA* (p<0.05).

Gene name	Co-culture	<i>R. palustris</i>	FC	Annotation
<u>[C] Energy production and conversion</u>				
RPA1435	10.2	0.0	-	alternative nitrogenase 3 subunit beta
RPA1379	46.5	11.4	4.08	vanadium dinitrogenase subunit delta
RPA1437	17.9	6.6	2.72	nitrogenase molybdenum-iron protein subunit alpha
RPA1373	10.2	7.5	1.36	nitrogenase cofactor synthesis protein
RPA2637	51.0	40.0	1.28	nitrogenase iron-molybdenum protein subunit nifK-like protein
RPA1911	27.4	6.7	4.08	nickel-dependent hydrogenase b-type cytochrome subunit
RPA1501	242.1	152.5	1.59	coenzyme F420 hydrogenase subunit beta
RPA2315	152.2	106.6	1.43	nickel-dependent hydrogenase b-type cytochrome subunit
RPA2306	54.5	46.7	1.17	nickel-dependent hydrogenase b-type cytochrome subunit
RPA0374	203.8	57.1	3.57	Zn-containing alcohol dehydrogenase
RPA2951	273.2	243.5	1.12	NADH dehydrogenase subunit B
RPA1578	131.4	46.0	2.86	ferredoxin-NADP reductase
RPA0042	38.4	25.1	1.53	4Fe-4S ferredoxin
RPA3009	381.3	21133.4	0.02	light harvesting protein B-800-850 beta subunit C
RPA0177	16.5	1722.4	0.01	ATP synthase F0F1 subunit gamma
RPA0845	0.0	1470.9	0.00	ATP synthase F0F1 subunit C
<u>[O] Posttranslational modification, protein turnover, chaperones</u>				
RPA4572	908.2	15.4	58.86	serine protease
RPA2895	9214.6	29.5	312.15	small heat shock protein
RPA0889	43758.3	381.8	114.60	heat shock protein Hsp20
<u>[P] Inorganic ion transport and metabolism</u>				
RPA0694	172.0	6.0	28.57	carbon-phosphorus lyase complex subunit
RPA4457	159.7	88.0	1.81	sulfide dehydrogenase

- Co-culture cells in PM vs NFM

The gene expression levels between co-culture and sole bacteria cells have been discussed. Up-regulation of hydrogenase and nitrogenase consisted with the hydrogen production result in the co-culture. Nitrogen source is an important factor both for cell growth and hydrogen production. This session, ammonium stress on the gene expression in co-culture will be evaluated. Co-culture cells were grown anaerobically in light in defined mineral medium containing ammonium sulfate

(PM) and nitrogen-fixing medium (NFM) that was the same medium without added ammonium sulfate. Cells in mid-exponential phase in NFM and PM were collected for RNA sequencing analysis.

The following hierarchical clustering map shows a clear different gene expression in NFM- and PM- grown cells in Figure 6.13. Genes in the circled area were related to functional category C for Energy production and conversion. Highly up-regulated genes in PM presented the existing effects on transcriptome expression by ammonium stress. DEG figures were used to identify the detailed genes related to hydrogen production in next session.

Figure 6.13 Heat map based on hierarchical clustering analysis in NFM- and PM-grown co-culture cells.

In this set comparison, RPKM nomination was recommended for the changes analysis in global gene expression. The co-culture set was both compared with the libraries of *C. butyricum* and *R. palustris*. Based on the library of *C. butyricum*, 81 genes for Energy production and conversion, 92 genes for Carbohydrate transport and metabolism, 20 genes for Lipid transport and metabolism and 78 genes for Inorganic ion transport and metabolism were up-regulated in PM-grown cells. While 246 genes for Energy production and conversion, 123 genes for Carbohydrate transport and metabolism, 146 genes for Lipid transport and metabolism and 219 genes for Inorganic ion transport and metabolism were up-regulated in PM-grown cells relative to NFM-grown cells from the library of *R. palustris*. From the DEG figure 6.14, it was clearly to see that more genes belonging to the selected categories in PM-grown cells were up-regulated in the library of *R. palustris*. Both *C. butyricum* (AQQF000000000) and *R. palustris* (BX571963) libraries were applied for the comparison. The detailed lists of genes for the RNA sequences identified in the NFM- and PM-grown cells are presented in **Appendix Table**.

Figure 6.14 Comparison of differential regulation in PM- relative to NFM- grown cells.

Photosynthesis is one of the most important pathways involved in hydrogen production. The KEEG pathway profile in PM/NFM-grown cells is presented in Figure 6.15. The significant changes in the gene expression were found in the ATP photosynthesis part, which produces ATP from ADP in the presence of a proton gradient across the membrane.

Figure 6.15 Photosynthesis metabolism expression changes in PM-/NFM- grown cells.

Table 6.6 presents the related genes in photosynthesis metabolism. ATP synthase were highly expressed in PM-grown cells indicating more energy was produced, which could utilize more ATP for hydrogen production in photo fermentative bacteria.

Table 6.6 Gene expression changes in PM- and NFM- grown cell.

Gene name	PM	NFM	FC	eggNOG category	Annotation
<i>Photosynthesis metabolism</i>					
RPA0176	111.3	49.3	2.3	C	ATP synthase F0F1 subunit beta
RPA0177	230.5	16.5	14.0	C	ATP synthase F0F1 subunit gamma
RPA0179	1587.1	53.2	29.9	C	ATP synthase F0F1 subunit delta
RPA0844	85.4	0.0	-	C	H ⁺ -transporting two-sector ATPase subunit B/B'
RPA0846	1619.4	117.0	13.8	C	ATP synthase F0F1 subunit A
<i>Nitrogen metabolism</i>					
RPA4145	138.3	0.0	-	Q	dissimilatory nitrite reductase27
RPA4209	57.5	591.9	0.1	E	glutamine synthetase
RPA4618	1345.8	270.3	5.0	C	nitrogenase molybdenum-iron protein subunit beta
RPA4619	1711.3	269.4	6.4	C	nitrogenase molybdenum-iron protein subunit alpha
RPA4620	1805.9	272.1	6.6	P	nitrogenase iron protein

Another important pathway was the nitrogen metabolism in the co-culture system (Figure 6.16). PM medium contained enough nitrogen sources, however, in NFM medium nitrogen gas was the sole nitrogen supply. Cells grew better as the nitrogen metabolism was up regulated in PM medium. RPA4620 nitrogenase reductase in charge of providing electrons to the nitrogenase complex was highly up regulated in PM-grown cells. The other genes expression changes were listed in Table 6.6.

Figure 6.16 Nitrogen metabolism expression changes in PM-/NFM- grown cells.

6.4 Conclusion

Sequential and combined dark and photo fermentation systems were investigated in this study by using *C. butyricum* and *R. palustris* NifA*. An overall hydrogen yield of 7.01 mol/mol glucose was achieved in the sequential dark and photo fermentation system, which was 58.5% of the maximum theoretical hydrogen yield. From the stage analysis, it was observed the hydrogen yield was highly affected by the dark fermentation due to its lower substrate conversion efficiency. Sequential study also revealed that initial inoculum concentration had less effect on hydrogen production compared to pH, and initial substrate concentration. The optical density range of 0.05 to 0.1 was optimal inoculum concentration for photo fermentative hydrogen production.

A stable co-culture group CR1 was obtained from the combined dark and photo fermentation study. RNAseq technology was applied to characterize the transcriptome in the co-culture of *C. butyricum* and *R. palustris* relative to the single *C. butyricum* and *R. palustris*. This was the first study to characterize co-culture effects on the hydrogen production metabolism of *C. butyricum* and *R. palustris* at the whole genome transcriptional level. Besides, the effects of ammonium stress on co-culture were investigated. Results presented that genes employed in hydrogen production were highly regulated in co-culture cells compared with the single culture cells. The positive effect of ammonium stress on hydrogen production was investigated. Meanwhile, significant regulation of genes for energy production and conversion, lipid transport, carbohydrate transport, and inorganic iron transport were found from the genomic study. These results provide new insights into the responses of *C. butyricum* and *R. palustris* to co-culture system. Notably, the findings not only provide a robust database for investigating the functions of genes induced in co-culture system, but also identify potential targets for genetic manipulations to further improve hydrogen production by co-culture of *C. butyricum* and *R. palustris*. Further studies are required both at the translational and enzyme activity level in order to clarify the co-operation mechanism involved in the co-culture system.

To sum up, integrated dark and photo fermentations have proved an overall high hydrogen yield compared to the other biological processes, however, challenges are still existing for the real scaling-up application. For sequential dark and photo fermentation, the system management and operation is complex because of two distinct bio-systems; as well, the imbalance of the fatty acids production and consumption affects the system stability. For combined dark and photo fermentation, the reaction rate balancing in the culture consortium is infrequent; light penetration is decreased along with the biomass accumulation; further research on the sustainable co-culture system is needed. Thus, efficient bioreactors for light harvesting and constructed engineering cultures are expected to overcome the above limitations in the future study.

Chapter 7 Conclusions and Recommendation

7.1 Conclusions

After intensive research, an efficient integrated dark and photo fermentative hydrogen production system was developed in this study. Food waste was evaluated as a good candidate for biological hydrogen production. Molecular biology studies provided important insights into the complex integration system. The major findings and conclusions have been summarized as follows:

Hydrogen production potential and characteristics of four dark fermentative bacteria (*C. butyricum*, *C. pasteurianum*, *C. beijerinckii*, and *E. aerogenes*) was investigated using glucose as substrate. Dark fermentative bacteria consumed more glucose by increasing initial glucose concentration, but the glucose utilization efficiencies were varied. *C. butyricum* had higher hydrogen yield and production rate compared to the other three bacteria. At 10 g/L of glucose, the highest hydrogen yield of 3.12 mol H₂/mol glucose was achieved, with a hydrogen production rate of 18.29 mL-H₂/L-medium/h and specific hydrogen production rate of 3.90 mL-H₂/g-biomass/h. In addition, it was found that the distribution of volatile fatty acids was different among the fermentative bacteria. *C. butyricum* and *C. pasteurianum* had higher acetate to butyrate ratio compared to the other two species, which favored hydrogen generation in dark fermentation.

The comprehensive characteristics of biodegradable food waste were quantitatively analyzed. Hydrogen production potential from food waste was evaluated by the selected dark fermentative cultures. The influence of the initial pH and autoclaving on hydrogen production was investigated as well. Fermentative studies showed that pure cultures like *C. beijerinckii* could directly utilize raw food waste to produce hydrogen, while other *Clostridium* species (*C. butyricum* and *C. pasteurianum*) could produce hydrogen only after pH adjustment. Autoclaving could further enhance hydrogen yields due to increased hydrolysis of food waste. The maximum hydrogen yield was achieved by *C. butyricum* (38.9 mL-H₂/g-VS_{added}) after autoclaving food waste with pH adjustment at 7. In addition, the decreased acetic to

butyric acid ratio indicated butyrate metabolic pathway was favored in the fermentation from autoclaved food waste.

Specific mixed-culture study presented the synergy among different bacteria could enhance hydrogen production and improve the system stability. In the glucose fermentation, the co-operation among *C. butyricum*, *C. pasteurianum*, and *C. beijerinckii* had a significant positive effect on the enhancement of hydrogen production. Cumulative hydrogen production was obviously increased in the co-culture consortium of *C. butyricum* + *C. pasteurianum* and *C. butyricum* + *C. beijerinckii*. More organic acids were produced in the mixed-cultures community, especially an increase of 50% acetate observed in the co-culture *C. butyricum* + *C. pasteurianum*. In food waste fermentation study, only the co-culture groups of *C. butyricum* + *C. beijerinckii*, *C. pasteurianum* + *C. beijerinckii*, and MIX group had considerable hydrogen evolution. In terms of the mixed-culture consortium, *C. beijerinckii* showed a critical role in the conversion of food waste to hydrogen, although *C. butyricum* produced most hydrogen among all the bacteria. Besides, a significant decrease in acetate to butyrate ratio was observed in food waste fermentation. MIX group with all the four dark fermentative bacteria did not have a good performance on hydrogen production that further indicated the synergistic and inhibitive effects between microorganisms. No significant changes on the hydrogen content in biogas, final pH, and ammonium production was observed from the present mixed-culture study.

In photo fermentation study, incandescent lighting system was more efficient for PNSB growth and hydrogen evolution than fluorescent lighting system. Substrate selection characteristics on hydrogen production were observed from four different PNSB (*R. palustris*, *R. capsulatus*, *R. sphaeroides* and *R. rubrum*). *R. palustris* grew faster and showed a high capacity to use wide range of light and carbon sources. Although pH self-adjustment was observed in some conditions, pH control was extremely important for photo fermentative hydrogen production. A mutant strain *R. palustris*NifA* was studied, which was capable of coping with the inhibition of ammonium existing to hydrogen production. Lower concentration of carbon sources could shorten the lag phase and was suggested to apply at the start-up stage. 1%

yeast extract had a 40 - 50% enhancement on hydrogen production, and also lag phase was obviously reduced. Substrate conversion efficiency of 78.3% was achieved in nitrogen starvation study by non-growing cells, while 42.0 – 59.8% more hydrogen was produced from acetate, butyrate and lactate. Besides, because of the stable low cell density lighting efficiency was highly improved. It was suggested to apply low N/C or sole carbon waste in the non-growing photo fermentation system.

A sequential dark and photo fermentation system was investigated by *C. butyricum* and *R. palustris* NifA*. An overall system hydrogen yield of 7.01 mol/mol glucose was achieved, which was 58.5% of the maximum theoretical hydrogen yield. From the stage analysis, it was observed the hydrogen yield was highly affected by the dark fermentation due to its lower substrate conversion efficiency. Sequential study also revealed that initial inoculum concentration had less effect on hydrogen production compared to pH, and initial substrate concentration. The optical density range of 0.05 to 0.1 was optimal inoculum concentration for photo fermentative hydrogen production.

The co-culture of *C. butyricum* and *R. palustris* NifA* was applied in the combined dark and photo fermentation to improve hydrogen production. After several generations cultivation, CR1 group (2:1 of *R. palustris* NifA*: *C. butyricum*) continued to produce hydrogen with higher overall hydrogen yield. An evaluation on how gene expression differs in co-culture system as opposed to single pure bacteria was conducted by using RNA sequencing technology. A large proportion of genes were consistently regulated in both the co-culture system and pure cultures. Genes regulated in hydrogen production were highly expressed in co-culture cells compared with the single culture cells. The positive effect of ammonium stress on hydrogen production was investigated. Meanwhile, significant regulation of genes for energy production and conversion, lipid transport, carbohydrate transport, and inorganic iron transport were found from the genomic study. These results provide new insights into the responses of *C. butyricum* and *R. palustris* to co-culture system. Taken together, this study reported the first transcriptome study on co-culture of dark and photo fermentative bacteria and

provided insights into the complex co-culture consortium system.

7.2 Recommendation

The following aspects are recommended for future studies:

- ✚ The relationship of hydrogen production and food waste characteristics study. In the present study, food waste has been proved as renewable source for hydrogen production. However, a further detailed characteristics study on food waste will definitely help improve hydrogen production and also extract other valuable byproducts. Food waste might be varied because of batch by batch collection, therefore, the control strategies for more fermentative hydrogen production should be adjusted accordingly.
- ✚ Design an effective outdoor fermentation reactor. The use of sunlight as the light source will award the photo fermentative hydrogen production and the integrated fermentation process. Since light is one of the most important factor affecting photo fermentation, an efficient reactor with more light penetration and compact configure will drive the hydrogen market.
- ✚ Further study on gene expression and transcriptome. Large information could be unveiled and obtained from transcriptome. From the environmental engineering perspective, it could help explore and improve the biological process. For biological hydrogen production process, it could help construct the more efficient bacteria consortium and optimize the process from the gene expression analysis. As well, qPCR test is highly recommended for quantitative study in the mixed cultures system.

REFERENCES

- Adessi, A. and De Philippis, R. (2014), "Photobioreactor design and illumination systems for H₂ production with anoxygenic photosynthetic bacteria: a review", International Journal of Hydrogen Energy, Vol. 39, No. 7, pp. 3127-3141.
- Aguilar, M. A. R., Fdez-Güelfo, L. A., *et al.* (2013), "Effect of HRT on hydrogen production and organic matter solubilization in acidogenic anaerobic digestion of OFMSW", Chemical Engineering Journal, Vol. 219, pp. 443-449.
- Ahn, Y., Park, E.-J., *et al.* (2005), "Biofilm microbial community of a thermophilic trickling biofilter used for continuous biohydrogen production", FEMS Microbiology Letters, Vol. 249, No. 1, pp. 31-38.
- Akkerman, I., Janssen, M., *et al.* (2002), "Photobiological hydrogen production: photochemical efficiency and bioreactor design", International Journal of Hydrogen Energy, Vol. 27, No. 11–12, pp. 1195-1208.
- Alshiyab, H., Kalil, M. S., *et al.* (2008), "Trace metal effect on hydrogen production using *C. acetobutylicum*", OnLine Journal of Biological Sciences, Vol. 8, No. 1, pp. 1-9.
- Alvarado-Cuevas, Z. D., Acevedo, L. G. O., *et al.* (2013), "Nitrogen sources impact hydrogen production by *Escherichia coli* using cheese whey as substrate", New Biotechnology, Vol. 30, No. 6, pp. 585-590.
- Androga, D. D., Özgür, E., *et al.* (2011), "Significance of carbon to nitrogen ratio on the long-term stability of continuous photofermentative hydrogen production", International Journal of Hydrogen Energy, Vol. 36, No. 24, pp. 15583-15594.
- Antal, T. K., Krendeleva, T. E., *et al.* (2011), "Acclimation of green algae to sulfur deficiency: underlying mechanisms and application for hydrogen production", Applied Microbiology and Biotechnology, Vol. 89, No. 1, pp. 3-15.
- Argun, H. and Kargi, F. (2010a), "Bio-hydrogen production from ground wheat starch by continuous combined fermentation using annular-hybrid bioreactor", International Journal of Hydrogen Energy, Vol. 35, No. 12, pp. 6170-6178.
- Argun, H. and Kargi, F. (2010b), "Effects of light source, intensity and lighting regime on bio-hydrogen production from ground wheat starch by combined dark and photo-fermentations", International Journal of Hydrogen Energy, Vol. 35, No. 4, pp. 1604-1612.
- Argun, H. and Kargi, F. (2011), "Bio-hydrogen production by different operational modes of dark and photo-fermentation: An overview", International Journal of Hydrogen Energy, Vol. 36, No. 13, pp. 7443-7459.

Argun, H., Kargi, F., *et al.* (2008a), "Light fermentation of dark fermentation effluent for bio-hydrogen production by different *Rhodobacter* species at different initial volatile fatty acid (VFA) concentrations", International Journal of Hydrogen Energy, Vol. 33, No. 24, pp. 7405-7412.

Argun, H., Kargi, F., *et al.* (2009a), "Effects of the substrate and cell concentration on bio-hydrogen production from ground wheat by combined dark and photo-fermentation", International Journal of Hydrogen Energy, Vol. 34, No. 15, pp. 6181-6188.

Argun, H., Kargi, F., *et al.* (2009b), "Hydrogen production by combined dark and light fermentation of ground wheat solution", International Journal of Hydrogen Energy, Vol. 34, No. 10, pp. 4305-4311.

Argun, H., Kargi, F., *et al.* (2008b), "Biohydrogen production by dark fermentation of wheat powder solution: Effects of C/N and C/P ratio on hydrogen yield and formation rate", International Journal of Hydrogen Energy, Vol. 33, No. 7, pp. 1813-1819.

Arimi, M. M., Knodel, J., *et al.* (2015), "Strategies for improvement of biohydrogen production from organic-rich wastewater: a review", Biomass and Bioenergy, Vol. 75, pp. 101-118.

Asada, Y., Tokumoto, M., *et al.* (2006), "Hydrogen production by co-cultures of *Lactobacillus* and a photosynthetic bacterium, *Rhodobacter sphaeroides* RV", International Journal of Hydrogen Energy, Vol. 31, No. 11, pp. 1509-1513.

Azwar, M., Hussain, M., *et al.* (2014), "Development of biohydrogen production by photobiological, fermentation and electrochemical processes: A review", Renewable and Sustainable Energy Reviews, Vol. 31, pp. 158-173.

Baghchehsaraee, B., Nakhla, G., *et al.* (2008), "The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures", International Journal of Hydrogen Energy, Vol. 33, No. 15, pp. 4064-4073.

Balat, M. (2008), "Potential importance of hydrogen as a future solution to environmental and transportation problems", International Journal of Hydrogen Energy, Vol. 33, No. 15, pp. 4013-4029.

Barbosa, M. J., Rocha, J. M. S., *et al.* (2001), "Acetate as a carbon source for hydrogen production by photosynthetic bacteria", Journal of Biotechnology, Vol. 85, No. 1, pp. 25-33.

Basak, N. and Das, D. (2007), "The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: The present state of the art", World Journal of Microbiology and Biotechnology, Vol. 23, No. 1, pp. 31-42.

Benemann, J. (1996), "Hydrogen biotechnology: progress and prospects", Nature Biotechnology, Vol. 14, pp. 1101-1103.

- Benemann, J. R. (1997), "Feasibility analysis of photobiological hydrogen production", International Journal of Hydrogen Energy, Vol. 22, pp. 979-987.
- Berberoğlu, H., Jay, J., *et al.* (2008), "Effect of nutrient media on photobiological hydrogen production by *Anabaena variabilis* ATCC 29413", International Journal of Hydrogen Energy, Vol. 33, No. 4, pp. 1172-1184.
- Bisaillon, A., Turcot, J., *et al.* (2006), "The effect of nutrient limitation on hydrogen production by batch cultures of *Escherichia coli*", International Journal of Hydrogen Energy, Vol. 31, No. 11, pp. 1504-1508.
- Bishop, N. I., Frick, M., *et al.* (1977), "Photohydrogen production in green algae: Water serves as the primary substrate for hydrogen and oxygen production", Biological Solar Energy Conversion, Vol. 1, pp. 3-22.
- Boran, E., Özgür, E., *et al.* (2009), "Investigation of influencing factors for biological hydrogen production by *R. capsulatus* in tubular photo-bioreactors", Chemical Engineering Transactions, Vol. 18, pp. 357-362.
- Brenner, D. J., Boone, D. R., *et al.* (2005). Bergey's manual of systematic bacteriology Boston, MA : Bergey's Manual Trust. Second Edition.
- Brentner, L. B., Peccia, J., *et al.* (2010), "Challenges in developing biohydrogen as a sustainable energy source: implications for a research agenda", Environmental Science & Technology, Vol. 44, No. 7, pp. 2243-2254.
- Brosseau, J. D., Kosaric, N., *et al.* (1980), "The effect of pH on hydrogen production with *Citrobacter intermedius*", Biotechnology Letters, Vol. 2, No. 3, pp. 93-98.
- Bueno, E., Mesa, S., *et al.* (2012), "Bacterial adaptation of respiration from oxic to microoxic and anoxic conditions: redox control", Antioxidants & Redox Signaling, Vol. 16, No. 8, pp. 819-852. .
- Buitrón, G. and Carvajal, C. (2010), "Biohydrogen production from Tequila vinasses in an anaerobic sequencing batch reactor: effect of initial substrate concentration, temperature and hydraulic retention time", Bioresource Technology, Vol. 101, No. 23, pp. 9071-9077.
- Cai, G., Jin, B., *et al.* (2010), "Metabolic flux analysis of hydrogen production network by *Clostridium butyricum* W5: Effect of pH and glucose concentrations", International Journal of Hydrogen Energy, Vol. 35, No. 13, pp. 6681-6690.
- Cai, M. L., Liu, J. X., *et al.* (2004), "Enhanced biohydrogen production from sewage sludge with alkaline pretreatment", Environmental Science & Technology, Vol. 38, No. 11, pp. 3195-3202.
- Cantrell, K. B., Ducey, T., *et al.* (2008), "Livestock waste-to-bioenergy generation opportunities", Bioresource Technology, Vol. 99, No. 17, pp. 7941-7953.

- Carlozzi, P. (2009), "The effect of irradiance growing on hydrogen photoevolution and on the kinetic growth in *Rhodospseudomonas palustris*, strain 42OL", International Journal of Hydrogen Energy, Vol. 34, No. 19, pp. 7949-7958.
- Carrieri, D., Wawrousek, K., *et al.* (2011), "The role of the bidirectional hydrogenase in cyanobacteria", Bioresource Technology, Vol. 102, No. 18, pp. 8368-8377.
- Chader, S., Hacene, H., *et al.* (2009), "Study of hydrogen production by three strains of *Chlorella* isolated from the soil in the Algerian Sahara", International Journal of Hydrogen Energy, Vol. 34, No. 11, pp. 4941-4946.
- Chang, F.-Y. and Lin, C.-Y. (2004), "Biohydrogen production using an up-flow anaerobic sludge blanket reactor", International Journal of Hydrogen Energy, Vol. 29, No. 1, pp. 33-39.
- Chang, J.-S., Lee, K.-S., *et al.* (2002), "Biohydrogen production with fixed-bed bioreactors", International Journal of Hydrogen Energy, Vol. 27, No. 11, pp. 1167-1174.
- Chen, C.-Y., Yang, M.-H., *et al.* (2008a), "Biohydrogen production using sequential two-stage dark and photo fermentation processes", International Journal of Hydrogen Energy, Vol. 33, No. 18, pp. 4755-4762.
- Chen, C. C., Lin, C. Y., *et al.* (2001), "Kinetics of hydrogen production with continuous anaerobic cultures utilizing sucrose as the limiting substrate", Applied Microbiology and Biotechnology, Vol. 57, No. 1-2, pp. 56-64.
- Chen, S., Lee, K., *et al.* (2008b), "Batch and continuous biohydrogen production from starch hydrolysate by *Clostridium* species", International Journal of Hydrogen Energy, Vol. 33, No. 7, pp. 1803-1812.
- Chen, W., Tseng, Z., *et al.* (2005), "Fermentative hydrogen production with CGS5 isolated from anaerobic sewage sludge", International Journal of Hydrogen Energy, Vol. 30, No. 10, pp. 1063-1070.
- Chen, X., Sun, Y., *et al.* (2006), "Stoichiometric analysis of biological hydrogen production by fermentative bacteria", International Journal of Hydrogen Energy, Vol. 31, No. 4, pp. 539-549.
- Cheng, J., Ding, L., *et al.* (2015), "Hydrogen production using amino acids obtained by protein degradation in waste biomass by combined dark-and photo-fermentation", Bioresource Technology, Vol. 179, pp. 13-19.
- Collet, C. (2004), "Hydrogen production by *Clostridium thermolacticum* during continuous fermentation of lactose", International Journal of Hydrogen Energy, Vol. 29, No. 14, pp. 1479-1485.

- Dabrock, B., Bahl, H., *et al.* (1992), "Parameters affecting solvent production by *Clostridium pasteurianum*", Applied and Environmental Microbiology, Vol. 58, No. 4, pp. 1233-1239.
- Das, D., Khanna, N., *et al.* (2014). Biohydrogen production: fundamentals and technology advances, CRC Press.
- De Beer, D., Huisman, J., *et al.* (1992), "The effect of pH profiles in methanogenic aggregates on the kinetics of acetate conversion", Water Research, Vol. 26, No. 10, pp. 1329-1336.
- Dillies, M.-A., Rau, A., *et al.* (2013), "A comprehensive evaluation of normalization methods for Illumina high-throughput RNA sequencing data analysis", Briefings in Bioinformatics, Vol. 14, No. 6, pp. 671-683.
- Ding, J., Liu, B.-F., *et al.* (2009), "Hydrogen production from glucose by co-culture of *Clostridium butyricum* and immobilized *Rhodopseudomonas faecalis* RLD-53", International Journal of Hydrogen Energy, Vol. 34, No. 9, pp. 3647-3652.
- Dubois, M., Gilles, K. A., *et al.* (1956), "Colorimetric method for determination of sugars and related substances", Analytical Chemistry, Vol. 28, No. 3, pp. 350-356.
- Dungey, J. W. (1951), "Photoreduction in algae", Nature, Vol. 167, pp. 1030.
- Dutta, S. (2014), "A review on production, storage of hydrogen and its utilization as an energy resource", Journal of Industrial and Engineering Chemistry, Vol. 20, No. 4, pp. 1148-1156.
- Eaton, A. D. and Franson, M. A. H. (2005). Standard methods for the examination of water & wastewater, American Public Health Association.
- Elbeshbishy, E., Hafez, H., *et al.* (2011), "Single and combined effect of various pretreatment methods for biohydrogen production from food waste", International Journal of Hydrogen Energy, Vol. 36, No. 17, pp. 11379-11387.
- Elbeshbishy, E., Hafez, H., *et al.* (2012), "Viability of ultrasonication of food waste for hydrogen production", International Journal of Hydrogen Energy, Vol. 37, No. 3, pp. 2960-2964.
- Eroglu, E. and Melis, A. (2011), "Photobiological hydrogen production: recent advances and state of the art", Bioresource Technology, Vol. 102, No. 18, pp. 8403-8413.
- Espinoza-Escalante, F. M., Pelayo-Ortiz, C., *et al.* (2008), "Multiple response optimization analysis for pretreatments of Tequila's stillages for VFAs and hydrogen production", Bioresource Technology, Vol. 99, No. 13, pp. 5822-5829.
- Evens, T. J., Chapman, D. J., *et al.* (2000), "An analytical flat-plate photobioreactor with a spectrally attenuated light source for the incubation of phytoplankton under dynamic light regimes", Hydrobiologia, Vol. 434, No. 1-3, pp. 55-62.

- Evvyernie, D., Morimoto, K., *et al.* (2001), "Conversion of chitinous wastes to hydrogen gas by *Clostridium paraputrificum* M-21", Journal of Bioscience and Bioengineering, Vol. 91, No. 4, pp. 339-343.
- Fang, H., Zhang, T., *et al.* (2002a), "Microbial diversity of a mesophilic hydrogen-producing sludge", Applied Microbiology and Biotechnology, Vol. 58, No. 1, pp. 112-118.
- Fang, H. H. P. and Liu, H. (2002b), "Effect of pH on hydrogen production from glucose by a mixed culture", Bioresource Technology, Vol. 82, No. 1, pp. 87-93.
- Fang, H. H. P., Zhu, H., *et al.* (2006), "Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides*", International Journal of Hydrogen Energy, Vol. 31, No. 15, pp. 2223-2230.
- Fascetti, E. and Todini, O. (1995), "*Rhodobacter sphaeroides* RV cultivation and hydrogen production in a one- and two-stage chemostat", Applied Microbiology and Biotechnology, Vol. 44, No. 3-4, pp. 300-305.
- Ferchichi, M., Crabbe, E., *et al.* (2005), "Influence of culture parameters on biological hydrogen production by *Clostridium saccharoperbutylacetonicum* ATCC 27021", World Journal of Microbiology and Biotechnology, Vol. 21, No. 6-7, pp. 855-862.
- Ferreira, A. F., Marques, A. C., *et al.* (2012), "Biological hydrogen production by *Anabaena* sp.–yield, energy and CO₂ analysis including fermentative biomass recovery", International Journal of Hydrogen Energy, Vol. 37, No. 1, pp. 179-190.
- Fukui, T., Atomi, H., *et al.* (2005), "Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes", Genome Research, Vol. 15, No. 3, pp. 352-363.
- Gaffron, H. (1940), "Carbon dioxide reduction with molecular hydrogen in green algae", American Journal of Botany, Vol. 27, pp. 273-283.
- Gardini, F., Martuscelli, M., *et al.* (2001), "Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*", International Journal of Food Microbiology, Vol. 64, No. 1, pp. 105-117.
- Gebicki, J., Modigell, M., *et al.* (2009), "Development of photobioreactors for anoxygenic production of hydrogen by purple bacteria", Chemical Engineering Transactions, Vol. 18, pp. 363-366.
- Ghimire, A., Frunzo, L., *et al.* (2015), "A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products", Applied Energy, Vol. 144, pp. 73-95.

Ginkel, S. V., Sung, S., *et al.* (2001), "Biohydrogen production as a function of pH and substrate concentration", Environmental Science & Technology, Vol. 35, No. 24, pp. 4726-4730.

Glass, C. and Bozzi, D. (2000), "Biogas production from anaerobic digestion of processed municipal solid waste", Hazardous and Industry Wastes, Vol. 32, pp. 422-431.

Guo, X. M., Trably, E., *et al.* (2010), "Hydrogen production from agricultural waste by dark fermentation: a review", International Journal of Hydrogen Energy, Vol. 35, No. 19, pp. 10660-10673.

Gürkan, M., Erkal, N. A., *et al.* (2015), "Transcriptional Profiling of Hydrogen Production Metabolism of *Rhodobacter capsulatus* under Temperature Stress by Microarray Analysis", International Journal of Molecular Sciences, Vol. 16, No. 6, pp. 13781-13797.

Hadicke, O., Grammel, H., *et al.* (2011), "Metabolic network modeling of redox balancing and biohydrogen production in purple nonsulfur bacteria", BMC Systems Biology, Vol. 5, pp. 150.

Hallenbeck, P. C. (2005), "Fundamentals of the fermentative production of hydrogen", Water Science and Technology, Vol. 52, No. 1-2, pp. 21-29.

Hallenbeck, P. C. and Benemann, J. R. (2002), "Biological hydrogen production: fundamentals and limiting processes", International Journal of Hydrogen Energy, Vol. 27, No. 11-12, pp. 1185-1193.

Hallenbeck, P. C. and Ghosh, D. (2012), "Improvements in fermentative biological hydrogen production through metabolic engineering", Journal of Environmental Management, Vol. 95, No., pp. S360-S364.

Han, S.-K. and Shin, H.-S. (2004a), "Biohydrogen production by anaerobic fermentation of food waste", International Journal of Hydrogen Energy, Vol. 29, No. 6, pp. 569-577.

Han, S.-K. and Shin, H.-S. (2004b), "Performance of an innovative two-stage process converting food waste to hydrogen and methane", Journal of the Air & Waste Management Association, Vol. 54, No. 2, pp. 242-249.

Hawkes, F. R., Dinsdale, R., *et al.* (2002), "Sustainable fermentative hydrogen production: challenges for process optimisation", International Journal of Hydrogen Energy, Vol. 27, No. 11, pp. 1339-1347.

Howarth, D. and Codd, G. A. (1985), "The uptake and production of molecular hydrogen by unicellular cyanobacteria", Journal of General Microbiology, Vol. 131, No. 7, pp. 1561-1569.

Hsiao, C.-L., Chang, J.-J., *et al.* (2009), "*Clostridium* strain co-cultures for biohydrogen production enhancement from condensed molasses fermentation

solubles", International Journal of Hydrogen Energy, Vol. 34, No. 17, pp. 7173-7181.

Hu, C. C., Giannis, A., *et al.* (2014), "Evaluation of hydrogen producing cultures using pretreated food waste", International Journal of Hydrogen Energy, Vol. 39, No. 33, pp. 19337-19342.

Huang, J. J., Heiniger, E. K., *et al.* (2010), "Production of hydrogen gas from light and the inorganic electron donor thiosulfate by *Rhodopseudomonas palustris*", Applied and Environmental Microbiology, Vol. 76, No. 23, pp. 7717-7722.

Hussy, I., Hawkes, F. R., *et al.* (2005), "Continuous fermentative hydrogen production from sucrose and sugarbeet", International Journal of Hydrogen Energy, Vol. 30, No. 5, pp. 471-483.

Hwang, J.-H., Choi, J.-A., *et al.* (2011), "Feasibility of hydrogen production from ripened fruits by a combined two-stage (dark/dark) fermentation system", Bioresource Technology, Vol. 102, No. 2, pp. 1051-1058.

Hwang, M. H., Jang, N. J., *et al.* (2004), "Anaerobic bio-hydrogen production from ethanol fermentation: the role of pH", Journal of Biotechnology, Vol. 111, No. 3, pp. 297-309.

Iacovidou, E., Ohandja, D.-G., *et al.* (2012), "Food waste disposal units in UK households: The need for policy intervention", Science of the Total Environment, Vol. 423, pp. 1-7.

Ibrahim, N., Yusoff, D., *et al.* (2011). Food waste characteristics after autoclaving treatment. Proceedings of International Conference on Biotechnology and Food Science (ICBFS 2011).

Jayasinghearachchi, H. S., Sarma, P. M., *et al.* (2009), "Fermentative hydrogen production by two novel strains of *Enterobacter aerogenes* HGN-2 and HT 34 isolated from sea buried crude oil pipelines", International Journal of Hydrogen Energy, Vol. 34, No. 17, pp. 7197-7207.

Jayasinghearachchi, H. S., Singh, S., *et al.* (2010), "Fermentative hydrogen production by new marine *Clostridium amygdalinum* strain C9 isolated from offshore crude oil pipeline", International Journal of Hydrogen Energy, Vol. 35, No. 13, pp. 6665-6673.

Jeong, T. Y., Cha, G. C., *et al.* (2008), "Comparison of hydrogen production by four representative hydrogen-producing bacteria", Journal of Industrial and Engineering Chemistry, Vol. 14, No. 3, pp. 333-337.

Jiao, Y., Navid, A., *et al.* (2012), "Syntrophic metabolism of a co-culture containing *Clostridium cellulolyticum* and *Rhodopseudomonas palustris* for hydrogen production", International Journal of Hydrogen Energy, Vol. 37, No. 16, pp. 11719-11726.

- Jo, J. H., Lee, D. S., *et al.* (2008), "Optimization of key process variables for enhanced hydrogen production by *Enterobacter aerogenes* using statistical methods", Bioresource Technology, Vol. 99, No. 6, pp. 2061-2066.
- Jung, G. Y., Kim, J. R., *et al.* (1999), "A new chemoheterotrophic bacterium catalyzing water-gas shift reaction", Biotechnology Letters, Vol. 21, No. 10, pp. 869-873.
- Jung, K.-W., Kim, D.-H., *et al.* (2010), "Continuous fermentative hydrogen production from coffee drink manufacturing wastewater by applying UASB reactor", International Journal of Hydrogen Energy, Vol. 35, No. 24, pp. 13370-13378.
- Jung, K.-W., Kim, D.-H., *et al.* (2011), "Fermentative hydrogen production from *Laminaria japonica* and optimization of thermal pretreatment conditions", Bioresource Technology, Vol. 102, No. 3, pp. 2745-2750.
- Kaidi, F., Rihani, R., *et al.* (2012), "Photobioreactor design for hydrogen production", Procedia Engineering, Vol. 33, pp. 492-498.
- Kalia, V. C., Jain, S. R., *et al.* (1994), "Fermentation of biowaste to H₂ by *Bacillus licheniformis*", World Journal of Microbiology and Biotechnology, Vol. 10, No. 2, pp. 224-227.
- Kanai, T., Imanaka, H., *et al.* (2005), "Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1", Journal of Biotechnology, Vol. 116, No. 3, pp. 271-282.
- Kapdan, I. K. and Kargi, F. (2006), "Bio-hydrogen production from waste materials", Enzyme and Microbial Technology, Vol. 38, No. 5, pp. 569-582.
- Kargi, F. and Ozmihci, S. (2010), "Effects of dark/light bacteria ratio on bio-hydrogen production by combined fed-batch fermentation of ground wheat starch", Biomass and Bioenergy, Vol. 34, No. 6, pp. 869-874.
- Kars, G., Gündüz, U., *et al.* (2008), "Improved hydrogen production by uptake hydrogenase deficient mutant strain of *Rhodobacter sphaeroides* OU 001", International Journal of Hydrogen Energy, Vol. 33, No. 12, pp. 3056-3060.
- Kataoka, N., Miya, A., *et al.* (1997), "Studies on hydrogen production by continuous culture system of hydrogen-producing anaerobic bacteria", Water Science and Technology, Vol. 36, No. 6, pp. 41-47.
- Keskin, T., Abo-Hashesh, M., *et al.* (2011), "Photofermentative hydrogen production from wastes", Bioresource Technology, Vol. 102, No. 18, pp. 8557-8568.
- Kessler, E. (1974). Hydrogenase, photoreduction, and anaerobic growth of algae. Oxford, University of California Press.

- Kim, D.-H., Han, S.-K., *et al.* (2006a), "Effect of gas sparging on continuous fermentative hydrogen production", International Journal of Hydrogen Energy, Vol. 31, No. 15, pp. 2158-2169.
- Kim, D.-H., Son, H., *et al.* (2012a), "Effect of substrate concentration on continuous photo-fermentative hydrogen production from lactate using *Rhodobacter sphaeroides*", International Journal of Hydrogen Energy, Vol. 37, No. 20, pp. 15483-15488.
- Kim, J. K., Nhat, L., *et al.* (2008a), "Hydrogen production conditions from food waste by dark fermentation with *Clostridium beijerinckii* KCTC 1785", Biotechnology and Bioprocess Engineering, Vol. 13, No. 4, pp. 499-504.
- Kim, M., Baek, J., *et al.* (2006b), "Hydrogen production from *Chlamydomonas reinhardtii* biomass using a two-step conversion process: Anaerobic conversion and photosynthetic fermentation", International Journal of Hydrogen Energy, Vol. 31, No. 6, pp. 812-816.
- Kim, M., Yang, Y., *et al.* (2012b), "Hydrogen production by anaerobic co-digestion of rice straw and sewage sludge", International Journal of Hydrogen Energy, Vol. 37, No. 4, pp. 3142-3149.
- Kim, M.-K. and Harwood, C. S. (1991), "Regulation of benzoate-CoA ligase in *Rhodospseudomonas palustris*", FEMS Microbiology Letters, Vol. 83, No. 2, pp. 199-203.
- Kim, S. (2004), "Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge", International Journal of Hydrogen Energy, Vol. 29, No. 15, pp. 1607-1616.
- Kim, S., Seol, E., *et al.* (2008b), "Various hydrogenases and formate-dependent hydrogen production in *Citrobacter amalonaticus* Y19", International Journal of Hydrogen Energy, Vol. 33, No. 5, pp. 1509-1515.
- Kim, S.-H., Han, S.-K., *et al.* (2004), "Feasibility of biohydrogen production by anaerobic co-digestion of food waste and sewage sludge", International Journal of Hydrogen Energy, Vol. 29, No. 15, pp. 1607-1616.
- Kim, Y., Jo, J. H., *et al.* (2006c), "Fermentative hydrogen production from food waste", Studies in Surface Science and Catalysis, Vol. 159, pp. 149-152.
- Kishida, N., Kim, J.-H., *et al.* (2003), "Effectiveness of oxidation-reduction potential and pH as monitoring and control parameters for nitrogen removal in swine wastewater treatment by sequencing batch reactors", Journal of Bioscience and Bioengineering, Vol. 96, No. 3, pp. 285-290.
- Kivistö, A., Santala, V., *et al.* (2010), "Hydrogen production from glycerol using halophilic fermentative bacteria", Bioresource Technology, Vol. 101, No. 22, pp. 8671-8677.

- Koku, H., Eroğlu, İ., *et al.* (2002), "Aspects of the metabolism of hydrogen production by *Rhodobacter Sphaeroides*", International Journal of Hydrogen Energy, Vol. 27, No. 11–12, pp. 1315-1329.
- Kotay, S. M. and Das, D. (2007), "Microbial hydrogen production with *Bacillus coagulans* IIT-BT S1 isolated from anaerobic sewage sludge", Bioresource Technology, Vol. 98, No. 6, pp. 1183-1190.
- Kraemer, J. T. and Bagley, D. M. (2007), "Improving the yield from fermentative hydrogen production", Biotechnology Letters, Vol. 29, No. 5, pp. 685-695.
- Kumar, G. R. and Chowdhary, N. (2016), "Biotechnological and bioinformatics approaches for augmentation of biohydrogen production: A review", Renewable and Sustainable Energy Reviews, Vol. 56, pp. 1194-1206.
- Kumar, N. and Das, D. (2000), "Enhancement of hydrogen production by *Enterobacter cloacae* IIT-BT 08", Process Biochemistry, Vol. 35, No. 6, pp. 589-593.
- Kumar, N., Ghosh, A., *et al.* (2001), "Redirection of biochemical pathways for the enhancement of H₂ production by *Enterobacter cloacae*", Biotechnology Letters, Vol. 23, No. 7, pp. 537-541.
- Lacey, A. L. d., Fernández, V. M., *et al.* (2005), "Native and mutant nickel–iron hydrogenases: unravelling structure and function", Coordination Chemistry Reviews, Vol. 249, No. 15, pp. 1596-1608.
- Laurinavichene, T. V., Fedorov, A. S., *et al.* (2006), "Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas reinhardtii* cells", International Journal of Hydrogen Energy, Vol. 31, No. 5, pp. 659-667.
- Lay, J.-J., Lee, Y.-J., *et al.* (1999), "Feasibility of biological hydrogen production from organic fraction of municipal solid waste", Water Research, Vol. 33, No. 11, pp. 2579-2586.
- Lazaro, C. Z., Varesche, M. B. A., *et al.* (2015), "Effect of inoculum concentration, pH, light intensity and lighting regime on hydrogen production by phototrophic microbial consortium", Renewable Energy, Vol. 75, No., pp. 1-7.
- Lee, D. J., Show, K. Y., *et al.* (2011), "Dark fermentation on biohydrogen production: pure culture", Bioresource Technology, Vol. 102, No. 18, pp. 8393-8402.
- Lee, K., Lin, P., *et al.* (2006), "Temperature effects on biohydrogen production in a granular sludge bed induced by activated carbon carriers", International Journal of Hydrogen Energy, Vol. 31, No. 4, pp. 465-472.
- Lee, Y. J., Miyahara, T., *et al.* (2001), "Effect of iron concentration on hydrogen fermentation", Bioresource Technology, Vol. 80, No. 3, pp. 227-231.

- Levin, D. (2004), "Biohydrogen production: prospects and limitations to practical application", International Journal of Hydrogen Energy, Vol. 29, No. 2, pp. 173-185.
- Levin, D., Islam, R., *et al.* (2006), "Hydrogen production by *Clostridium thermocellum* 27405 from cellulosic biomass substrates", International Journal of Hydrogen Energy, Vol. 31, No. 11, pp. 1496-1503.
- Li, C. L. and Fang, H. H. P. (2007a), "Inhibition of heavy metals on fermentative hydrogen production by granular sludge", Chemosphere, Vol. 67, No. 4, pp. 668-673.
- Li, C. L. and Fang, H. H. P. (2007b), "Fermentative hydrogen production from wastewater and solid wastes by mixed cultures", Critical Reviews in Environmental Science and Technology, Vol. 37, No. 1, pp. 1-39.
- Lin, C.-N., Wu, S.-Y., *et al.* (2007), "Integration of fermentative hydrogen process and fuel cell for on-line electricity generation", International Journal of Hydrogen Energy, Vol. 32, No. 7, pp. 802-808.
- Lin, C.-Y. (1993), "Effect of heavy metals on acidogenesis in anaerobic digestion", Water Research, Vol. 27, No. 1, pp. 147-152.
- Lin, C.-Y. and Shei, S.-H. (2008), "Heavy metal effects on fermentative hydrogen production using natural mixed microflora", International Journal of Hydrogen Energy, Vol. 33, No. 2, pp. 587-593.
- Lin, P.-Y., Whang, L.-M., *et al.* (2007b), "Biological hydrogen production of the genus *Clostridium*: metabolic study and mathematical model simulation", International Journal of Hydrogen Energy, Vol. 32, No. 12, pp. 1728-1735.
- Liu, B.-F., Ren, N.-Q., *et al.* (2010a), "Bio-hydrogen production by mixed culture of photo- and dark-fermentation bacteria", International Journal of Hydrogen Energy, Vol. 35, No. 7, pp. 2858-2862.
- Liu, B.-F., Ren, N.-Q., *et al.* (2009a), "Hydrogen production by immobilized *R. faecalis* RLD-53 using soluble metabolites from ethanol fermentation bacteria *E. harbinense* B49", Bioresource Technology, Vol. 100, No. 10, pp. 2719-2723.
- Liu, B. F., Ren, N. Q., *et al.* (2010b), "Enhanced bio-hydrogen production by the combination of dark- and photo-fermentation in batch culture", Bioresource Technology, Vol. 101, No. 14, pp. 5325-5329.
- Liu, B. F., Ren, N. Q., *et al.* (2009b), "Hydrogen production by immobilized *R. faecalis* RLD-53 using soluble metabolites from ethanol fermentation bacteria *E. harbinense* B49", Bioresource Technology, Vol. 100, No. 10, pp. 2719-2723.
- Liu, I. C., Whang, L.-M., *et al.* (2011), "The effect of pH on the production of biohydrogen by *Clostridia*: Thermodynamic and metabolic considerations", International Journal of Hydrogen Energy, Vol. 36, No. 1, pp. 439-449.

- Lo, Y.-C., Chen, S.-D., *et al.* (2008a), "Combining enzymatic hydrolysis and dark-photo fermentation processes for hydrogen production from starch feedstock: A feasibility study", International Journal of Hydrogen Energy, Vol. 33, No. 19, pp. 5224-5233.
- Lo, Y.-C., Lee, K.-S., *et al.* (2009), "Bioreactors configured with distributors and carriers enhance the performance of continuous dark hydrogen fermentation", Bioresource Technology, Vol. 100, No. 19, pp. 4381-4387.
- Lo, Y. C., Chen, C. Y., *et al.* (2010), "Sequential dark-photo fermentation and autotrophic microalgal growth for high-yield and CO₂-free biohydrogen production", International Journal of Hydrogen Energy, Vol. 35, No. 20, pp. 10944-10953.
- Lo, Y. C., Chen, W. M., *et al.* (2008), "Dark H₂ fermentation from sucrose and xylose using H₂-producing indigenous bacteria: feasibility and kinetic studies", Water Research, Vol. 42, No. 4-5, pp. 827-842.
- Logan, B. E. (2004), "Peer reviewed: extracting hydrogen and electricity from renewable resources", Environmental Science & Technology, Vol. 38, No. 9, pp. 160A-167A.
- Lu, Y., Wang, L., *et al.* (2011), "Characteristics of hydrogen production of an *Enterobacter aerogenes* mutant generated by a new atmospheric and room temperature plasma (ARTP)", Biochemical Engineering Journal, Vol. 55, No. 1, pp. 17-22.
- Lu, Y., Zhao, H., *et al.* (2009), "Perturbation of formate pathway for hydrogen production by expressions of formate hydrogen lyase and its transcriptional activator in wild *Enterobacter aerogenes* and its mutants", International Journal of Hydrogen Energy, Vol. 34, No. 12, pp. 5072-5079.
- Maeda, I., Miyasaka, H., *et al.* (2003), "Maximization of hydrogen production ability in high - density suspension of *Rhodovulum sulfidophilum* cells using intracellular poly (3 - hydroxybutyrate) as sole substrate", Biotechnology and Bioengineering, Vol. 81, No. 4, pp. 474-481.
- Málek, I. and Fenel, Z. (2013). Theoretical and methodological basis of continuous culture of microorganisms, Elsevier.
- Mandal, B., Nath, K., *et al.* (2006), "Improvement of biohydrogen production under decreased partial pressure of H₂ by *Enterobacter cloacae*", Biotechnology Letters, Vol. 28, No. 11, pp. 831-835.
- Mandal, T. and Mandal, N. (1998), "Biomethanation of some waste materials with pure metallic magnesium catalyst: improved biogas yields", Energy Conversion and Management, Vol. 39, No. 11, pp. 1177-1179.

- Mangayil, R., Santala, V., *et al.* (2011), "Fermentative hydrogen production from different sugars by *Citrobacter* sp. CMC-1 in batch culture", International Journal of Hydrogen Energy, Vol. 36, No. 23, pp. 15187-15194.
- Manish, S. and Banerjee, R. (2008), "Comparison of biohydrogen production processes", International Journal of Hydrogen Energy, Vol. 33, No. 1, pp. 279-286.
- Mars, A. E., Veuskens, T., *et al.* (2010), "Biohydrogen production from untreated and hydrolyzed potato steam peels by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*", International Journal of Hydrogen Energy, Vol. 35, No. 15, pp. 7730-7737.
- Masset, J., Calusinska, M., *et al.* (2012), "Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium* spp", Biotechnology for Biofuels, Vol. 5, No. 1, pp. 35.
- Masset, J., Hiligsmann, S., *et al.* (2010), "Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently isolated strain of hydrogen-producing *Clostridium butyricum* CWBI1009", International Journal of Hydrogen Energy, Vol. 35, No. 8, pp. 3371-3378.
- Mathews, J. and Wang, G. (2009), "Metabolic pathway engineering for enhanced biohydrogen production", International Journal of Hydrogen Energy, Vol. 34, No. 17, pp. 7404-7416.
- Maza, E., Frasse, P., *et al.* (2013), "Comparison of normalization methods for differential gene expression analysis in RNA-Seq experiments: A matter of relative size of studied transcriptomes", Communicative & Integrative Biology, Vol. 6, No. 6, pp. e25849.
- McKinlay, J. B. and Harwood, C. S. (2010a), "Photobiological production of hydrogen gas as a biofuel", Current Opinion in Biotechnology, Vol. 21, No. 3, pp. 244-251.
- McKinlay, J. B. and Harwood, C. S. (2010b), "Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria", Proceedings of the National Academy of Sciences, Vol. 107, No. 26, pp. 11669-11675.
- McKinlay, J. B. and Harwood, C. S. (2011), "Calvin cycle flux, pathway constraints, and substrate oxidation state together determine the H₂ biofuel yield in photoheterotrophic bacteria", MBio, Vol. 2, No. 2, pp. e00323-00310.
- McKinlay, J. B., Oda, Y., *et al.* (2014), "Non-growing *Rhodospseudomonas palustris* increases the hydrogen gas yield from acetate by shifting from the glyoxylate shunt to the tricarboxylic acid cycle", Journal of Biological Chemistry, Vol. 289, No. 4, pp. 1960-1970.
- Melis, A. and Melnicki, M. (2006), "Integrated biological hydrogen production", International Journal of Hydrogen Energy, Vol. 31, No. 11, pp. 1563-1573.

MEWR (2015), "Key Environmental Statistics 2015", Ministry of the Environment and Water Resources.

Miyake, J. U. N., Mao, X.-Y., *et al.* (1984), "Photoproduction of hydrogen from glucose by a co-culture of a photosynthetic bacterium and *Clostridium butyricum*", Journal of Fermentation Technology, Vol. 62, No. 6, pp. 531-535.

Mizuno, O., Dinsdale, R., *et al.* (2000), "Enhancement of hydrogen production from glucose by nitrogen gas sparging", Bioresource Technology, Vol. 73, No. 1, pp. 59-65.

Moezelaar, R., Bijvank, S. M., *et al.* (1996), "Fermentation and sulfur reduction in the mat-building cyanobacterium *Microcoleus chthonoplastes*", Applied and Environmental Microbiology, Vol. 62, No. 5, pp. 1752-1758.

Murtagh, F. and Contreras, P. (2012), "Algorithms for hierarchical clustering: an overview", Wiley Interdisciplinary Reviews: Data Mining and Knowledge Discovery, Vol. 2, No. 1, pp. 86-97.

Nath, K. and Das, D. (2004a), "Improvement of fermentative hydrogen production: various approaches", Applied Microbiology and Biotechnology, Vol. 65, No. 5, pp. 520-529.

Nath, K. and Das, D. (2004b), "Biohydrogen production as a potential energy resource-present state-of-art", Journal of Scientific and Industrial Research, Vol. 63, pp. 729-738.

Nath, K., Kumar, A., *et al.* (2006), "Effect of some environmental parameters on fermentative hydrogen production by *Enterobacter cloacae* DM11", Canadian Journal of Microbiology, Vol. 52, No. 6, pp. 525-532.

Ng, B. J. H., Mao, Y., *et al.* (2015), "Municipal food waste management in Singapore: practices, challenges and recommendations", Journal of Material Cycles and Waste Management, pp. 1-10.

Oh, S. E., Iyer, P., *et al.* (2004a), "Biological hydrogen production using a membrane bioreactor", Biotechnology and Bioengineering, Vol. 87, No. 1, pp. 119-127.

Oh, Y.-K., Park, M., *et al.* (2003b), "Isolation of hydrogen-producing bacteria from granular sludge of an upflow anaerobic sludge blanket reactor", Biotechnology and Bioprocess Engineering, Vol. 8, No. 1, pp. 54-57.

Oh, Y.-K., Seol, E.-H., *et al.* (2002), "Fermentative hydrogen production by a new chemoheterotrophic bacterium *Rhodopseudomonas Palustris* P4", International Journal of Hydrogen Energy, Vol. 27, No. 11, pp. 1373-1379.

Oh, Y. F., Park, S., *et al.* (2008b), "Carbon and energy balances of glucose fermentation with hydrogenproducing bacterium *Citrobacter amalonaticus* Y19", Journal of Microbiology and Biotechnology, Vol. 18, No. 3, pp. 532-538.

- Oh, Y. K., H., S. E., *et al.* (2003a), "Fermentative biohydrogen production by a new chemoheterotrophic bacterium *Citrobacter* sp. Y19", International Journal of Hydrogen Energy, Vol. 28, No. 12, pp. 1353-1359.
- Oh, Y. K., Kim, H. J., *et al.* (2008), "Metabolic-flux analysis of hydrogen production pathway in *Citrobacter amalonaticus* Y19", International Journal of Hydrogen Energy, Vol. 33, No. 5, pp. 1471-1482.
- Oh, Y. K., Kim, S. H., *et al.* (2004b), "Thermophilic biohydrogen production from glucose with trickling biofilter", Biotechnology and Bioengineering, Vol. 88, No. 6, pp. 690-698.
- Oh, Y. K., Raj, S. M., *et al.* (2011), "Current status of the metabolic engineering of microorganisms for biohydrogen production", Bioresource Technology, Vol. 102, No. 18, pp. 8357-8367.
- Ohta, S., Miyamoto, K., *et al.* (1987), "Hydrogen evolution as a consumption mode of reducing equivalents in green algal fermentation", Plant Physiology, Vol. 83, No. 4, pp. 1022-1026.
- Ohta, Y., Frank, J., *et al.* (1981), "Hydrogen production by marine photosynthetic bacteria: Effect of environmental factors and substrate specificity on the growth of a hydrogen-producing marine photosynthetic bacterium, *Chromatium* sp. Miami PBS 1071", International Journal of Hydrogen Energy, Vol. 6, No. 5, pp. 451-460.
- Özgür, E., Mars, A. E., *et al.* (2010), "Biohydrogen production from beet molasses by sequential dark and photofermentation", International Journal of Hydrogen Energy, Vol. 35, No. 2, pp. 511-517.
- Özkan, E., Uyar, B., *et al.* (2012), "Photofermentative hydrogen production using dark fermentation effluent of sugar beet thick juice in outdoor conditions", International Journal of Hydrogen Energy, Vol. 37, No. 2, pp. 2044-2049.
- Ozmihci, S. and Kargi, F. (2010a), "Bio-hydrogen production by photo-fermentation of dark fermentation effluent with intermittent feeding and effluent removal", International Journal of Hydrogen Energy, Vol. 35, No. 13, pp. 6674-6680.
- Ozmihci, S. and Kargi, F. (2010b), "Comparison of different mixed cultures for bio-hydrogen production from ground wheat starch by combined dark and light fermentation", Journal of Industrial Microbiology & Biotechnology, Vol. 37, No. 4, pp. 341-347.
- Ozmihci, S. and Kargi, F. (2010c), "Effects of starch loading rate on performance of combined fed-batch fermentation of ground wheat for bio-hydrogen production", International Journal of Hydrogen Energy, Vol. 35, No. 3, pp. 1106-1111.
- Peddie, C. C., Mavinic, D. S., *et al.* (1990), "Use of ORP for monitoring and control of aerobic sludge digestion", Journal of Environmental Engineering.

Penaud, V., Delgenes, J. P., *et al.* (1999), "Thermo-chemical pretreatment of a microbial biomass: influence of sodium hydroxide addition on solubilization and anaerobic biodegradability", Enzyme and Microbial Technology, Vol. 25, No. 3, pp. 258-263.

Peng, Y., Stevens, P., *et al.* (1987), "Relation between pH, hydrogenase and nitrogenase activity, NH_4^+ concentration and hydrogen production in cultures of *Rhodobacter sulfidophilus*", Journal of General Microbiology, Vol. 133, No., pp. 1243-1247.

Perera, K. R. J., Ketheesan, B., *et al.* (2012), "Fermentative biohydrogen production II: Net energy gain from organic wastes", International Journal of Hydrogen Energy, Vol. 37, No. 1, pp. 167-178.

Phlips, E. J. and Mitsui, A. (1983), "Role of light intensity and temperature in the regulation of hydrogen photoproduction by the marine cyanobacterium *Oscillatoria* sp. strain Miami BG7", Applied and Environmental Microbiology, Vol. 45, No. 4, pp. 1212-1220.

Prachanurak, P., Chiemchaisri, C., *et al.* (2014), "Biomass production from fermented starch wastewater in photo-bioreactor with internal overflow recirculation", Bioresource Technology, Vol. 165, No., pp. 129-136.

Qin, J., Wang, X., *et al.* (2015), "Comparative transcriptome analysis reveals different molecular mechanisms of *Bacillus coagulans* 2-6 response to sodium lactate and calcium lactate during lactic acid production", PLoS One, Vol. 10, No. 4, pp.

Rachman, M. A., Furutani, Y., *et al.* (1997), "Enhanced hydrogen production in altered mixed acid fermentation of glucose by *Enterobacter aerogenes*", Journal of Fermentation and Bioengineering, Vol. 83, No. 4, pp. 358-363.

Ramos, C., Buitrón, G., *et al.* (2012), "Effect of the initial total solids concentration and initial pH on the bio-hydrogen production from cafeteria food waste", International Journal of Hydrogen Energy, Vol. 37, No. 18, pp. 13288-13295.

Ravi Kumar Parihar and Upadhyay, K. (2016), "Production of biohydrogen gas from dairy industry wastewater by anaerobic fermentation process", International Journal of Applied Research, Vol. 2, No. 3, pp. 4.

Ravinder, T., Ramesh, B., *et al.* (2000), "Fermentative production of acetic acid from various pure and natural cellulosic materials by *Clostridium lentocellum* SG6", World Journal of Microbiology and Biotechnology, Vol. 16, No. 6, pp. 507-512.

Redondas, V., Gomez, X., *et al.* (2012), "Hydrogen production from food wastes and gas post-treatment by CO_2 adsorption", Waste Management, Vol. 32, No. 1, pp. 60-66.

- Ren, N.-Q., Guo, W.-Q., *et al.* (2008), "Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production", International Journal of Hydrogen Energy, Vol. 33, No. 16, pp. 4318-4324.
- Ren, N.-Q., Liu, B.-F., *et al.* (2009), "Hydrogen production with *R. faecalis* RLD-53 isolated from freshwater pond sludge", Bioresource Technology, Vol. 100, No. 1, pp. 484-487.
- Rey, F. E., Oda, Y., *et al.* (2006), "Regulation of uptake hydrogenase and effects of hydrogen utilization on gene expression in *Rhodopseudomonas palustris*", Journal of Bacteriology, Vol. 188, No. 17, pp. 6143-6152.
- Rollin, J. A., del Campo, J. M., *et al.* (2015), "High-yield hydrogen production from biomass by *in vitro* metabolic engineering: Mixed sugars coutilization and kinetic modeling", Proceedings of the National Academy of Sciences, Vol. 112, No. 16, pp. 4964-4969.
- Sagnak, R. and Kargi, F. (2011), "Hydrogen gas production from acid hydrolyzed wheat starch by combined dark and photo-fermentation with periodic feeding", International Journal of Hydrogen Energy, Vol. 36, No. 17, pp. 10683-10689.
- Sasikala, K., Ramana, C. V., *et al.* (1992), "Photoproduction of hydrogen from the waste water of a distillery by *Rhodobacter sphaeroides* O.U. 001", International Journal of Hydrogen Energy, Vol. 17, No. 1, pp. 23-27.
- Schröder, C., Selig, M., *et al.* (1994), "Glucose fermentation to acetate, CO₂ and H₂ in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: involvement of the Embden-Meyerhof pathway", Archives of Microbiology, Vol. 161, No. 6, pp. 460-470.
- Seibert, M., King, P. W., *et al.* (2008). Photosynthetic water-splitting for hydrogen production. Bioenergy, ASM Press.
- Sen, U., Shakdwipee, M., *et al.* (2008), "Status of biological hydrogen production", Journal of Scientific and Industrial Research, Vol. 67, No. 11, pp. 980.
- Serebryakova, L. T., Sheremetieva, M. E., *et al.* (2000), "H₂-Uptake and evolution in the unicellular cyanobacterium *Chroococcidiopsis thermalis* CALU 758", Plant Physiology and Biochemistry, Vol. 38, No. 6, pp. 525-530.
- Shi, X. and Yu, H. (2006), "Continuous production of hydrogen from mixed volatile fatty acids with *Rhodopseudomonas capsulata*", International Journal of Hydrogen Energy, Vol. 31, No. 12, pp. 1641-1647.
- Shin, H.-S. and Youn, J.-H. (2005), "Conversion of food waste into hydrogen by thermophilic acidogenesis", Biodegradation, Vol. 16, No. 1, pp. 33-44.
- Shin, H.-S., Youn, J.-H., *et al.* (2004), "Hydrogen production from food waste in anaerobic mesophilic and thermophilic acidogenesis", International Journal of Hydrogen Energy, Vol. 29, No. 13, pp. 1355-1363.

- Shin, J., Hyunyon, J., *et al.* (2007), "Fermentative hydrogen production by the newly isolated *Enterobacter asburiae* SNU-1", International Journal of Hydrogen Energy, Vol. 32, No. 2, pp. 192-199.
- Show, K.-Y., Zhang, Z.-P., *et al.* (2007), "Production of hydrogen in a granular sludge-based anaerobic continuous stirred tank reactor", International Journal of Hydrogen Energy, Vol. 32, No. 18, pp. 4744-4753.
- Show, K. Y., Lee, D. J., *et al.* (2011), "Bioreactor and process design for biohydrogen production", Bioresource Technology, Vol. 102, No. 18, pp. 8524-8533.
- Singh, L. and Wahid, Z. A. (2015), "Methods for enhancing bio-hydrogen production from biological process: a review", Journal of Industrial and Engineering Chemistry, Vol. 21, No., pp. 70-80.
- Sinha, P. and Pandey, A. (2011), "An evaluative report and challenges for fermentative biohydrogen production", International Journal of Hydrogen Energy, Vol. 36, No. 13, pp. 7460-7478.
- Sreela-or, C., Plangklang, P., *et al.* (2011), "Co-digestion of food waste and sludge for hydrogen production by anaerobic mixed cultures: Statistical key factors optimization", International Journal of Hydrogen Energy, Vol. 36, No. 21, pp. 14227-14237.
- Srirangan, K., Pyne, M. E., *et al.* (2011), "Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria", Bioresource Technology, Vol. 102, No. 18, pp. 8589-8604.
- Su, H. B., Cheng, J., *et al.* (2009), "Combination of dark- and photo-fermentation to enhance hydrogen production and energy conversion efficiency", International Journal of Hydrogen Energy, Vol. 34, No. 21, pp. 8846-8853.
- Sun, Q., Xiao, W., *et al.* (2010), "Statistical optimization of biohydrogen production from sucrose by a co-culture of *Clostridium acidisoli* and *Rhodobacter sphaeroides*", International Journal of Hydrogen Energy, Vol. 35, No. 9, pp. 4076-4084.
- Sung, S. and Liu, T. (2003), "Ammonia inhibition on thermophilic anaerobic digestion", Chemosphere, Vol. 53, No. 1, pp. 43-52.
- Tada, C., Yang, Y., *et al.* (2005), "Effect of natural zeolite on methane production for anaerobic digestion of ammonium rich organic sludge", Bioresource Technology, Vol. 96, No. 4, pp. 459-464.
- Tamagnini, P., Leitão, E., *et al.* (2007), "Cyanobacterial hydrogenases: diversity, regulation and applications", FEMS Microbiology Reviews, Vol. 31, No. 6, pp. 692-720.

- Tanisho, S., Suzuki, Y., *et al.* (1987), "Fermentative hydrogen evolution by *Enterbacter aerogenese* strain E. 82005", International Journal of Hydrogen Energy, Vol. 12, No. 9, pp. 623-627.
- Tao, Y. (2008), "Characteristics of a new photosynthetic bacterial strain for hydrogen production and its application in wastewater treatment", International Journal of Hydrogen Energy, Vol. 33, No. 3, pp. 963-973.
- Tawfik, A. and El-Qelish, M. (2012), "Continuous hydrogen production from co-digestion of municipal food waste and kitchen wastewater in mesophilic anaerobic baffled reactor", Bioresource Technology, Vol. 114, pp. 270-274.
- Timothy, L. (2011), "An overview of hydrogen production and storage systems with renewable hydrogen case studies", A Clean Energy State Alliance Report.
- Tseng, C.-H., Chiang, P.-W., *et al.* (2015), "Prokaryotic assemblages and metagenomes in pelagic zones of the South China Sea", BMC Genomics, Vol. 16, No. 1, pp. 219.
- Ueno, Y., Haruta, S., *et al.* (2001), "Microbial community in anaerobic hydrogen-producing microflora enriched from sludge compost", Applied Microbiology and Biotechnology, Vol. 57, No. 4, pp. 555-562.
- Ueno, Y., Kawai, T., *et al.* (1995), "Biological production of hydrogen from cellulose by natural anaerobic microflora", Journal of Fermentation and Bioengineering, Vol. 79, No. 4, pp. 395-397.
- Ueno, Y., Otsuka, S., *et al.* (1996), "Hydrogen production from industrial wastewater by anaerobic microflora in chemostat culture", Journal of Fermentation and Bioengineering, Vol. 82, No. 2, pp. 194-197.
- Ugwu, C. U., Aoyagi, H., *et al.* (2008), "Photobioreactors for mass cultivation of algae", Bioresource Technology, Vol. 99, No. 10, pp. 4021-4028.
- Urbaniec, K. and Bakker, R. R. (2015), "Biomass residues as raw material for dark hydrogen fermentation--A review", International Journal of Hydrogen Energy, Vol. 40, No. 9, pp. 3648-3658.
- Uyar, B., Eroglu, I., *et al.* (2007), "Effect of light intensity, wavelength and illumination protocol on hydrogen production in photobioreactors", International Journal of Hydrogen Energy, Vol. 32, No. 18, pp. 4670-4677.
- Valdez-Vazquez, I. and Poggi-Varaldo, H. M. (2009), "Hydrogen production by fermentative consortia", Renewable and Sustainable Energy Reviews, Vol. 13, No. 5, pp. 1000-1013.
- van Niel, E. W. J., Budde, M. A. W., *et al.* (2002), "Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*", International Journal of Hydrogen Energy, Vol. 27, No. 11, pp. 1391-1398.

- van Niel, E. W. J., Claassen, P. A., *et al.* (2003), "Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*", Biotechnology and bioengineering, Vol. 81, No. 3, pp. 255-262.
- Vardar-Schara, G., Maeda, T., *et al.* (2008), "Metabolically engineered bacteria for producing hydrogen via fermentation", Microbial Biotechnology, Vol. 1, No. 2, pp. 107-125.
- Vijaya Bhaskar, Y., Venkata Mohan, S., *et al.* (2008), "Effect of substrate loading rate of chemical wastewater on fermentative biohydrogen production in biofilm configured sequencing batch reactor", Bioresource Technology, Vol. 99, No. 15, pp. 6941-6948.
- Wang, A. J., Sun, D., *et al.* (2011), "Integrated hydrogen production process from cellulose by combining dark fermentation, microbial fuel cells, and a microbial electrolysis cell", Bioresource Technology, Vol. 102, No. 5, pp. 4137-4143.
- Wang, B., Wan, W., *et al.* (2009), "Effect of ammonia concentration on fermentative hydrogen production by mixed cultures", Bioresource Technology, Vol. 100, No. 3, pp. 1211-1213.
- Wang, C. C., Chang, C. W., *et al.* (2003), "Producing hydrogen from wastewater sludge by *Clostridium bifermentans*", Journal of Biotechnology, Vol. 102, No. 1, pp. 83-92.
- Wang, J. and Wan, W. (2009), "Factors influencing fermentative hydrogen production: A review", International Journal of Hydrogen Energy, Vol. 34, No. 2, pp. 799-811.
- Wang, J., Zhang, H., *et al.* (2005), "Comparison of lab-scale and pilot-scale hybrid anaerobic solid-liquid systems operated in batch and semi-continuous modes", Process Biochemistry, Vol. 40, No. 11, pp. 3580-3586.
- Wang, J. L. and Wan, W. (2008), "Influence of Ni^{2+} concentration on biohydrogen production", Bioresource Technology, Vol. 99, No. 18, pp. 8864-8868.
- Wang, S., Huang, H., *et al.* (2010a), "NADP⁺ reduction with reduced ferredoxin and NADP⁺ reduction with NADH are coupled via an electron-bifurcating enzyme complex in *Clostridium kluyveri*", Journal of Bacteriology, Vol. 192, No. 19, pp. 5115-5123.
- Wang, X., Ding, J., *et al.* (2010b), "A hydrodynamics-reaction kinetics coupled model for evaluating bioreactors derived from CFD simulation", Bioresource Technology, Vol. 101, No. 24, pp. 9749-9757.
- Wang, X. J., Ren, N. Q., *et al.* (2007), "Influence of gaseous end-products inhibition and nutrient limitations on the growth and hydrogen production by hydrogen-producing fermentative bacterial B49", International Journal of Hydrogen Energy, Vol. 32, No. 6, pp. 748-754.

Wei, H., Fu, Y., *et al.* (2014), "Comparison of transcriptional profiles of *Clostridium thermocellum* grown on cellobiose and pretreated yellow poplar using RNA-Seq", Frontiers in Microbiology, Vol. 5, No. 142, pp. 1-6.

Winter, C.-J. (2005), "Into the hydrogen energy economy-milestones", International Journal of Hydrogen Energy, Vol. 30, No. 7, pp. 681-685.

Wu, S., Hung, C., *et al.* (2008), "HRT-dependent hydrogen production and bacterial community structure of mixed anaerobic microflora in suspended, granular and immobilized sludge systems using glucose as the carbon substrate", International Journal of Hydrogen Energy, Vol. 33, No. 5, pp. 1542-1549.

Wu, X. B., Li, Q. Y., *et al.* (2010), "Enhanced H₂ gas production from bagasse using adhE inactivated *Klebsiella oxytoca* HP1 by sequential dark-photo fermentations", Bioresource Technology, Vol. 101, No. 24, pp. 9605-9611.

Xie, G.-J., Liu, B.-F., *et al.* (2012), "The kinetic characterization of photofermentative bacterium *Rhodopseudomonas faecalis* RLD-53 and its application for enhancing continuous hydrogen production", International Journal of Hydrogen Energy, Vol. 37, No. 18, pp. 13718-13724.

Xie, G. J., Feng, L. B., *et al.* (2010), "Control strategies for hydrogen production through co-culture of *Ethanoligenens harbinense* B49 and immobilized *Rhodopseudomonas faecalis* RLD-53", International Journal of Hydrogen Energy, Vol. 35, No. 5, pp. 1929-1935.

Xu, L. Y., Ren, N. Q., *et al.* (2008), "Biohydrogen production by *Ethanoligenens harbinense* B49: nutrient optimization", International Journal of Hydrogen Energy, Vol. 33, No. 23, pp. 6962-6967.

Yerushalmi, L., Volesky, B., *et al.* (1985), "Effect of increased hydrogen partial pressure on the acetone-butanol fermentation by *Clostridium acetobutylicum*", Applied Microbiology and Biotechnology, Vol. 22, No. 2, pp. 103-107.

Yokoi, H., Maki, R., *et al.* (2002), "Microbial production of hydrogen from starch-manufacturing wastes", Biomass and Bioenergy, Vol. 22, No. 5, pp. 389-395.

Yokoi, H., Mori, S., *et al.* (1998a), "H₂ production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M19", Biotechnology Letters, Vol. 20, No. 9, pp. 895-899.

Yokoi, H., Tokushige, T., *et al.* (1998b), "H₂ production from starch by a mixed culture of *Clostridium butyricum* and *Enterobacter aerogenes*", Biotechnology Letters, Vol. 20, No. 2, pp. 143-147.

Yu, H. Q., Zhu, Z. H., *et al.* (2002), "Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures", International Journal of Hydrogen Energy, Vol. 27, No. 11, pp. 1359-1365.

Zhang, C., Lv, F. X., *et al.* (2011), "Bioengineering of the *Enterobacter aerogenes* strain for biohydrogen production", Bioresource Technology, Vol. 102, No. 18, pp. 8344-8349.

Zhang, C., Ma, K., *et al.* (2009), "Regulation of hydrogen production by *Enterobacter aerogenes* by external NADH and NAD⁺", International Journal of Hydrogen Energy, Vol. 34, No. 3, pp. 1226-1232.

Zhang, D., Xiao, N., *et al.* (2015a), "Bioprocess modelling of biohydrogen production by *Rhodopseudomonas palustris*: Model development and effects of operating conditions on hydrogen yield and glycerol conversion efficiency", Chemical Engineering Science, Vol. 130, pp. 68-78.

Zhang, D., Xiao, N., *et al.* (2015b), "Bioprocess modelling of biohydrogen production by *Rhodopseudomonas palustris*: Model development and effects of operating conditions on hydrogen yield and glycerol conversion efficiency", Chemical Engineering Science, Vol. 130, pp. 68-78.

Zhang, H., Bruns, M. A., *et al.* (2006), "Biological hydrogen production by *Clostridium acetobutylicum* in an unsaturated flow reactor", Water Research, Vol. 40, No. 4, pp. 728-734.

Zhang, L. G., Ban, Q. Y., *et al.* (2014a), "Assessment of effects of yeast extract on bio-hydrogen production from anaerobic activated sludge", International Journal of Agriculture and Biology, Vol. 16, No. 6, pp. 1189-1193.

Zhang, R. H., El-Mashad, H. M., *et al.* (2007a), "Characterization of food waste as feedstock for anaerobic digestion", Bioresource Technology, Vol. 98, No. 4, pp. 929-935.

Zhang, T., Liu, H., *et al.* (2003), "Biohydrogen production from starch in wastewater under thermophilic condition", Journal of Environmental Management, Vol. 69, No. 2, pp. 149-156.

Zhang, Y. F. and Shen, J. Q. (2006), "Effect of temperature and iron concentration on the growth and hydrogen production of mixed bacteria", International Journal of Hydrogen Energy, Vol. 31, No. 4, pp. 441-446.

Zhang, Z., Wu, Q., *et al.* (2014b), "Effect of inlet velocity on heat transfer process in a novel photo-fermentation biohydrogen production bioreactor using computational fluid dynamics simulation", BioResources, Vol. 10, No. 1, pp. 469-481.

Zhang, Z. P., Tay, J. H., *et al.* (2007b), "Biohydrogen production in a granular activated carbon anaerobic fluidized bed reactor", International Journal of Hydrogen Energy, Vol. 32, No. 2, pp. 185-191.

Zhao, X., Xing, D., *et al.* (2011), "Hydrogen production by the newly isolated *Clostridium beijerinckii* RZF-1108", Bioresource Technology, Vol. 102, No. 18, pp. 8432-8436.

Zhao, X., Xing, D. F., *et al.* (2012), "The effects of metal ions and l-cysteine on *hydA* gene expression and hydrogen production by *Clostridium beijerinckii* RZF-1108", International Journal of Hydrogen Energy, Vol. 37, No. 18, pp. 13711-13717.

Zhou, P., Elbeshbishy, E., *et al.* (2013), "Optimization of biological hydrogen production for anaerobic co-digestion of food waste and wastewater biosolids", Bioresource Technology, Vol. 130, No., pp. 710-718.

Zhu, H. G., Parker, W., *et al.* (2008), "Biohydrogen production by anaerobic co-digestion of municipal food waste and sewage sludges", International Journal of Hydrogen Energy, Vol. 33, No. 14, pp. 3651-3659.

Zurrer, H. and Bachofen, R. (1979), "Hydrogen production by the photosynthetic bacterium *Rhodospirillum rubrum*", Applied and Environmental Microbiology, Vol. 37, No. 5, pp. 789-793.

Zürer, H. and Bachofen, R. (1982), "Aspects of growth and hydrogen production of the photosynthetic bacterium *Rhodospirillum rubrum* in continuous culture", Biomass, Vol. 2, No. 3, pp. 165-174.

APPENDIX TABLE

Table A1. Genes Expression changes according to Library AQQF00000000.

Gene name	PM	NFM	FC	Annotation
<i>[C]Energy production and conversion</i>				
K670_RS07485	2.7	0.0	-	Fumarate reductase flavoprotein subunit
K670_RS08995	131.7	11.6	11.4	adenylsulfate reductase
K670_RS12955	286.9	26.3	10.9	pyridine nucleotide-disulfide oxidoreductase
K670_RS09960	9093.5	1109.3	8.2	Reverse rubrerythrin-1
K670_RS03000	191.0	23.4	8.2	Acylphosphatase
K670_RS00805	228.0	31.5	7.2	NADPH-dependent butanol dehydrogenase Electron transfer protein for anaerobic sulfite reductase subunit A
K670_RS15545	35.7	5.5	6.5	Reverse rubrerythrin-1
K670_RS20640	6048.2	960.3	6.3	Reverse rubrerythrin-1
K670_RS06855	142.1	25.5	5.6	L-cystine uptake protein TcyP
K670_RS08310	8987.1	1630.5	5.5	hypothetical protein
K670_RS11265	237.3	45.0	5.3	NADPH-dependent butanol dehydrogenase
K670_RS18885	782.5	148.8	5.3	malate dehydrogenase
K670_RS05810	82.7	16.6	5.0	NADPH-dependent butanol dehydrogenase
K670_RS04245	5136.7	1033.2	5.0	hypothetical protein
K670_RS12455	90.5	18.5	4.9	Putative NADH dehydrogenase/NAD(P)H nitroreductase
K670_RS08990	64.3	13.1	4.9	adenylsulfate reductase
K670_RS02310	221.4	57.2	3.9	isocitrate dehydrogenase
K670_RS10050	653.0	175.4	3.7	oxidoreductase
K670_RS12375	872.4	249.2	3.5	L-cystine uptake protein TcyP
K670_RS05495	487.6	141.0	3.5	Cobalt-dependent inorganic pyrophosphatase
K670_RS08675	263.8	77.5	3.4	(Fe-S)-binding protein
K670_RS14785	449.5	133.2	3.4	ATP synthase F0F1 subunit gamma
K670_RS18720	623.7	188.7	3.3	pyruvate carboxylase
K670_RS09685	691.6	225.9	3.1	Pyruvate-flavodoxin oxidoreductase
K670_RS09040	24.6	8.4	2.9	glycerol dehydrogenase
K670_RS00795	130.2	44.4	2.9	NADPH-dependent butanol dehydrogenase
K670_RS01085	975.4	332.9	2.9	fumarate hydratase
K670_RS07040	94.6	32.8	2.9	lactate dehydrogenase
K670_RS16360	375.5	133.4	2.8	Iron hydrogenase 1
K670_RS05025	66.2	23.7	2.8	glycerol dehydrogenase
K670_RS08480	47.3	17.4	2.7	lactate dehydrogenase
K670_RS01080	469.0	176.0	2.7	hydrolyase tartrate subunit alpha/fumarate domain-containing protein, Fe-S type
K670_RS06465	1251.6	486.7	2.6	glycerol-3-phosphate dehydrogenase
K670_RS07400	2.2	0.9	2.5	2-enoate reductase FldZ
K670_RS15540	23.0	9.4	2.5	sulfite reductase
K670_RS08135	52.9	21.6	2.5	Anaerobic sulfite reductase subunit C
K670_RS19085	33.4	13.7	2.4	lactate dehydrogenase
K670_RS18780	6885.8	2818.1	2.4	nitrogen-fixing protein NifU
K670_RS13700	4.3	1.8	2.4	glycerol-3-phosphate dehydrogenase
K670_RS17065	38.4	16.7	2.3	sodium:proton antiporter
K670_RS19145	29.3	12.9	2.3	molybdopterin oxidoreductase
K670_RS12320	172.4	76.8	2.2	ferredoxin-NADP+ reductase
K670_RS11280	130.0	57.9	2.2	flavodoxin
K670_RS06640	3525.6	1615.2	2.2	Bifunctional protein NifU/MnmA
K670_RS14800	3604.5	1677.6	2.1	F0F1 ATP synthase subunit B
K670_RS14790	2467.6	1164.3	2.1	ATP F0F1 synthase subunit alpha
K670_RS14805	563.4	271.7	2.1	F0F1 ATP synthase subunit C

K670_RS17160	1145.5	561.3	2.0	Hydro-lyase with broad substrate specificity
K670_RS03405	57.9	29.1	2.0	hypothetical protein
K670_RS14780	3251.4	1658.6	2.0	F0F1 ATP synthase subunit beta
K670_RS19500	53.4	27.3	2.0	glycerophosphodiester phosphodiesterase
K670_RS10530	2347.6	1215.4	1.9	ferredoxin
K670_RS13725	40.3	21.1	1.9	xanthine dehydrogenase molybdopterin-binding subunit
K670_RS09790	478.6	260.4	1.8	hypothetical protein
K670_RS14165	13.8	7.5	1.8	oxidoreductase
K670_RS05030	57.1	31.5	1.8	ferredoxin--nitrite reductase
K670_RS16760	721.8	405.0	1.8	desulfoferredoxin
K670_RS06475	530.2	297.5	1.8	Fe-S oxidoreductase
K670_RS16570	138.6	79.8	1.7	ATP synthase subunit A
K670_RS08700	78.9	48.3	1.6	flavodoxin
K670_RS14810	528.3	334.4	1.6	F0F1 ATP synthase subunit A
K670_RS10770	34.7	22.1	1.6	UPF0313 protein CPE1196
K670_RS14795	3451.9	2244.6	1.5	ATP synthase subunit delta Dihydroorotate dehydrogenase B (NAD(+)), electron transfer subunit
K670_RS07075	37.0	25.1	1.5	NAD(P)H nitroreductase
K670_RS08945	552.5	375.8	1.5	NAD(P)H nitroreductase
K670_RS02700	1873.5	1284.6	1.5	NADPH-dependent butanol dehydrogenase
K670_RS09945	156.2	114.3	1.4	lactate dehydrogenase
K670_RS19480	36.3	26.6	1.4	(Fe-S)-binding protein
K670_RS00005	899.8	671.9	1.3	flavodoxin Putative diflavin flavoprotein A 6
K670_RS13560	172.4	132.8	1.3	D-lactate dehydrogenase (acceptor)
K670_RS05785	136.7	105.8	1.3	D-3-phosphoglycerate dehydrogenase
K670_RS01160	575.5	452.6	1.3	butyrate kinase
K670_RS16580	202.1	161.3	1.3	ATP synthase subunit C
K670_RS04845	24.6	20.1	1.2	Uncharacterized oxidoreductase YcsN
K670_RS20210	42.9	35.0	1.2	Nitrite reductase [NAD(P)H]
K670_RS15820	4.2	3.4	1.2	Glycerol dehydrogenase
K670_RS13715	5.8	4.8	1.2	molybdopterin dehydrogenase
K670_RS16595	121.0	100.6	1.2	ATP synthase subunit I
K670_RS12800	73.4	61.3	1.2	NADH oxidase
K670_RS16575	911.0	763.4	1.2	V-type ATP synthase subunit F
K670_RS17500	32.9	28.8	1.1	pyridine nucleotide-disulfide oxidoreductase
K670_RS15130	2705.1	2461.0	1.1	protein fixB
K670_RS16605	15.8	15.1	1.1	radical SAM protein
K670_RS16765	389.1	375.9	1.0	rubrerythrin Rubrerythrin-1
K670_RS08625	23.4	22.9	1.0	glyceraldehyde 3-phosphate reductase
K670_RS14070	101.6	100.7	1.0	Putative NADH dehydrogenase/NAD(P)H nitroreductase
K670_RS16590	355.1	352.4	1.0	V-type sodium ATPase K subunit
K670_RS02105	4329.5	4315.3	1.0	NADPH-dependent butanol dehydrogenase
K670_RS03220	56.9	58.1	1.0	B12-binding protein
K670_RS17990	54.8	56.9	1.0	NADPH dehydrogenase
K670_RS15800	74.6	82.0	0.9	(Fe-S)-binding protein
K670_RS13885	24.1	27.1	0.9	4Fe-4S-binding domain protein
K670_RS03830	1646.8	1867.9	0.9	NADPH-dependent butanol dehydrogenase
K670_RS01155	622.2	713.0	0.9	Phosphate butyryltransferase
K670_RS18450	139.9	171.2	0.8	formate acetyltransferase
K670_RS13870	6.0	7.4	0.8	4Fe-4S ferredoxin, iron-sulfur-binding domain protein
K670_RS19455	3.9	4.7	0.8	1,3-propanediol dehydrogenase
K670_RS05140	5007.6	6155.2	0.8	pyruvate:ferredoxin (flavodoxin) oxidoreductase
K670_RS15135	685.1	865.0	0.8	Electron transfer flavoprotein subunit beta
K670_RS14775	109.1	143.5	0.8	ATP synthase subunit epsilon
K670_RS06310	875.9	1165.8	0.8	Phosphate acetyltransferase
K670_RS08750	133.2	187.7	0.7	Glycerol dehydrogenase

K670_RS06305	1748.9	2526.1	0.7	acetate kinase
K670_RS18545	5.4	8.1	0.7	formate acetyltransferase
K670_RS19715	1554.6	2344.7	0.7	CoA-disulfide reductase
K670_RS16565	80.4	122.1	0.7	ATP synthase subunit B
K670_RS00825	3729.0	5723.5	0.7	NADPH-dependent butanol dehydrogenase
K670_RS04990	101.4	169.0	0.6	Glycerol-1-phosphate dehydrogenase [NAD(P)+]
K670_RS05630	8.6	15.1	0.6	hypothetical protein
K670_RS00695	5.9	10.8	0.5	glycerol kinase
K670_RS02725	59.9	110.6	0.5	dehydrogenase
K670_RS11785	91.8	170.6	0.5	Fatty aldehyde dehydrogenase
K670_RS19905	47.9	89.3	0.5	Sulfite reductase, assimilatory-type
K670_RS15535	9.2	18.8	0.5	Anaerobic sulfite reductase subunit C
K670_RS14100	3.3	6.7	0.5	FAD-linked oxidase
K670_RS15650	39.5	80.6	0.5	rubrerythrin Rubrerythrin-1
K670_RS07060	13.7	30.4	0.5	formate acetyltransferase
K670_RS03530	214.5	493.4	0.4	potassium transporter
K670_RS16585	71.1	170.8	0.4	V-type ATP synthase subunit E 1
K670_RS16175	4.4	11.6	0.4	Artemisinic aldehyde Delta(11(13)) reductase
K670_RS05135	1475.7	4326.5	0.3	An electron-transfer protein
K670_RS08180	10.0	32.7	0.3	NADH oxidase
K670_RS03425	34.8	117.3	0.3	Epoxyqueuosine reductase
K670_RS15520	3.6	13.3	0.3	NADH-rubredoxin oxidoreductase
K670_RS08935	87.1	329.5	0.3	Protein FixA
K670_RS12710	15.7	60.2	0.3	oxidoreductase FAD/NAD
K670_RS08930	91.4	359.2	0.3	electron transfer flavoprotein subunit alpha
K670_RS16560	7.2	29.5	0.2	V-type ATP synthase subunit D
K670_RS16390	25.6	128.2	0.2	sodium:proton antiporter
K670_RS08925	43.0	218.8	0.2	L-lactate permease
K670_RS08920	40.2	215.7	0.2	FAD/FMN-containing dehydrogenase
K670_RS13620	4.9	29.2	0.2	hydrogen dehydrogenase
K670_RS13350	4.9	33.3	0.1	pyruvate:ferredoxin (flavodoxin) oxidoreductase
K670_RS15260	3.2	21.8	0.1	glycolate oxidase subunit GlcD
K670_RS15110	41.7	299.9	0.1	iron hydrogenase
K670_RS02095	18.2	132.6	0.1	NADPH-dependent butanol dehydrogenase
K670_RS03370	8.7	63.6	0.1	rubrerythrin
K670_RS01455	27.1	208.5	0.1	Long-chain-alcohol dehydrogenase 1
K670_RS02015	4.0	30.9	0.1	NAD-dependent methanol dehydrogenase
K670_RS15270	11.6	92.1	0.1	electron transfer flavoprotein subunit beta
K670_RS03965	38.9	330.4	0.1	Iron hydrogenase 1
K670_RS17705	4.8	45.1	0.1	lactate dehydrogenase
K670_RS15265	18.7	179.0	0.1	Electron transfer flavoprotein subunit alpha
K670_RS18075	20.0	195.9	0.1	Fe-S oxidoreductase
K670_RS13615	11.1	124.5	0.1	NADP-reducing hydrogenase subunit HndC
K670_RS16085	86.1	994.3	0.1	Nitrogenase molybdenum-iron protein alpha chain
K670_RS10910	15.8	226.0	0.1	NAD-dependent 4-hydroxybutyrate dehydrogenase
K670_RS05840	4.0	62.6	0.1	NAD-dependent 4-hydroxybutyrate dehydrogenase
K670_RS04760	2.7	48.7	0.1	hydroxylamine reductase
K670_RS05860	3.0	67.1	0.0	Betaine aldehyde dehydrogenase
K670_RS09050	18.2	750.0	0.0	hypothetical protein
K670_RS16080	13.0	1095.2	0.0	nitrogenase molybdenum-iron protein subunit beta
K670_RS00685	0.0	8.7	0.0	hypothetical protein
K670_RS00690	0.0	5.1	0.0	(2Fe-2S)-binding protein
K670_RS00720	0.0	9.4	0.0	Phosphatidylglycerol phospholipase C
K670_RS00815	0.0	6.3	0.0	ferredoxin-NADP+ reductase subunit alpha
K670_RS05925	0.0	6.4	0.0	hypothetical protein

K670_RS05955	0.0	31.6	0.0	reverse rubrerythrin-2
K670_RS07050	0.0	63.4	0.0	Anaerobic nitric oxide reductase
K670_RS09480	0.0	88.0	0.0	xanthine dehydrogenase
K670_RS09485	0.0	8.6	0.0	Glyceraldehyde dehydrogenase medium chain
K670_RS09490	0.0	40.8	0.0	Indole-3-acetaldehyde oxidase
K670_RS09495	0.0	94.9	0.0	aldehyde oxidase
K670_RS12715	0.0	11.9	0.0	(R,R)-butanediol dehydrogenase
K670_RS13610	0.0	11.5	0.0	NADH-quinone oxidoreductase subunit E
K670_RS13690	0.0	1.3	0.0	Glycerol-1-phosphate dehydrogenase [NAD(P)+]
K670_RS15525	0.0	9.5	0.0	nitrate reductase subunit beta
K670_RS15530	0.0	21.6	0.0	Periplasmic nitrate reductase
K670_RS16075	0.0	38.0	0.0	Nitrogenase iron-molybdenum cofactor biosynthesis protein NifE
K670_RS16720	0.0	5.3	0.0	Anaerobic sulfite reductase subunit C
K670_RS16730	0.0	1.8	0.0	Electron transfer protein for anaerobic sulfite reductase subunit A
K670_RS17130	0.0	49.9	0.0	ferredoxin
K670_RS19465	0.0	6.8	0.0	pyruvate formate-lyase
K670_RS19680	0.0	15.8	0.0	Hydroxylamine reductase
[G]Carbohydrate transport and metabolism				
K670_RS02495	11.4	0.0	-	phosphopentomutase
K670_RS02705	8.3	0.0	-	Transketolase 1
K670_RS09555	8.1	0.0	-	Alpha-glucoside transport ATP-binding protein AgIK
K670_RS09635	22.5	0.0	-	Probable transketolase
K670_RS10695	31.1	0.0	-	PTS mannose transporter subunit IIA
K670_RS12445	3.4	0.0	-	Lactose-binding protein
K670_RS13275	0.0	0.0	-	2-dehydro-3-deoxygluconokinase
K670_RS13280	3.1	0.0	-	altronate oxidoreductase
K670_RS13680	36.3	0.0	-	Putative phosphotransferase enzyme IIB component SgcB
K670_RS00580	46.1	1.9	24.5	ABC transporter substrate-binding protein
K670_RS16315	1059.4	101.1	10.5	methylglyoxal synthase
K670_RS17370	47.4	4.6	10.4	drug resistance transporter EmrB/QacA family Lincomycin resistance protein LmrB
K670_RS16280	52.7	5.4	9.8	Transketolase
K670_RS10705	27.1	2.8	9.8	PTS lactose/cellobiose family IIC component
K670_RS01855	2233.4	245.5	9.1	PTS system glucose-specific EIICB component
K670_RS02290	18.8	2.6	7.4	Mannan endo-1,4-beta-mannosidase A
K670_RS00500	44.3	6.0	7.4	sugar ABC transporter substrate-binding protein
K670_RS08165	80.5	12.1	6.6	6-phosphogluconolactonase
K670_RS03725	194.6	33.0	5.9	6-phosphofructokinase
K670_RS13515	51.6	9.0	5.7	phosphoglycerate kinase
K670_RS20815	912.5	159.9	5.7	transketolase
K670_RS11240	6.5	1.3	4.9	PTS system beta-glucoside-specific EIIBC component
K670_RS15920	10.2	2.1	4.9	sugar ABC transporter permease
K670_RS11255	9.6	2.0	4.9	PfkB domain protein
K670_RS11165	19.1	3.9	4.9	N-acetylgalactosamine-specific phosphotransferase enzyme IIB component 1
K670_RS07520	4.0	0.8	4.9	Alpha-galactosidase 1
K670_RS20820	3361.3	770.6	4.4	transketolase
K670_RS09245	15.5	3.6	4.3	glycogen debranching protein
K670_RS07430	44.1	10.8	4.1	Sialic acid-binding periplasmic protein SiaP
K670_RS10060	302.7	80.2	3.8	Beta-glucanase
K670_RS18485	9.1	2.5	3.7	PTS beta-glucoside IIABC component
K670_RS07425	13.7	4.2	3.3	Ectoine TRAP transporter large permease protein TeaC
K670_RS09255	21.4	6.6	3.3	sugar ABC transporter permease
K670_RS09265	369.2	113.7	3.2	sugar-binding protein
K670_RS07020	55.8	17.5	3.2	SPBc2 prophage-derived uncharacterized protein YomE

K670_RS17470	61.9	19.4	3.2	Peptidoglycan-N-acetylmuramic acid deacetylase PdaC
K670_RS09250	166.5	54.1	3.1	peripheral protein
K670_RS13400	81.0	28.4	2.8	Alpha-amylase
K670_RS13285	3.0	1.2	2.5	Altronate dehydratase
K670_RS01340	3.2	1.3	2.5	PTS glucitol transporter subunit IIA
K670_RS01270	2.8	1.2	2.5	4-alpha-glucanotransferase
K670_RS07555	3.5	1.4	2.5	sugar ABC transporter substrate-binding protein
K670_RS18030	3.7	1.5	2.5	transporter
K670_RS12545	10.5	4.3	2.5	ABC transporter substrate-binding protein
K670_RS11180	11.9	4.9	2.5	PTS sugar transporter
K670_RS10700	16.7	6.8	2.5	cytochrome C biogenesis protein CcmE
K670_RS16035	3.6	1.5	2.5	galactoside permease
K670_RS10295	6.6	2.7	2.5	N-acetylglucosamine-6-phosphate 2-epimerase
K670_RS01360	4.8	2.0	2.5	tagatose-6-phosphate kinase
K670_RS12645	4.0	1.6	2.5	Multiple sugar-binding periplasmic receptor ChvE
K670_RS10325	45.9	18.7	2.5	Cyclomaltodextrin glucanotransferase
K670_RS15175	24.5	10.0	2.5	PTS lactose/cellobiose family IIC component
K670_RS12770	107.6	47.4	2.3	exopolysaccharide biosynthesis protein
K670_RS02985	116.2	52.1	2.2	spermidine/putrescine ABC transporter permease
K670_RS06005	7707.9	3483.0	2.2	PTS sugar transporter subunit IIA
K670_RS06345	169.2	77.0	2.2	melibiose:sodium symporter 6-phospho-beta-glucosidase 6-phospho-beta-glucosidase GmuD
K670_RS07600	25.4	11.7	2.2	glucose-6-phosphate isomerase
K670_RS15055	496.9	235.0	2.1	glucose-6-phosphate isomerase
K670_RS01850	4978.1	2411.3	2.1	PTS system glucose-specific EIIBC component
K670_RS17540	27.4	14.4	1.9	sugar ABC transporter substrate-binding protein
K670_RS16625	9.2	5.0	1.8	Aryl-phospho-beta-D-glucosidase BglC
K670_RS11430	87.8	47.8	1.8	inorganic polyphosphate/ATP-NAD kinase
K670_RS10315	71.4	39.6	1.8	hypothetical protein
K670_RS01255	111.4	63.4	1.8	ABC transporter substrate-binding protein PTS system arbutin-, cellobiose-, and salicin-specific EIIBC component
K670_RS00020	15.7	9.0	1.7	fructose 1,6-bisphosphatase
K670_RS07615	111.9	66.7	1.7	fructose 1,6-bisphosphatase
K670_RS17420	15.0	9.2	1.6	arabinose ABC transporter permease
K670_RS08295	24.4	14.9	1.6	Phosphopentomutase Probable starch degradation products transport system permease protein AmyC
K670_RS01265	20.1	12.3	1.6	Fructose-bisphosphate aldolase 2,3-bisphosphoglycerate-independent phosphoglycerate mutase
K670_RS03500	692.5	443.2	1.6	Mannose-6-phosphate isomerase
K670_RS07605	32.2	20.6	1.6	Sucrose-6-phosphate hydrolase
K670_RS11250	21.3	13.7	1.6	N-acetylglucosamine-6-phosphate deacetylase
K670_RS17865	39.2	25.6	1.5	aryl-phospho-beta-D-glucosidase
K670_RS10715	9.5	6.4	1.5	transketolase
K670_RS02535	14.6	10.0	1.5	antiporter
K670_RS15220	9.4	6.4	1.5	Oligo-1,6-glucosidase 1
K670_RS01280	7.9	5.4	1.5	galactose mutarotase
K670_RS17170	56.9	40.1	1.4	PTS system beta-glucoside-specific EIIBC component
K670_RS15735	2.4	1.9	1.2	Aryl-phospho-beta-D-glucosidase BglC
K670_RS09450	3.1	2.5	1.2	ABC transporter substrate-binding protein
K670_RS07540	3.4	2.8	1.2	sugar ABC transporter substrate-binding protein
K670_RS12610	4.2	3.4	1.2	MFS transporter
K670_RS14135	3.5	2.8	1.2	Glucosamine-6-phosphate deaminase
K670_RS00055	175.6	143.3	1.2	transaldolase
K670_RS12675	21.5	17.6	1.2	endonuclease
K670_RS04435	34.7	28.3	1.2	Phosphopentomutase
K670_RS12660	11.8	9.6	1.2	

K670_RS11220	7.9	6.5	1.2	alpha-ribazole-5-phosphate phosphatase
K670_RS16030	9.0	7.4	1.2	sucrose-6-phosphate hydrolase
K670_RS04195	16.5	14.2	1.2	Maltase-glucoamylase, intestinal
K670_RS20695	400.7	350.2	1.1	ATP-dependent 6-phosphofructokinase
K670_RS15665	50.4	44.1	1.1	1,4-alpha-glucan branching enzyme GlgB 1
K670_RS18245	16.8	14.9	1.1	PTS system alpha-glucoside-specific EIICB component
K670_RS12510	177.4	158.5	1.1	N-acetylmannosamine kinase
K670_RS14050	225.9	202.8	1.1	PTS alpha-glucoside transporter subunit IIBC
K670_RS11920	74.7	67.2	1.1	cytochrome C oxidase subunit II Alpha-amylase
K670_RS20690	929.2	837.2	1.1	Pyruvate kinase
K670_RS15925	87.4	81.9	1.1	hypothetical protein
K670_RS01120	1678.7	1632.5	1.0	phosphoenolpyruvate-protein phosphotransferase
K670_RS12600	4.9	4.8	1.0	alpha-amylase
K670_RS20840	316.7	311.1	1.0	Bifunctional NAD(P)H-hydrate repair enzyme Nnr
K670_RS03130	371.9	368.5	1.0	Pyruvate kinase
K670_RS02565	6.1	6.2	1.0	PTS ascorbate transporter subunit IIC
K670_RS15845	15.0	15.3	1.0	hypothetical protein
K670_RS05775	71.4	72.8	1.0	4-alpha-glucanotransferase
K670_RS12565	49.7	54.5	0.9	galactokinase
K670_RS16630	23.3	25.7	0.9	PTS system beta-glucoside-specific EIIBCBA component
K670_RS01285	10.7	12.0	0.9	PTS system glucose-specific EIICBA component
K670_RS16195	36.1	40.5	0.9	fucose isomerase
K670_RS07255	40.9	46.4	0.9	acyltransferase
K670_RS01430	41.3	47.3	0.9	Transketolase
K670_RS00495	33.1	38.2	0.9	sugar ABC transporter substrate-binding protein
K670_RS19300	314.7	366.0	0.9	glycogen branching protein
K670_RS03870	75.3	91.3	0.8	glycogen-debranching protein
K670_RS08100	3.5	4.2	0.8	Mannan endo-1,4-beta-mannosidase
K670_RS16705	13.6	16.6	0.8	ribose 5-phosphate isomerase A
K670_RS09440	9.9	12.2	0.8	PTS cellobiose transporter subunit IIC
K670_RS15935	6.4	7.8	0.8	beta-galactosidase
K670_RS17570	8.6	10.6	0.8	Autoinducer 2 import system permease protein LsrD
K670_RS07410	3.7	4.5	0.8	Inner membrane transport protein YdiN
K670_RS19080	5.9	7.2	0.8	Xylulose kinase
K670_RS00015	2.7	3.3	0.8	Trehalose-6-phosphate hydrolase
K670_RS00410	1.9	2.3	0.8	alpha-glucosidase
K670_RS10110	2351.5	2906.8	0.8	ABC transporter permeas
K670_RS03490	983.8	1255.6	0.8	phosphoglycerate kinase glycerol-3-phosphate ABC-transporter substrate-binding protein
K670_RS18380	16.5	21.5	0.8	glucose-1-phosphate adenylyltransferase
K670_RS19320	287.3	376.5	0.8	beta-glucanase
K670_RS12585	409.2	539.4	0.8	polysaccharide deacetylase
K670_RS10825	32.6	43.7	0.7	6-phospho-alpha-glucosidase
K670_RS15825	20.0	27.2	0.7	phosphoglucosamine mutase
K670_RS01205	141.8	193.8	0.7	PTS system mannose-specific EIIBAB component
K670_RS14055	539.1	754.0	0.7	Probable 6-phospho-beta-glucosidase
K670_RS10160	20.3	29.0	0.7	triosephosphate isomerase
K670_RS03495	625.5	893.4	0.7	D-galactose-binding periplasmic protein
K670_RS09645	11.7	16.8	0.7	ABC transporter substrate-binding protein
K670_RS10120	806.0	1157.4	0.7	Maltodextrin phosphorylase
K670_RS01390	32.9	47.4	0.7	Glyceraldehyde-3-phosphate dehydrogenase
K670_RS03485	25002.0	37174.4	0.7	Galactose-1-phosphate uridylyltransferase
K670_RS12575	29.6	44.7	0.7	D-galactose-binding periplasmic protein
K670_RS15700	21.3	33.0	0.6	L-arabinose isomerase
K670_RS17555	30.2	48.1	0.6	Probable arabinogalactan endo-beta-1,4-galactanase A
K670_RS13035	1.2	1.9	0.6	

K670_RS13005	5.0	8.1	0.6	hypothetical protein
K670_RS13685	3.5	5.7	0.6	PTS galactitol transporter subunit IIC
K670_RS01640	7.9	12.8	0.6	Glucan 1,6- α -glucosidase
K670_RS10170	3.1	5.1	0.6	6-phospho- β -glucosidase BglA
K670_RS13270	7.2	11.7	0.6	2-dehydro-3-deoxy-phosphogluconate aldolase
K670_RS13965	6.6	10.8	0.6	Transketolase
K670_RS17810	43.6	71.1	0.6	glycosyl hydrolase
K670_RS17875	9.9	16.2	0.6	carbohydrate ABC transporter substrate-binding protein
K670_RS04215	8.3	13.6	0.6	Transketolase 1
K670_RS14045	148.8	244.9	0.6	PTS mannose transporter subunit IID
K670_RS17685	10.3	18.2	0.6	ABC transporter substrate-binding protein
K670_RS07175	66.3	119.7	0.6	Phosphoglucomutase
K670_RS05760	36.3	65.9	0.6	sugar ABC transporter ATP-binding protein
K670_RS19315	195.8	368.2	0.5	glucose-1-phosphate adenylyltransferase
K670_RS14110	23.1	44.0	0.5	MFS transporter
K670_RS19305	55.4	106.8	0.5	glycogen synthase
K670_RS19310	132.0	255.6	0.5	Amylopullulanase
K670_RS05780	659.2	1325.5	0.5	maltodextrin phosphorylase
K670_RS20660	4.4	9.1	0.5	Endoglucanase D
K670_RS02550	6.7	13.6	0.5	ribulose 5-phosphate epimerase
K670_RS13335	20.0	40.8	0.5	1-phosphofructokinase
K670_RS12690	5.6	11.4	0.5	Putative transketolase N-terminal section
K670_RS09560	5.5	11.2	0.5	L-arabinose transport system permease protein AraQ
K670_RS18395	4.0	8.2	0.5	Fe(3+) ions import ATP-binding protein FbpC 1
K670_RS17530	47.7	103.9	0.5	PTS lactose IIa component
K670_RS00090	21.8	47.4	0.5	hypothetical protein
K670_RS10125	69.3	152.1	0.5	PTS cellobiose transporter subunit IIC
K670_RS19940	167.4	373.3	0.4	galactose-1-phosphate uridylyltransferase
K670_RS06375	28.7	64.5	0.4	Ribulose-phosphate 3-epimerase
K670_RS18385	9.7	21.7	0.4	ABC transporter permease
K670_RS12405	5.8	13.0	0.4	β -galactosidase
K670_RS05715	599.3	1354.2	0.4	Pyruvate, phosphate dikinase
K670_RS10275	24.1	56.4	0.4	Phospho-cellobiase
K670_RS00220	7.5	18.5	0.4	Inner membrane transport protein YajR
K670_RS18690	7.3	17.8	0.4	sugar ABC transporter
K670_RS17545	6.7	16.4	0.4	ribulose 5-phosphate epimerase
K670_RS13975	5.0	12.2	0.4	D-ribose transporter subunit RbsB
K670_RS09240	2.2	6.4	0.4	Pullulanase
K670_RS13340	4.6	13.1	0.4	PTS system fructose-specific EIIABC component
K670_RS05045	3.5	10.0	0.3	ABC transporter substrate-binding protein
K670_RS13910	2.6	7.5	0.3	polysaccharide ABC transporter substrate-binding protein
K670_RS03605	87.8	256.1	0.3	dihydroxyacetone kinase subunit DhaM
K670_RS17870	9.4	28.9	0.3	N-acetyl-D-glucosamine kinase
K670_RS15295	10.2	31.2	0.3	Putative polysaccharide deacetylase YxkH
K670_RS15205	4.0	13.0	0.3	N-acetylglucosamine-6-phosphate deacetylase
K670_RS14560	4.0	13.0	0.3	Glycerate kinase
K670_RS03600	29.6	96.7	0.3	PTS-dependent dihydroxyacetone kinase, ADP-binding subunit DhaL
K670_RS18705	2.6	8.3	0.3	Cyclomaltodextrinase
K670_RS16290	8.4	30.5	0.3	transaldolase
K670_RS09390	10.9	39.9	0.3	Fructose permease IID component
K670_RS09550	5.7	20.9	0.3	sugar phosphate isomerase
K670_RS10280	13.1	49.5	0.3	6-phospho- β -glucosidase
K670_RS18540	6.8	27.6	0.2	Fructose-6-phosphate aldolase 1
K670_RS10190	3.4	14.0	0.2	PTS lactose/cellobiose family IIC component
K670_RS03675	12.6	53.9	0.2	Peptidoglycan-N-acetylmuramic acid deacetylase PdaC

K670_RS19935	42.3	189.7	0.2	Pullulanase 1, chloroplastic
K670_RS12050	566.3	2542.1	0.2	Uncharacterized protein YrkC
K670_RS01400	5.1	23.9	0.2	Maltogenic alpha-amylase
K670_RS12685	9.8	51.9	0.2	Putative transketolase C-terminal section
K670_RS00700	6.5	34.6	0.2	Glycerol uptake facilitator protein
K670_RS05930	42.2	238.3	0.2	Macrolide efflux protein A
K670_RS07105	36.7	216.5	0.2	ATP-dependent 6-phosphofructokinase 2
K670_RS16140	3.4	21.1	0.2	enolase
K670_RS13605	5.2	32.0	0.2	sugar ABC transporter permease
K670_RS19740	3.9	25.6	0.2	Peptidoglycan-N-acetylglucosamine deacetylase
K670_RS18340	33.3	225.4	0.1	Peptidoglycan-N-acetylglucosamine deacetylase Bifunctional UDP-N-acetylglucosamine 2-epimerase/N- acetylmannosamine kinase
K670_RS12805	4.9	34.3	0.1	Ribokinase
K670_RS13995	4.9	42.2	0.1	glycosyl transferase
K670_RS20665	5.0	55.3	0.1	arabinose ABC transporter permease
K670_RS12535	5.5	85.2	0.1	Pyruvate kinase I
K670_RS20620	65.5	1090.9	0.1	Enolase
K670_RS03515	31.0	644.6	0.0	beta-glucosidase
K670_RS00415	0.0	5.9	0.0	PTS fructose transporter subunit IIB
K670_RS01335	0.0	20.4	0.0	galactose-6-phosphate isomerase
K670_RS01350	0.0	11.2	0.0	ructose-bisphosphate aldolase
K670_RS01355	0.0	4.3	0.0	galactose mutarotase
K670_RS01365	0.0	4.2	0.0	transketolase
K670_RS02265	0.0	15.8	0.0	Ribulose-phosphate 3-epimerase, chromosomal
K670_RS02525	0.0	5.5	0.0	transketolase
K670_RS02540	0.0	2.3	0.0	Putative L-ribulose-5-phosphate 3-epimerase SgbU
K670_RS02545	0.0	6.4	0.0	transaldolase
K670_RS04230	0.0	18.4	0.0	transketolase
K670_RS04235	0.0	16.1	0.0	hypothetical protein
K670_RS04465	0.0	51.6	0.0	ATPase
K670_RS04865	0.0	2.1	0.0	Cellobiose-specific phosphotransferase enzyme IIA component
K670_RS04875	0.0	20.4	0.0	Probable 6-phospho-beta-glucosidase
K670_RS04880	0.0	15.2	0.0	PTS system glucose-specific EIIBC component
K670_RS06050	0.0	1.0	0.0	peptidoglycan N-acetylglucosamine deacetylase
K670_RS07240	0.0	10.0	0.0	N-acetylmannosamine-6-phosphate epimerase
K670_RS07500	0.0	8.1	0.0	glucokinase
K670_RS07505	0.0	3.9	0.0	sugar ABC transporter permease
K670_RS07565	0.0	2.2	0.0	glycosylase
K670_RS07570	0.0	12.6	0.0	Beta-glucoside kinase
K670_RS07585	0.0	2.0	0.0	ABC transporter substrate-binding protein
K670_RS07590	0.0	8.5	0.0	PTS sorbose transporter subunit IIC
K670_RS09395	0.0	4.3	0.0	hypothetical protein
K670_RS09400	0.0	3.9	0.0	Oligo-beta-mannoside-specific phosphotransferase enzyme IIA component
K670_RS09435	0.0	19.9	0.0	Beta-glucanase
K670_RS09460	0.0	2.9	0.0	Part of the ABC transporter complex UgpABCE involved in sn-glycerol-3-phosphate import
K670_RS09570	0.0	4.2	0.0	PTS cellobiose transporter subunit IIB
K670_RS10130	0.0	13.6	0.0	TOLigo-beta-mannoside-specific phosphotransferase enzyme IIA component
K670_RS10135	0.0	6.4	0.0	sugar kinase
K670_RS10175	0.0	4.1	0.0	Cellobiose-specific phosphotransferase enzyme IIB component
K670_RS10180	0.0	6.7	0.0	6-phospho-beta-glucosidase BglA
K670_RS10200	0.0	2.5	0.0	sugar ABC transporter substrate-binding protein
K670_RS10980	0.0	24.1	0.0	4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase 1
K670_RS11150	0.0	2.2	0.0	

K670_RS11175	0.0	11.4	0.0	PTS N-acetylglucosamine transporter subunit IIBC
K670_RS11270	0.0	7.3	0.0	NUDIX hydrolase
K670_RS12550	0.0	3.3	0.0	Alpha-galactosidase 1
K670_RS12560	0.0	7.7	0.0	sugar phosphorylase
K670_RS12635	0.0	3.0	0.0	Autoinducer 2 import system permease protein LsrC
K670_RS12695	0.0	7.5	0.0	phosphopentomutase
K670_RS12810	0.0	299.3	0.0	L-fucose phosphate aldolase
K670_RS12875	0.0	0.9	0.0	Pullulanase
K670_RS13265	0.0	14.2	0.0	glucuronate isomerase
K670_RS13315	0.0	9.6	0.0	MFS transporter
K670_RS13590	0.0	2.2	0.0	Alpha-glucosidase
K670_RS13595	0.0	4.4	0.0	ABC transporter substrate-binding protein
K670_RS13675	0.0	4.1	0.0	PTS galactitol transporter subunit IIA
K670_RS13695	0.0	2.3	0.0	Phosphoglycolate phosphatase
K670_RS13930	0.0	6.4	0.0	N-acetylmannosamine kinase
K670_RS13970	0.0	3.2	0.0	transaldolase
K670_RS13990	0.0	10.1	0.0	D-ribose pyranase
K670_RS15195	0.0	6.8	0.0	PTS cellobiose transporter subunit IIB
K670_RS15740	0.0	5.1	0.0	6-phospho-beta-glucosidase
K670_RS15930	0.0	5.1	0.0	hypothetical protein
K670_RS15940	0.0	3.6	0.0	arabinogalactan endo-1,4-beta-galactosidase
K670_RS16015	0.0	1.8	0.0	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)
K670_RS16270	0.0	2.2	0.0	polysaccharide ABC transporter substrate-binding protein
K670_RS17405	0.0	4.9	0.0	Chitoooligosaccharide deacetylase
K670_RS17580	0.0	16.3	0.0	LacI family transcriptional regulator
K670_RS17585	0.0	21.5	0.0	LacI family transcriptional regulator
K670_RS17740	0.0	5.0	0.0	PTS system N-acetylglucosamine-specific EIICB component
K670_RS17830	0.0	2.2	0.0	Fructose-bisphosphate aldolase
K670_RS17835	0.0	9.1	0.0	PTS mannose transporter subunit IIAB
K670_RS17850	0.0	6.6	0.0	Fructose permease IID component
K670_RS17855	0.0	2.4	0.0	Putative N-acetylglactosamine permease
K670_RS17885	0.0	2.1	0.0	ABC transporter
K670_RS18010	0.0	2.1	0.0	Pectate lyase C
K670_RS18465	0.0	1.1	0.0	Beta-xylosidase
K670_RS18525	0.0	0.7	0.0	alpha-mannosidase
K670_RS18710	0.0	7.1	0.0	haloacid dehalogenase
K670_RS18740	0.0	12.0	0.0	2-dehydro-3-deoxy-phosphogluconate aldolase
K670_RS19075	0.0	4.8	0.0	N-acetylmannosamine kinase
[I]Lipid transport and metabolism				
K670_RS16370	1357.7	312.2	4.3	Phosphatidylserine decarboxylase proenzyme 1
K670_RS06285	11914.5	2761.9	4.3	acyl carrier protein
K670_RS06730	1261.5	353.9	3.6	3-oxoacyl-ACP synthase III
K670_RS07345	347.0	120.1	2.9	cardiolipin synthase
K670_RS11125	1260.4	453.2	2.8	1-acyl-sn-glycerol-3-phosphate acyltransferase 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (flavodoxin)
K670_RS06145	132.6	54.1	2.5	undecaprenyl pyrophosphate synthetase 1
K670_RS06170	156.1	63.7	2.5	undecaprenyl pyrophosphate synthetase 1
K670_RS08085	1724.3	709.3	2.4	hypothetical protein
K670_RS18865	54.3	24.6	2.2	acyl-ACP thioesterase
K670_RS06725	3766.5	2022.9	1.9	acyl carrier protein
K670_RS07720	411.2	253.9	1.6	oxidoreductase CDP-diacylglycerol--glycerol-3-phosphate3- phosphatidyltransferase
K670_RS06025	213.2	132.1	1.6	phosphatidyltransferase
K670_RS14640	22.2	15.6	1.4	2-hydroxyglutaryl-CoA dehydratase
K670_RS06165	86.1	60.9	1.4	CDP-diglyceride synthetase

K670_RS15305	51.1	37.5	1.4	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, chloroplastic
K670_RS03385	1745.3	1295.6	1.3	CDP-diacylglycerol--serineO-phosphatidy
K670_RS03085	305.0	233.4	1.3	hypothetical protein
K670_RS03390	122.0	97.5	1.3	lipid kinase
K670_RS06680	333.6	288.1	1.2	acetyl-CoA carboxylase subunit alpha
K670_RS17690	78.2	68.9	1.1	Acetyl esterase
K670_RS15140	1926.6	1851.0	1.0	Acyl-CoA dehydrogenase, short-chain specific
K670_RS06690	246.2	238.5	1.0	Biotin carboxylase
K670_RS06715	178.0	172.7	1.0	ACP S-malonyltransferase
K670_RS06685	1686.9	1732.4	1.0	acetyl-CoA carboxylase carboxyltransferase subunit beta
K670_RS03430	71.8	77.2	0.9	1-acylglycerol-3-phosphate O-acyltransferase
K670_RS11745	43.9	50.1	0.9	phosphoesterase
K670_RS14910	37.7	44.0	0.9	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
K670_RS20845	91.9	112.5	0.8	4'-phosphopantetheinyl transferase
K670_RS12260	655.1	859.6	0.8	acetyl-xylan esterase
K670_RS06695	90.1	119.5	0.8	3-hydroxyacyl-[acyl-carrier-protein] dehydratase FabZ
K670_RS06290	76.4	104.5	0.7	Phosphate acyltransferase
K670_RS06155	361.9	526.3	0.7	1-deoxy-D-xylulose 5-phosphate reductoisomerase
K670_RS10465	547.0	865.0	0.6	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase
K670_RS15125	1487.5	2465.2	0.6	3-hydroxybutyryl-CoA dehydrogenase
K670_RS14820	2274.6	3998.1	0.6	acetyl-CoA acetyltransferase
K670_RS15145	400.8	900.7	0.4	crotonase
K670_RS09680	22.4	56.1	0.4	phospholipase D
K670_RS06700	130.8	339.4	0.4	Biotin carboxyl carrier protein of acetyl-CoA carboxylase
K670_RS13025	11.5	31.3	0.4	lysophospholipase
K670_RS12815	5.6	16.1	0.3	Acetate CoA-transferase YdiF
K670_RS06705	180.8	543.0	0.3	3-oxoacyl-ACP synthase
K670_RS08150	7.2	47.1	0.2	dihydroorotate dehydrogenase
K670_RS19895	21.9	148.4	0.1	lysophospholipase
K670_RS08915	7.8	60.7	0.1	Acyl-CoA dehydrogenase, short-chain specific
K670_RS00190	0.0	27.1	0.0	CDP-diacylglycerol--serine O-phosphatidyltransferase
K670_RS00710	0.0	3.5	0.0	Acetate CoA-transferase YdiF
K670_RS01605	0.0	84.6	0.0	biotin attachment protein
K670_RS01610	0.0	141.3	0.0	Pyruvate carboxylase subunit A
K670_RS03765	0.0	126.8	0.0	D-alanine--poly(phosphoribitol) ligase subunit 2
[P]Inorganic ion transport and metabolism				
K670_RS06480	31.2	0.0	-	hypothetical protein
K670_RS07420	19.9	0.0	-	C4-dicarboxylate transport system
K670_RS09420	6.7	0.0	-	molybdate ABC transporter permease
K670_RS12640	2.9	0.0	-	L-arabinose transport ATP-binding protein AraG
K670_RS13555	20.9	0.0	-	Methionine import system permease protein MetP
K670_RS13915	5.1	0.0	-	sugar ABC transporter permease
K670_RS16830	5.4	0.0	-	ATP-binding protein
K670_RS18455	23.9	0.0	-	formate/nitrite transporter family protein
K670_RS18700	10.3	0.0	-	Probable ABC transporter permease protein YurM
K670_RS03925	38.6	2.6	14.7	Trk system potassium uptake protein TrkA homolog 1
K670_RS08140	16.1	1.6	9.8	arsenic transporter
K670_RS02585	40.6	4.1	9.8	Uncharacterized sodium-dependent transporter HI_1690
K670_RS08985	72.6	8.4	8.6	Sulfate adenyltransferase subunit 2
K670_RS09260	22.3	2.6	8.6	sugar ABC transporter permease
K670_RS01370	101.5	13.8	7.3	carbonate dehydratase
K670_RS09000	105.7	15.5	6.8	sulfate ABC transporter ATP-binding protein
K670_RS10665	40.6	7.4	5.5	phosphate transporter
K670_RS03930	125.4	23.7	5.3	potassium transporter KefA
K670_RS08465	18.9	3.9	4.9	iron ABC transporter permease

K670_RS19515	907.5	192.8	4.7	Na(+)/H(+)-K(+) antiporter GerN Na(+)/H(+) antiporter
K670_RS09015	191.0	40.8	4.7	Sulfate-binding protein
K670_RS02650	36.2	8.4	4.3	heme ABC transporter ATP-binding protein
K670_RS13550	21.8	5.4	4.1	methionine import ATP-binding protein MetN 1
K670_RS01050	170.1	44.0	3.9	transporter
K670_RS01045	561.1	150.5	3.7	cobalt ABC transporter ATP-binding protein
K670_RS17915	755.7	204.9	3.7	peptide ABC transporter permease
K670_RS05560	26.0	7.1	3.7	Calcium-transporting ATPase 1
K670_RS08565	143.4	39.0	3.7	iron transporter FeoA
K670_RS08980	271.1	75.3	3.6	Sulfate adenyltransferase subunit 1
K670_RS01040	441.2	128.6	3.4	obalt import ATP-binding protein CbiO 1
K670_RS14320	619.0	196.3	3.2	periplasmic solute-binding protein
K670_RS02440	99.5	31.9	3.1	Ktr system potassium uptake protein A
K670_RS09010	37.5	13.1	2.9	sulfate ABC transporter permease CysT
K670_RS01070	726.3	257.4	2.8	hypothetical protein
K670_RS17720	127.3	46.3	2.7	H ⁺ -transporting ATPase
K670_RS17920	1844.2	678.5	2.7	Oligopeptide transport system permease protein AmiD
K670_RS02090	249.6	94.8	2.6	hemolysin
K670_RS10570	143.3	56.5	2.5	magnesium transporter
K670_RS13600	5.3	2.1	2.5	sugar ABC transporter permease
K670_RS02655	5.5	2.2	2.5	membrane protein
K670_RS10080	4.9	2.0	2.5	DNA-binding protein
K670_RS11755	74.0	30.2	2.5	hemerythrin
K670_RS14010	239.1	97.6	2.5	iron transporter FeoA
K670_RS20015	64.0	26.1	2.5	Sodium-dependent acetylcholine transporter
K670_RS12435	5.4	2.2	2.5	Lactose transport system permease protein LacG
K670_RS13985	2.9	1.2	2.4	Arabinose import ATP-binding protein AraG 1
K670_RS02980	75.5	33.0	2.3	ABC transporter permease
K670_RS14175	86.9	39.0	2.2	Transport of potassium into the cell
K670_RS09005	49.5	22.4	2.2	sulfate ABC transporter permease
K670_RS19290	62.9	29.9	2.1	bacitracin ABC transporter permease
K670_RS20330	345.8	167.9	2.1	hypothetical protein
K670_RS15870	50.0	25.5	2.0	iron ABC transporter
K670_RS06825	49.4	25.9	1.9	chloride channel protein
K670_RS03895	8.0	4.3	1.8	Na/Pi cotransporter
K670_RS17305	32.1	17.5	1.8	sulfate permease
K670_RS19875	42.5	23.1	1.8	ArsC family transcriptional regulator
K670_RS02445	26.5	14.8	1.8	TrkH family potassium uptake protein
K670_RS20375	30.4	18.6	1.6	cation diffusion facilitator transporter
K670_RS16045	27.4	17.4	1.6	MDR-type permease
K670_RS10860	29.7	18.8	1.6	ATP synthase subunit J
K670_RS08560	121.9	78.2	1.6	iron transporter FeoB
K670_RS13405	55.5	36.0	1.5	magnesium-importing ATPase
K670_RS09720	50.4	32.9	1.5	arsenic ABC transporter ATPase
K670_RS17715	55.5	36.8	1.5	potassium uptake protein
K670_RS03745	20.6	14.0	1.5	metal ABC transporter ATP-binding protein
K670_RS12440	15.2	10.3	1.5	lactose ABC transporter permease
K670_RS14610	18.2	12.4	1.5	ABC transporter ATP-binding protein
K670_RS03095	70.4	49.3	1.4	ABC transporter substrate-binding protein Uncharacterized ABC transporter substrate-binding
K670_RS15865	28.5	21.3	1.3	lipoprotein YvrC
K670_RS01895	30.3	22.7	1.3	Dipeptide transport system permease protein DppC
K670_RS06900	41.9	31.7	1.3	ABC transporter permease
K670_RS14600	35.5	27.2	1.3	ABC transporter substrate-binding protein
K670_RS20465	81.2	66.3	1.2	copper ion-binding protein

K670_RS18275	17.3	14.1	1.2	Putative oligopeptide transport system permease protein oppB2
K670_RS16820	16.1	13.1	1.2	cobalt ABC transporter
K670_RS08460	4.7	3.9	1.2	iron ABC transporter permease
K670_RS13545	92.0	79.5	1.2	methionine ABC transporter ATPase
K670_RS20470	12.5	11.6	1.1	ATPase
K670_RS16770	94.4	89.9	1.1	membrane protein
K670_RS03410	128.5	128.2	1.0	Magnesium transporter MgtE
K670_RS11855	67.4	68.7	1.0	sulfurtransferase
K670_RS12015	67.5	68.9	1.0	Magnesium and cobalt efflux protein CorC
K670_RS10855	83.3	93.4	0.9	potassium transporter Trk
K670_RS03740	62.6	74.3	0.8	metal ABC transporter permease
K670_RS11130	633.7	760.8	0.8	carbonate dehydratase
K670_RS01680	5.7	7.0	0.8	ABC transporter ATP-binding protein
K670_RS14195	22.2	27.2	0.8	cobalt transporter
K670_RS18315	17.6	21.5	0.8	ZIP zinc transporter
K670_RS00210	25.0	30.6	0.8	Copper homeostasis protein CutC
K670_RS08375	35.2	45.0	0.8	cation transporte
K670_RS13525	42.9	57.8	0.7	cation transporter
K670_RS10115	859.1	1174.1	0.7	ABC transporter
K670_RS01900	65.6	91.7	0.7	Oligopeptide transport system permease protein AmiC
K670_RS19810	62.5	88.6	0.7	Sodium-dependent proline transporter
K670_RS04670	106.7	158.5	0.7	Uncharacterized protein YqeW
K670_RS13470	28.4	44.9	0.6	Calcium-transporting ATPase
K670_RS11795	11.7	19.1	0.6	potassium channel protein
K670_RS14200	2.6	4.2	0.6	cobalt ABC transporter ATP-binding protein
K670_RS15225	12.9	22.8	0.6	ABC transporter substrate-binding protein
K670_RS14605	24.6	45.2	0.5	ABC transporter permease
K670_RS01620	35.3	64.8	0.5	chromate transporter
K670_RS16825	73.6	135.1	0.5	cobalt transporter CbiM
K670_RS17950	9.6	17.7	0.5	Dipeptide transport system permease protein DppB
K670_RS01305	120.8	230.4	0.5	multidrug transporter
K670_RS07560	4.8	9.8	0.5	Multiple sugar-binding transport system permease protein MsmF
K670_RS11930	4.7	9.6	0.5	Inner membrane protein YrbG
K670_RS01625	9.2	18.7	0.5	chromate transporter
K670_RS15350	35.7	75.3	0.5	formate/nitrite transporter
K670_RS06905	117.2	250.1	0.5	ABC transporter substrate-binding protein
K670_RS09565	10.0	22.4	0.4	ABC transporter permease
K670_RS01670	17.4	40.3	0.4	taurine ABC transporter permease
K670_RS03560	114.5	267.0	0.4	methionine ABC transporter substrate-binding protein
K670_RS09935	35.6	86.1	0.4	Uncharacterized protein YqeW
K670_RS10535	27.6	67.6	0.4	hypothetical protein
K670_RS18390	5.3	12.9	0.4	glycerol-3-phosphate ABC transporter
K670_RS06500	5.1	12.5	0.4	phosphate ABC transporter permease
K670_RS09730	25.7	66.3	0.4	arsenic transporter
K670_RS10055	22.7	59.2	0.4	Calcium-transporting ATPase 1
K670_RS03905	28.8	77.0	0.4	Calcium-transporting ATPase 2
K670_RS16105	13.8	37.5	0.4	molybdate-binding protein
K670_RS14000	18.3	49.9	0.4	iron transporter FeoB
K670_RS08275	5.6	15.9	0.4	potassium transporter
K670_RS02595	5.9	16.7	0.4	Zinc transporter ZupT
K670_RS19910	8.7	24.8	0.3	chromate transporter
K670_RS03670	68.9	203.8	0.3	tryptophan transporter
K670_RS14075	169.7	503.0	0.3	acriflavin resistance protein
K670_RS10660	7.4	24.3	0.3	UPF0111 protein YkaA

K670_RS04740	106.6	365.3	0.3	tellurium resistance protein
K670_RS12835	26.3	92.2	0.3	Inner membrane protein alx
K670_RS15160	12.4	47.7	0.3	Cadmium, zinc and cobalt-transporting ATPase
K670_RS16120	722.8	2818.4	0.3	molybdate ABC transporter substrate-binding protein
K670_RS06490	61.1	239.2	0.3	Phosphate import ATP-binding protein PstB
K670_RS14155	4.8	19.4	0.2	ferrichrome ABC transporter substrate-binding protein
K670_RS08450	4.8	19.6	0.2	ABC transporter
K670_RS13030	3.7	14.9	0.2	Chloride/fluoride channel protein
K670_RS16130	81.3	342.1	0.2	Spermidine/putrescine import ATP-binding protein PotA
K670_RS01260	52.3	256.3	0.2	ABC transporter permease
K670_RS16125	20.2	104.5	0.2	molybdate ABC transporter permease
K670_RS08765	15.9	88.7	0.2	Fe ³⁺ ABC transporter permease
K670_RS18060	35.9	232.8	0.2	cobalamin biosynthesis protein CbiM Spermidine/putrescine transport system permease protein
K670_RS05870	16.9	122.1	0.1	PotC homolog
K670_RS12500	2.8	20.8	0.1	alkaline phosphatase
K670_RS03610	76.0	565.1	0.1	Potassium-transporting ATPase A chain
K670_RS03555	21.8	162.8	0.1	methionine ABC transporter permease
K670_RS07270	17.6	144.0	0.1	spermidine/putrescine ABC transporter ATP-binding protein
K670_RS13415	78.2	720.6	0.1	magnesium ABC transporter ATPase
K670_RS05505	66.8	616.1	0.1	calcium-translocating P-type ATPase PMCA-type
K670_RS03615	116.7	1153.2	0.1	potassium-transporting ATPase subunit B
K670_RS06495	5.1	52.2	0.1	phosphate ABC transporter permease
K670_RS03620	30.7	407.7	0.1	potassium-transporting ATPase C chain
K670_RS07265	69.1	1058.0	0.1	sulfonate ABC transporter permease
K670_RS06505	4.8	77.1	0.1	phosphate-binding protein
K670_RS18345	39.7	717.7	0.1	Magnesium transporter
K670_RS19970	16.3	357.9	0.0	iron transporter FeoB
K670_RS17315	64.3	1956.8	0.0	ammonia permease
K670_RS07275	22.1	846.4	0.0	nitrate ABC transporter substrate-binding protein
K670_RS18090	7.5	415.3	0.0	superoxide dismutase
K670_RS05875	5.3	463.0	0.0	spermidine/putrescine ABC transporter permease
K670_RS10245	2.8	273.1	0.0	ammonium transporter
K670_RS16100	5.5	885.8	0.0	Nitrogenase iron protein
K670_RS01675	0.0	1.8	0.0	taurine ABC transporter substrate-binding protein
K670_RS06485	0.0	28.5	0.0	PhoU family transcriptional regulator
K670_RS07460	0.0	1.9	0.0	peptide ABC transporter permease
K670_RS08455	0.0	4.9	0.0	iron ABC transporter ATP-binding protein
K670_RS08775	0.0	7.1	0.0	ABC transporter substrate-binding protein
K670_RS09760	0.0	32.1	0.0	sulfurtransferase
K670_RS09780	0.0	41.5	0.0	cation diffusion facilitator transporter
K670_RS10670	0.0	12.0	0.0	Protein PhnA
K670_RS12920	0.0	141.8	0.0	protein CotJC
K670_RS12985	0.0	1029.6	0.0	Protein CotJC
K670_RS14005	0.0	50.4	0.0	iron transporter FeoA
K670_RS14145	0.0	1.8	0.0	iron ABC transporter permease
K670_RS14150	0.0	1.9	0.0	ferrichrome ABC transporter permease
K670_RS15875	0.0	2.4	0.0	ABC transporter
K670_RS16835	0.0	4.8	0.0	cobalt ABC transporter permease
K670_RS17575	0.0	1.2	0.0	sugar ABC transporter ATP-binding protein
K670_RS17890	0.0	4.2	0.0	ABC transporter permease
K670_RS17955	0.0	4.5	0.0	Oligopeptide transport system permease protein AppC
K670_RS18065	0.0	27.9	0.0	cobalt ABC transporter permease
K670_RS18070	0.0	61.9	0.0	cobalt ABC transporter ATP-binding protein
K670_RS18145	0.0	6.9	0.0	Putative thiamine biosynthesis protein HI_0357
K670_RS18150	0.0	26.7	0.0	ABC transporter permease

K670_RS18155	0.0	7.2	0.0	ABC transporter permease
K670_RS18160	0.0	29.0	0.0	Sulfate/thiosulfate import ATP-binding protein CysA
K670_RS18270	0.0	12.5	0.0	peptide ABC transporter permease
K670_RS18675	0.0	10.1	0.0	ABC transporter ATP-binding protein
K670_RS18695	0.0	7.8	0.0	sugar ABC transporter permease
K670_RS19900	0.0	11.7	0.0	ArsC family transcriptional regulator
K670_RS19915	0.0	61.1	0.0	chromate transporter
K670_RS19975	0.0	186.4	0.0	ferrous iron transporter A

Table A2. Genes Expression changes according to Library BX571963.

Gene name	NFM	PM	FC	Annotation
<i>[C]Energy production and conversion</i>				
RPA0107	6.7	0.0	-	Zn-binding dehydrogenase
RPA0112	6.9	0.0	-	betaine aldehyde dehydrogenase
RPA0552	15.9	0.0	-	C4-dicarboxylate transporter DctA
RPA0670	15.1	0.0	-	4-hydroxybenzoyl-CoA reductase subunit
RPA0674	18.9	0.0	-	alcohol dehydrogenase
RPA0732	31.7	0.0	-	formate dehydrogenase subunit gamma
RPA0733	51.0	0.0	-	NADH dehydrogenase (quinone)
RPA0735	47.0	0.0	-	formate dehydrogenase accessory protein
RPA0736	80.1	0.0	-	NAD-dependent formate dehydrogenase subunit delta
RPA0744	14.2	0.0	-	high potential iron sulfur protein at residue 38
RPA0746	6.5	0.0	-	deca-heme c-type cytochrome
RPA0832	61.5	0.0	-	cytochrome-c oxidase
RPA0843	83.0	0.0	-	H ⁺ -transporting two-sector ATPase subunit B/B'
RPA0844	85.4	0.0	-	H ⁺ -transporting two-sector ATPase subunit B/B'
RPA0845	168.1	0.0	-	ATP synthase F0F1 subunit C
RPA0962	34.6	0.0	-	hydrogenase small chain
RPA0964	39.3	0.0	-	Ni/Fe-hydrogenase 1 B-type cytochrome subunit
RPA0965	16.6	0.0	-	hydrogenase maturation protein HupD
RPA0970	0.0	0.0	-	rubredoxin hupI
RPA1104	45.1	0.0	-	FAD-dependent pyridine nucleotide-disulfide oxidoreductase
RPA1131	30.8	0.0	-	FAD linked oxidase-like protein
RPA1225	224.0	0.0	-	pyruvate ferredoxin
RPA1227	201.0	0.0	-	2-oxoglutarate ferredoxin oxidoreductase subunit beta
RPA1255	11.3	0.0	-	formamide amidohydrolase
RPA1310	28.2	0.0	-	NADH:flavin oxidoreductase
RPA1319	20.5	0.0	-	cytochrome bd-I oxidase subunit II
RPA1331	6.9	0.0	-	malate dehydrogenase
RPA1372	10.0	0.0	-	nitrogenase cofactor synthesis protein
RPA1380	94.9	0.0	-	nitrogenase vanadium-iron protein subunit VnfK
RPA1392	40.1	0.0	-	nitroreductase family proteins
RPA1436	17.1	0.0	-	dinitrogenase 3 subunit delta
RPA1455	24.8	0.0	-	nitric-oxide reductase subunit C
RPA1582	40.5	0.0	-	ferredoxin:adenylate/guanylate cyclase
RPA1654	249.5	0.0	-	oxido-reductase
RPA1711	87.5	0.0	-	nitroreductase
RPA1714	29.5	0.0	-	hypothetical protein
RPA1726	31.1	0.0	-	oxidoreductase
RPA1733	18.7	0.0	-	ferredoxin
RPA1872	0.0	0.0	-	rhodocoxin
RPA1955	44.1	0.0	-	glutathione dependent formaldehyde dehydrogenase
RPA2018	68.7	0.0	-	alcohol dehydrogenase
RPA2093	48.2	0.0	-	tricarballoylate dehydrogenase
RPA2117	92.5	0.0	-	flavodoxin FldA

RPA2136	100.6	0.0	-	hypothetical protein
RPA2324	11.9	0.0	-	succinate-semialdehyde dehydrogenase
RPA2415	7.1	0.0	-	acetamidase/formamidase
RPA2493	5.7	0.0	-	P-methylase
RPA2537	325.2	0.0	-	ETC complex I subunit region nitrogenase iron-molybdenum cofactor biosynthesis protein NifE-like protein
RPA2616	2.6	0.0	-	vanadium nitrogenase associated protein N
RPA2617	44.5	0.0	-	NADH dehydrogenase subunit K
RPA2940	25.4	0.0	-	NADH dehydrogenase I subunit F
RPA2945	47.1	0.0	-	NADH-ubiquinone/plastoquinone (complex I)
RPA2995	2.2	0.0	-	glycerophosphoryl diester phosphodiesterase
RPA3138	124.6	0.0	-	racemase
RPA3485	8.8	0.0	-	hypothetical protein
RPA3566	11.5	0.0	-	alcohol dehydrogenase
RPA3655	10.0	0.0	-	glycerol kinase
RPA3705	135.7	0.0	-	ferredoxin-nitrite reductase
RPA3710	27.2	0.0	-	malate dehydrogenase
RPA3756	41.9	0.0	-	phenylacetate-CoA oxygenase/reductase subunit PaaK
RPA3764	19.3	0.0	-	acetoin dehydrogenase (TPP-dependent) subunit beta
RPA3921	3.3	0.0	-	acetoin dehydrogenase (TPP-dependent) subunit beta
RPA3922	13.3	0.0	-	acetoin dehydrogenase (TPP-dependent) subunit alpha
RPA3923	32.4	0.0	-	ferredoxin
RPA3956	270.4	0.0	-	diheme cytochrome c-553 nicotinamide nucleotide transhydrogenase subunit alpha2
RPA3972	22.8	0.0	-	NADH dehydrogenase (quinone)
RPA4181	291.8	0.0	-	NADH-ubiquinone oxidoreductase subunit 4L
RPA4252	17.8	0.0	-	NADH dehydrogenase subunit I
RPA4255	51.4	0.0	-	respiratory-chain NADH dehydrogenase subunit I
RPA4257	84.8	0.0	-	NADH dehydrogenase subunit E
RPA4258	36.7	0.0	-	flavocytochrome C sulfide dehydrogenase flavoprotein subunit
RPA4261	121.6	0.0	-	cytochrome subunit of sulfide dehydrogenase
RPA4459	112.9	0.0	-	cytochrome
RPA4461	482.8	0.0	-	carbon monoxide dehydrogenase subunit C
RPA4463	14.2	0.0	-	L-carnitine dehydratase/bile acid-inducible protein F
RPA4668	0.0	0.0	-	cytochrome bd ubiquinol oxidase subunit I
RPA4669	9.4	0.0	-	chlorophyllide reductase subunit Z
RPA4793	221.7	0.0	-	photosynthetic reaction center subunit L
RPA1524	1366.5	19.5	70.3	elongator protein 3/MiaB/NifB
RPA1527	6335.1	104.0	60.9	phosphoribulokinase
RPA4507	464.3	7.8	59.2	light harvesting protein B-800-850 beta subunit C
RPA4644	2098.8	49.4	42.5	chlorophyllide reductase subunit Y
RPA3009	15894.6	381.3	41.7	bifunctional NADH:ubiquinone oxidoreductase subunit C/D
RPA1523	663.3	17.7	37.5	ATP synthase F0F1 subunit delta
RPA4262	587.3	16.0	36.6	photosynthetic reaction center subunit M
RPA0179	1587.1	53.2	29.9	NADH dehydrogenase subunit L
RPA1528	4078.3	155.6	26.2	NADH dehydrogenase subunit B
RPA2939	130.8	6.7	19.6	
RPA4263	439.7	23.6	18.7	

RPA1668	2661.1	148.6	17.9	magnesium-protoporphyrin IX monomethyl ester cyclase
RPA2003	117.5	6.7	17.4	hypothetical protein
RPA3748	446.7	26.6	16.8	radical SAM family protein
RPA3013	4153.2	254.2	16.3	light harvesting protein B-800-850 beta subunit D
RPA4631	12064.1	745.4	16.2	4Fe-4S ferredoxin
RPA0734	77.7	4.9	16.0	formate dehydrogenase subunit alpha nitrogenase molybdenum-cofactor biosynthesis protein
RPA4616	651.2	41.1	15.8	NifN
RPA0015	1448.0	96.6	15.0	4Fe-4S ferredoxin
RPA0134	214.2	15.0	14.3	hydrogenase gamma-fused hydrogenase large and small subunit
RPA0177	230.5	16.5	14.0	ATP synthase F0F1 subunit gamma
RPA0846	1619.4	117.0	13.8	ATP synthase F0F1 subunit A
RPA1542	730.4	54.9	13.3	light-independent protochlorophyllide reductase subunit N
RPA0537	234.6	18.0	13.0	acetyl-CoA hydrolase
RPA1228	307.0	26.6	11.5	pyruvate ferredoxin/ flavodoxin oxidoreductase
RPA1192	1249.7	117.8	10.6	cytochrome b6-F complex iron-sulfur subunit
RPA3195	440.8	43.6	10.1	indolepyruvate ferredoxin oxidoreductase
RPA0188	113.8	11.3	10.1	dihydrolipoamide succinyltransferase
RPA0019	3491.1	349.3	10.0	cbb3-type cytochrome c oxidase subunit I
RPA2938	84.2	9.3	9.1	NADH dehydrogenase subunit M
RPA4259	93.2	10.5	8.8	NADH dehydrogenase subunit G
RPA1543	522.7	60.5	8.6	light-independent protochlorophyllide reductase subunit B
RPA3042	51.4	6.0	8.6	malic enzyme
RPA4612	444.9	51.7	8.6	4Fe-4S ferredoxin
RPA0216	788.2	92.7	8.5	succinate dehydrogenase iron-sulfur subunit
RPA0489	3873.9	469.3	8.3	ferredoxin II
RPA3550	62.2	7.9	7.9	coenzyme B12-binding elongator protein 3/MiaB/NifB
RPA0191	180.6	23.7	7.6	succinyl-CoA synthetase subunit beta
RPA2866	350.5	50.0	7.0	pyruvate dehydrogenase subunit beta
RPA4629	541.0	78.7	6.9	4Fe-4S ferredoxin
RPA0017	4695.5	703.5	6.7	cbb3-type cytochrome oxidase subunit
RPA4206	122.0	18.4	6.6	3-hydroxybutyrate dehydrogenase
RPA1535	4587.6	697.0	6.6	class I cytochrome c
RPA3450	182.5	28.2	6.5	methylmalonate-semialdehyde dehydrogenase
RPA0190	104.0	16.3	6.4	succinyl-CoA synthetase subunit alpha
RPA4619	1711.3	269.4	6.4	nitrogenase molybdenum-iron protein subunit alpha nitrogenase molybdenum-cofactor biosynthesis protein
RPA4617	416.0	67.3	6.2	NifE
RPA3500	120.7	19.7	6.1	fumarate hydratase
RPA2907	979.9	162.6	6.0	type II citrate synthase
RPA1928	470.6	79.1	5.9	ferredoxin
RPA0380	106.4	18.0	5.9	oxidoreductase
RPA0018	2305.9	396.9	5.8	cbb3-type cytochrome c oxidase subunit II
RPA0679	195.5	34.3	5.7	hypothetical protein
RPA1037	191.1	33.8	5.7	electron-transferring-flavoprotein dehydrogenase
RPA2863	112.1	19.8	5.7	dihydrolipoamide dehydrogenase
RPA2950	661.7	120.2	5.5	NADH dehydrogenase subunit C
RPA4394	839.3	154.5	5.4	isocitrate lyase

RPA0175	204.9	37.9	5.4	ATP synthase F0F1 subunit epsilon
RPA2065	146.3	27.1	5.4	hypothetical protein
RPA1532	566.6	105.9	5.4	geranylgeranyl reductase
RPA4603	919.2	173.0	5.3	electron-transferring-flavoprotein dehydrogenase
RPA2194	138.9	27.7	5.0	carbon-monoxide dehydrogenase large subunit
RPA4604	1093.8	218.7	5.0	electron transfer flavoprotein subunit beta
RPA4618	1345.8	270.3	5.0	nitrogenase molybdenum-iron protein subunit beta
RPA4566	58.3	11.9	4.9	acetate kinase
RPA4253	48.0	9.8	4.9	proton-translocating NADH-quinone oxidoreductase subunit M
RPA1560	348.7	72.8	4.8	ribulose-bisphosphate carboxylase small chain
RPA0217	403.2	84.4	4.8	succinate dehydrogenase flavoprotein subunit
RPA4693	114.0	24.4	4.7	FMN oxidoreductase
RPA3782	54.4	11.7	4.7	FAD-dependent pyridine nucleotide-disulfide oxidoreductase
RPA4460	101.7	22.4	4.5	flavocytochrome C sulfide dehydrogenase flavoprotein subunit
RPA3067	59.6	13.5	4.4	NAD-dependent alcohol dehydrogenase
RPA4254	87.7	20.2	4.3	proton-translocating NADH-quinone oxidoreductase subunit L
RPA2002	41.8	10.0	4.2	FAD linked oxidase
RPA1940	61.8	14.8	4.2	quinone oxidoreductase
RPA3974	98.6	24.0	4.1	carbon-monoxide dehydrogenase
RPA2952	685.6	171.7	4.0	NADH dehydrogenase subunit A
RPA2867	824.2	207.1	4.0	pyruvate dehydrogenase E1 subunit alpha
RPA4240	281.3	71.3	3.9	GCN5-like N-acetyltransferase
RPA4235	2565.2	650.0	3.9	cytochrome c class I
RPA1611	51.2	13.0	3.9	L-carnitine dehydratase/bile acid-inducible protein F
RPA1226	240.0	61.0	3.9	2-oxoglutarate ferredoxin oxidoreductase subunit alpha
RPA0711	85.9	21.9	3.9	nitroreductase family protein
RPA2944	26.3	6.7	3.9	NADH dehydrogenase subunit G
RPA1224	287.8	76.6	3.8	indolepyruvate ferredoxin oxidoreductase subunit alpha
RPA1193	351.4	94.5	3.7	cytochrome cI
RPA2942	114.0	30.9	3.7	NADH dehydrogenase subunit I
RPA2080	89.5	24.3	3.7	zinc-containing dehydrogenase
RPA1579	43.3	11.7	3.7	L-carnitine dehydratase/bile acid-inducible protein F
RPA0185	37.0	10.1	3.7	dihydrolipoamide dehydrogenase
RPA1132	195.7	53.8	3.6	glycolate oxidase subunit (Fe-S)protein GlcF
RPA2061	129.3	36.0	3.6	nitrous-oxide reductase
RPA4291	2880.2	832.2	3.5	light harvesting protein B-800-850 beta subunit B
RPA4168	44.5	12.9	3.4	FAD dependent oxidoreductase
RPA3973	116.7	33.9	3.4	class II cytochrome c
RPA2949	121.1	35.2	3.4	NADH dehydrogenase subunit D
RPA3215	292.4	86.6	3.4	nitroreductase
RPA0016	1678.2	506.6	3.3	cytochrome c oxidase cbb3-type subunit III
RPA0178	380.9	119.3	3.2	ATP synthase F0F1 subunit alpha
RPA1492	663.3	207.7	3.2	light harvesting protein B-800-850 alpha subunit E
RPA4216	119.6	38.4	3.1	malate synthase G
RPA0781	974.3	315.5	3.1	cytochrome c552
RPA4605	411.2	136.6	3.0	electron transfer flavoprotein subunit beta

RPA4602	3087.2	1042.6	3.0	ferredoxin like protein fixX
RPA2636	27.5	9.3	2.9	nitrogenase molybdenum-iron protein subunit alpha
RPA2153	87.1	29.6	2.9	aldehyde dehydrogenase
RPA3463	34.3	11.7	2.9	hypothetical protein
RPA1772	111.4	39.4	2.8	phosphoenolpyruvate carboxylase
RPA4264	225.5	79.8	2.8	NADH-ubiquinone/plastoquinone oxidoreductase subunit 3
RPA4320	141.0	49.9	2.8	L-lactate dehydrogenase
RPA4403	143.1	51.8	2.8	morphinone reductase
RPA1945	28.8	10.7	2.7	formyl-coenzyme A transferase
RPA3802	97.1	35.9	2.7	carbon monoxide dehydrogenase medium subunit
RPA0467	78.4	29.0	2.7	NAD-binding D-isomer specific 2-hydroxyacid dehydrogenase
RPA0671	16.1	6.0	2.7	4-hydroxybenzoyl-CoA reductase subunit
RPA2943	71.9	27.9	2.6	NADH dehydrogenase subunit H
RPA4794	139.1	56.6	2.5	cytochrome d ubiquinol oxidase subunit II
RPA2394	40.4	16.5	2.5	2-methylcitrate synthase
RPA2947	47.7	19.4	2.5	NADH dehydrogenase subunit E
RPA4557	58.3	23.7	2.5	hypothetical protein
RPA1501	569.2	242.1	2.4	coenzyme F420 hydrogenase subunit beta
RPA4653	28.1	12.0	2.3	biotin sulfoxide reductase
RPA1491	1616.5	693.1	2.3	light harvesting protein B-800-850 beta subunit E
RPA4609	370.8	159.6	2.3	NifU protein
RPA2674	101.1	44.1	2.3	quinone oxidoreductase
RPA0176	111.3	49.3	2.3	ATP synthase F0F1 subunit beta
RPA0963	17.3	7.8	2.2	hydrogenase large chain
RPA2941	102.0	46.1	2.2	NADH dehydrogenase subunit J
RPA0254	64.0	28.9	2.2	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
RPA1911	60.6	27.4	2.2	nickel-dependent hydrogenase b-type cytochrome subunit
RPA0836	37.3	16.9	2.2	cytochrome c oxidase subunit III
RPA4721	115.3	52.7	2.2	pyruvate-flavodoxin oxidoreductase
RPA3012	433.7	207.7	2.1	light harvesting protein B-800-850 alpha subunit D
RPA2870	76.4	37.3	2.0	L-carnitine dehydratase/bile acid-inducible protein F
RPA0192	120.0	59.2	2.0	malate dehydrogenase
RPA2348	18.0	9.2	2.0	nitrogenase molybdenum-iron protein subunit alpha
RPA1076	38.8	19.7	2.0	FAD linked oxidase-like protein
RPA1572	23.5	11.9	2.0	carbon-monoxide dehydrogenase large subunit
RPA0360	1149.8	608.9	1.9	phosphoenolpyruvate carboxykinase
RPA4182	46.5	25.2	1.8	NAD(P)(+) transhydrogenase
RPA2951	500.4	273.2	1.8	NADH dehydrogenase subunit B
RPA1578	235.3	131.4	1.8	ferredoxin-NADP reductase
RPA2448	111.6	63.4	1.8	C4-dicarboxylic acid transport protein
RPA0202	265.7	153.0	1.7	aconitate hydratase
RPA1373	17.6	10.2	1.7	nitrogenase cofactor synthesis protein
RPA3271	25.6	14.9	1.7	NAD-binding D-isomer specific 2-hydroxyacid dehydrogenase
RPA1261	15.1	8.8	1.7	FAD linked oxidase
RPA1378	17.1	9.9	1.7	nitrogenase vanadium-iron protein subunit alpha
RPA3503	78.0	48.1	1.6	D-lactate dehydrogenase oxidoreductase

RPA2937	62.5	39.2	1.6	NADH dehydrogenase subunit N
RPA2731	124.3	78.8	1.6	membrane-bound proton-translocating pyrophosphatase
RPA4336	71.2	45.7	1.6	aldo/keto reductase
RPA4667	17.4	11.8	1.5	carbon-monoxide dehydrogenase large subunit
RPA0411	452.3	306.8	1.5	inorganic pyrophosphatase
RPA1136	120.0	84.2	1.4	L-lactate permease
RPA4750	276.7	194.2	1.4	electron transfer flavoprotein subunit beta
RPA0461	173.6	122.5	1.4	succinate-semialdehyde dehydrogenase
RPA0189	216.2	154.4	1.4	2-oxoglutarate dehydrogenase E1 component
RPA4749	63.5	45.6	1.4	electron transfer flavoprotein subunit alpha
RPA0831	107.5	84.1	1.3	cytochrome-c oxidase
RPA4552	14.0	11.4	1.2	4-hydroxybutyrate-CoA transferase/hydrolase
RPA0219	477.7	388.9	1.2	succinate dehydrogenase cytochrome b subunit
RPA1687	36.2	29.5	1.2	aldehyde dehydrogenase
RPA1130	129.6	113.1	1.1	glycolate oxidase subunit GlcD
RPA0218	375.6	335.6	1.1	succinate dehydrogenase cytochrome b subunit
RPA3621	31.9	28.8	1.1	vanillate O-demethylase oxidoreductase
RPA0422	189.5	171.4	1.1	2-hydroxyacid dehydrogenase
RPA0507	57.1	51.7	1.1	cytochrome b561
RPA3876	120.7	110.1	1.1	fumarate hydratase class I
RPA1957	138.2	126.6	1.1	alkanal monooxygenase
RPA1013	271.1	263.7	1.0	nitrogen fixation protein FixG
RPA0959	28.1	28.6	1.0	uracil phosphoribosyltransferase
RPA4410	63.0	64.1	1.0	glycerol-3-phosphate dehydrogenase
RPA3760	26.9	27.4	1.0	5-carboxy-2-hydroxymuconate semialdehyde dehydrogenase
RPA3803	91.8	93.4	1.0	carbon-monoxide dehydrogenase small subunit
RPA1488	11.6	11.8	1.0	L-carnitine dehydratase/bile acid-inducible protein F
RPA3834	613.3	635.6	1.0	isocitrate dehydrogenase
RPA2421	461.9	488.8	0.9	NADH dehydrogenase
RPA4231	37.3	39.6	0.9	oxidoreductase
RPA2637	47.6	51.0	0.9	nitrogenase iron-molybdenum protein subunit nifK-like protein
RPA2016	253.9	285.1	0.9	methyltransferase-like protein
RPA0059	161.9	188.3	0.9	L-carnitine dehydratase/bile acid-inducible protein F
RPA3700	82.9	98.4	0.8	luciferase-like protein
RPA3408	174.1	206.7	0.8	nitroreductase family protein
RPA2653	2474.6	3011.4	0.8	light harvesting protein B-800-850 alpha subunit A
RPA1806	24.8	30.3	0.8	L-carnitine dehydratase/bile acid-inducible protein F
RPA0042	31.4	38.4	0.8	4Fe-4S ferredoxin
RPA4822	39.7	49.8	0.8	alcohol dehydrogenase
RPA2654	28063.2	35499.8	0.8	light harvesting protein B-800-850 beta subunit A
RPA0739	18.5	25.1	0.7	cytochrome c
RPA1329	14.2	19.3	0.7	fumarate hydratase
RPA2364	7.5	10.2	0.7	nitrogenase iron-molybdenum cofactor biosynthesis protein NifE-like protein
RPA0223	51.0	69.2	0.7	hypothetical protein
RPA0672	10.7	14.6	0.7	4-hydroxybenzoyl-CoA reductase subunit
RPA0113	7.5	10.1	0.7	oxidoreductase

RPA2347	7.5	10.1	0.7	vanadium nitrogenase associated protein N
RPA2458	359.2	505.1	0.7	cytochrome B561
RPA4213	41.6	59.3	0.7	sulfite reductase hemoprotein subunit nickel-dependent hydrogenase b-type cytochrome subunit
RPA2315	106.8	152.2	0.7	alcohol dehydrogenase
RPA0400	28.9	44.1	0.7	NADPH quinone oxidoreductase
RPA0915	42.3	71.7	0.6	Zn-containing alcohol dehydrogenase
RPA0374	107.3	203.8	0.5	alcohol dehydrogenase
RPA0656	6.9	14.0	0.5	carbon-monoxide dehydrogenase small subunit
RPA4666	31.7	64.5	0.5	alternative nitrogenase 3 subunit beta
RPA1435	5.0	10.2	0.5	alcohol dehydrogenase
RPA1205	6.1	12.3	0.5	hypothetical protein
RPA1991	6.6	13.4	0.5	glutathione reductase
RPA1983	135.1	295.3	0.5	NADH oxidoreductase nickel-dependent hydrogenase b-type cytochrome subunit
RPA2873	55.5	125.5	0.4	aldo/keto reductase
RPA2306	20.1	54.5	0.4	NAD(P) transhydrogenase subunit beta
RPA3023	57.1	159.6	0.4	cytochrome c556
RPA4180	44.4	130.6	0.3	nitrogenase molybdenum-iron protein subunit alpha
RPA2314	136.5	486.1	0.3	denitrification protein NorE NADH-ubiquinone/plastoquinone oxidoreductase subunit 6
RPA1437	4.4	17.9	0.2	cytochrome c
RPA1453	11.9	48.6	0.2	hypothetical protein
RPA4256	7.3	29.7	0.2	hypothetical protein
RPA3693	211.5	1049.1	0.2	nifU-like protein
RPA0908	433.8	2825.0	0.2	aldehyde dehydrogenase ubiquinol-cytochrome-c reductase Rieske iron-sulfur protein
RPA4285	12.3	100.5	0.1	vanadium dinitrogenase subunit delta
RPA3392	463.5	4177.5	0.1	nitrogenase molybdenum-iron protein subunit alpha
RPA1206	2.3	55.5	0.0	
RPA1016	96.5	5753.2	0.0	
RPA1379	0.0	46.5	0.0	
RPA2363	0.0	9.3	0.0	
[G]Carbohydrate transport and metabolism				
RPA0126	10.9	0.0	-	sugar ABC transporter sugar binding protein
RPA0127	8.5	0.0	-	sugar ABC transporter permease
RPA0129	0.0	0.0	-	sugar ABC transporter substrate-binding protein
RPA0379	127.8	0.0	-	6-phosphofructokinase
RPA0457	118.2	0.0	-	PfkB protein
RPA0619	107.0	0.0	-	E. coli beta lactamase (AmpC) regulator
RPA0737	20.2	0.0	-	MFS superfamily oxalate/formate antiporter
RPA0993	44.4	0.0	-	alpha-ribazole-5'-phosphate phosphatase
RPA1181	62.2	0.0	-	HAD-superfamily hydrolase
RPA1397	12.4	0.0	-	sugar ABC transporter permease
RPA1399	12.9	0.0	-	transport system ATP-binding protein
RPA1559	33.2	0.0	-	ribulose bisphosphate carboxylase
RPA1753	32.0	0.0	-	hypothetical protein
RPA1784	8.0	0.0	-	TRAP dicarboxylate family transporter DctM subunit
RPA1944	18.1	0.0	-	oxalate/formate antiporter
RPA2048	56.2	0.0	-	TRAP dicarboxylate family transporter subunit DctQ

RPA2099	6.3	0.0	-	MFS family diterpenoid transporter
RPA2271	20.6	0.0	-	pyruvate kinase
RPA2386	26.9	0.0	-	hypothetical protein
RPA2395	57.7	0.0	-	carboxyphosphoenolpyruvate phosphonmutase
RPA2459	8.6	0.0	-	permease
RPA2485	14.9	0.0	-	aquaporin Z
RPA2874	111.0	0.0	-	phosphopyruvate hydratase
RPA2878	11.4	0.0	-	MDR-like permease
RPA2888	38.2	0.0	-	triose-phosphate isomerase
RPA2999	16.8	0.0	-	nitrogen regulatory IIA protein
RPA3174	36.8	0.0	-	hypothetical protein
RPA3262	2.9	0.0	-	transporter
RPA3454	5.4	0.0	-	MFS family transporter
RPA3468	19.1	0.0	-	sugar ABC transporter permease
RPA3469	25.9	0.0	-	binding-protein-dependent transport system inner membrane protein
RPA3636	116.4	0.0	-	glucose-6-phosphate 1-dehydrogenase
RPA3638	64.9	0.0	-	carbohydrate kinase thermoresistant glucokinase
RPA3755	9.2	0.0	-	HpcH/HpaI aldolase
RPA3815	46.3	0.0	-	ribulose-phosphate 3-epimerase
RPA3965	46.1	0.0	-	major facilitator transporter
RPA3987	37.2	0.0	-	phosphoheptose isomerase
RPA4066	0.0	0.0	-	hypothetical protein
RPA4406	83.8	0.0	-	binding-protein-dependent transport system inner membrane protein
RPA4554	29.7	0.0	-	TRAP dicarboxylate family transporter subunit DctM
RPA4559	8.4	0.0	-	citrate lyase subunit beta
RPA4656	7.3	0.0	-	sugar kinase
RPA4800	37.4	0.0	-	transporter
RPA0378	491.4	11.1	44.5	alpha-D-galactoside galactohydrolase
RPA4642	499.9	13.1	38.1	fructose-1,6-bisphosphate aldolase
RPA1531	269.0	10.7	25.1	light harvesting pigment major facilitator family (MFS) transporter Bch2
RPA0382	426.7	19.4	22.0	glycogen synthase
RPA4641	432.8	20.4	21.3	ribulose bisphosphate carboxylase
RPA3641	112.4	7.2	15.7	4-alpha-glucanotransferase
RPA0381	1057.9	78.5	13.5	glucose-1-phosphate adenylyltransferase
RPA1547	1294.4	98.3	13.2	photosynthetic complex (LH1) assembly protein LhaA
RPA2014	141.1	12.8	11.1	family 1 extracellular solute-binding protein
RPA3342	80.6	7.6	10.6	glycosyl hydrolase
RPA3642	308.3	33.7	9.1	alpha amylase
RPA3635	121.4	13.7	8.8	6-phosphogluconate dehydrogenase
RPA1051	216.1	25.3	8.5	pyruvate phosphate dikinase
RPA1736	256.9	30.8	8.4	beta-glucosidase
RPA2047	234.4	28.5	8.2	TRAP dicarboxylate family transporter subunit DctP
RPA3643	87.4	12.6	7.0	trehalose synthase
RPA1395	52.8	9.0	5.9	trehalose/maltose binding protein
RPA4645	772.3	138.5	5.6	fructose-1,6-bisphosphatase
RPA3645	181.0	33.5	5.4	glycogen debranching protein GlgX

RPA3644	133.7	25.9	5.2	glycogen branching protein
RPA0943	55.4	11.9	4.7	phosphoglycerate kinase
RPA3188	28.3	6.4	4.4	alcohol dehydrogenase
RPA3646	35.0	7.9	4.4	maltooligosyltrehalose trehalohydrolase
RPA0026	86.8	20.2	4.3	major facilitator transporter
RPA3164	301.2	72.1	4.2	polysaccharide deacetylase
RPA1925	117.9	28.8	4.1	transmembrane protein chlorophyll major facilitator superfamily (MFS)
RPA1493	821.7	215.8	3.8	exporter PucC
RPA4404	59.5	15.6	3.8	family 1 extracellular solute-binding protein
RPA4584	46.1	12.5	3.7	endoglucanase
RPA0233	56.4	16.4	3.4	Citryl-CoA lyase
RPA0058	104.5	31.9	3.3	family 1 extracellular solute-binding protein
RPA0157	195.0	61.0	3.2	hypothetical protein
RPA2505	87.5	28.5	3.1	fructose 1,6-bisphosphatase II
RPA3634	274.5	89.9	3.1	bifunctional transaldolase/phosphoglucose isomerase
RPA0937	280.2	92.0	3.0	extragenic suppressor protein SuhB
RPA1484	77.2	25.5	3.0	phosphoglucomutase
RPA4106	233.8	81.6	2.9	multidrug efflux pump
RPA3039	218.0	76.5	2.8	polysaccharide deacetylase
RPA1880	264.2	93.9	2.8	MFS family transporter
RPA1639	125.8	45.5	2.8	major facilitator superfamily protein
RPA4556	37.7	13.9	2.7	TRAP dicarboxylate family transporter subunit DctP
RPA4655	57.7	21.3	2.7	L-fucose phosphate aldolase
RPA2657	158.2	60.1	2.6	beta (1-6) glucans synthase
RPA4801	89.3	34.1	2.6	major facilitator superfamily permease
RPA1143	60.6	23.5	2.6	major facilitator transporter
RPA0851	107.6	42.1	2.6	general substrate transporter
RPA1156	399.4	156.7	2.5	myo-inositol monophosphatase 2 family protein
RPA3647	37.9	14.9	2.5	malto-oligosyltrehalose synthase
RPA1692	56.4	22.9	2.5	major facilitator transporter
RPA2852	133.5	55.7	2.4	beta-N-acetylhexosaminidase
RPA4661	303.6	127.2	2.4	trehalose-6-phosphate phosphatase
RPA3927	97.1	43.9	2.2	phosphoheptose isomerase
RPA4643	355.6	163.2	2.2	transketolase
RPA3640	32.3	15.5	2.1	glycoside hydrolase family protein
RPA4660	653.5	313.5	2.1	alpha,alpha-trehalose-phosphate synthase
RPA4662	95.3	47.3	2.0	major facilitator transporter
RPA2965	55.3	28.2	2.0	hypothetical protein
RPA4727	317.3	179.9	1.8	glycogen/starch/alpha-glucan phosphorylase
RPA3405	21.9	12.7	1.7	transporter
RPA4788	36.6	21.3	1.7	family 1 extracellular solute-binding protein
RPA0612	127.8	76.5	1.7	PfkB protein
RPA3311	155.4	94.9	1.6	glycosyl hydrolase
RPA3374	142.6	87.1	1.6	inorganic polyphosphate/ATP-NAD kinase
RPA0944	341.8	212.9	1.6	glyceraldehyde-3-phosphate dehydrogenase
RPA2342	34.5	21.6	1.6	transmembrane transport protein
RPA4759	53.3	34.7	1.5	glycosyl hydrolase family protein

RPA2781	45.6	29.7	1.5	TrapT dctQ-M fusion permease dicarboxylate transport
RPA0738	14.7	10.0	1.5	L-sorbose dehydrogenase
RPA4785	284.8	202.2	1.4	polysaccharide deacetylase
RPA1862	163.1	119.5	1.4	polysaccharide deacetylase
RPA2049	28.8	21.3	1.4	TRAP dicarboxylate family transporter subunit DctM
RPA2541	12.8	10.4	1.2	TRAP dicarboxylate family transporter subunit DctM
RPA3286	23.7	19.3	1.2	phosphoglycerate mutase
RPA0560	72.6	59.1	1.2	malonyl-CoA decarboxylase
RPA1981	154.4	125.7	1.2	ribose-5-phosphate isomerase A
RPA4193	140.1	118.0	1.2	pyruvate kinase
RPA3781	95.0	80.6	1.2	PfkB protein
RPA0354	388.5	335.4	1.2	PTS system phosphocarrier protein HPr nitrogen regulation
RPA0940	369.7	331.4	1.1	fructose-bisphosphate aldolase
RPA3321	30.0	28.2	1.1	phosphomannomutase
RPA2198	63.4	62.3	1.0	multidrug efflux protein
RPA4317	74.4	73.1	1.0	major facilitator superfamily protein
RPA2169	25.3	25.7	1.0	ribulose bisphosphate carboxylase-like protein
RPA1797	10.9	11.1	1.0	hypothetical protein
RPA2543	41.3	42.1	1.0	TRAP dicarboxylate family transporter subunit DctP
RPA3624	15.2	15.5	1.0	gluconolactonase
RPA4511	11.0	11.2	1.0	major facilitator transporter
RPA2782	37.9	42.1	0.9	TRAP dicarboxylate family transporter subunit DctP
RPA3637	53.4	59.3	0.9	6-phosphogluconolactonase
RPA3165	961.7	1294.5	0.7	polysaccharide deacetylase
RPA1851	270.8	425.0	0.6	aldolase II superfamily protein
RPA0355	606.2	963.9	0.6	PTS system fructose subfamily transporter subunit IIA
RPA4318	118.0	198.4	0.6	phosphoglucosamine mutase
RPA0087	22.4	41.5	0.5	major facilitator transporter
RPA1673	197.5	366.5	0.5	phosphoketolase
RPA1079	10.8	22.0	0.5	4-methylmuconolactone transporter
RPA1783	11.1	22.5	0.5	TRAP dicarboxylate family transporter subunit DctQ
RPA3094	5.4	11.0	0.5	major facilitator transporter
RPA1782	52.2	113.2	0.5	TRAP dicarboxylate family transporter subunit DctP
RPA4333	111.0	293.0	0.4	glucose sorbose dehydrogenase
RPA3470	7.9	21.3	0.4	sugar uptake ABC transporter periplasmic solute-binding protein
RPA3825	25.1	68.1	0.4	hypothetical protein
RPA4808	8.1	22.0	0.4	hexuronate transporter
RPA2754	60.0	189.8	0.3	polysaccharide deacetylase
RPA0052	194.3	692.1	0.3	PTS transporter subunit IIA-like nitrogen-regulatory protein PtsN
RPA0340	251.0	993.6	0.3	phosphoglyceromutase
RPA2542	6.7	27.1	0.2	TRAP dicarboxylate family transporter subunit DctQ
RPA0262	37.5	152.5	0.2	ribulose bisphosphate carboxylase-like protein
RPA1200	43.0	224.8	0.2	phosphoglycerate mutase
RPA3128	100.3	594.0	0.2	transport protein
RPA0945	112.2	745.0	0.2	transketolase
RPA0226	5.9	47.8	0.1	major facilitator transporter

<i>[I]Lipid transport and metabolism</i>			
RPA0271	76.8	0.0	- acyl-CoA thioesterase
RPA0416	30.6	0.0	- enoyl-CoA hydratase
RPA0464	12.7	0.0	- long-chain fatty acid CoA ligase (AMP-binding)
RPA0480	9.9	0.0	- acyl-CoA dehydrogenase
RPA0481	30.1	0.0	- acyl-CoA dehydrogenase
RPA0482	8.8	0.0	- enoyl-CoA hydratase
RPA0650	13.8	0.0	- cyclohex-1-ene-1-carboxyl-CoA hydratase
RPA0651	12.6	0.0	- cyclohexanecarboxylate-CoA ligase
RPA0652	3.0	0.0	- cyclohexanecarboxyl-CoA dehydrogenase
RPA0740	12.6	0.0	- acyl-CoA dehydrogenase
RPA0995	42.7	0.0	- acyl-CoA dehydrogenase
RPA1029	23.3	0.0	- CoA transferase subunit A
RPA1030	38.0	0.0	- CoA transferase subunit B
RPA1234	4.1	0.0	- long-chain-fatty-acid--CoA ligase
RPA1238	16.7	0.0	- enoyl-CoA hydratase
RPA1368	12.3	0.0	- hypothetical protein
RPA1411	9.0	0.0	- enoyl-CoA hydratase
RPA1450	11.3	0.0	- pyruvate carboxylase
RPA1513	229.8	0.0	- squalene/phytoene synthase
RPA1699	7.7	0.0	- acyl-CoA dehydrogenase
RPA1706	0.0	0.0	- enoyl-CoA hydratase
RPA1708	12.4	0.0	- acyl-CoA dehydrogenase
RPA1709	5.6	0.0	- acyl-CoA dehydrogenase
RPA1710	14.0	0.0	- acyl-CoA dehydrogenase
RPA1712	41.0	0.0	- enoyl-CoA hydratase
RPA1725	21.8	0.0	- bifunctional aldehyde dehydrogenase/enoyl-CoA hydratase
RPA1756	13.7	0.0	- acetyl-CoA acetyltransferase
RPA1761	9.2	0.0	- enoyl-CoA hydratase
RPA1762	28.7	0.0	- acyl-CoA dehydrogenase
RPA1776	18.4	0.0	- lipid transfer protein
RPA1786	4.4	0.0	- enoyl-CoA hydratase
RPA1787	10.9	0.0	- feruloyl-CoA synthase
RPA2138	21.2	0.0	- acyl-CoA dehydrogenase
RPA2144	8.7	0.0	- carbamoyl-phosphate synthase L chain ATP-binding
RPA2145	4.6	0.0	- enoyl-CoA hydratase
RPA2436	135.0	0.0	- acetyl-CoA carboxylase biotin carboxyl carrier protein
RPA2454	31.7	0.0	- dehydrogenase
RPA2588	118.0	0.0	- cyclase
RPA2627	10.1	0.0	- carboxylesterase
RPA2680	41.7	0.0	- hypothetical protein
RPA2695	51.2	0.0	- 4'-phosphopantetheinyl transferase
RPA2828	28.4	0.0	- phytoene synthase
RPA2987	4.2	0.0	- hypothetical protein
RPA3192	16.5	0.0	- acyl dehydratase
RPA3299	11.1	0.0	- long-chain-fatty-acid CoA ligase
RPA3301	0.0	0.0	- lipid-transfer protein

RPA3341	58.3	0.0	-	acetyltransferase
RPA3441	49.4	0.0	-	enoyl-CoA hydratase
RPA3445	24.0	0.0	-	AMP-dependent synthetase/ligase
RPA3446	19.9	0.0	-	3-hydroxyisobutyrate dehydrogenase
RPA3453	4.6	0.0	-	enoyl-CoA hydratase
RPA3713	21.4	0.0	-	pimeloyl-CoA dehydrogenase small subunit
RPA3715	17.6	0.0	-	acetyl-CoA acetyltransferase
RPA3717	3.3	0.0	-	enoyl-CoA hydratase
RPA3742	71.9	0.0	-	squalene/phytoene synthase
RPA3795	14.3	0.0	-	enoyl-CoA hydratase
RPA4339	0.0	0.0	-	enoyl-CoA hydratase
RPA4456	6.8	0.0	-	hypothetical protein
RPA4598	45.3	0.0	-	acyl-CoA dehydrogenase
RPA4798	18.4	0.0	-	acyl-CoA dehydrogenase
RPA4823	15.6	0.0	-	acyl-CoA synthetase
RPA0780	915.1	33.6	27.3	acyl-CoA dehydrogenase
RPA1298	247.6	12.8	19.4	3-oxoacyl-ACP synthase
RPA0932	457.5	27.4	16.7	hypothetical protein
RPA4155	361.1	22.6	16.0	3-oxoacid CoA-transferase subunit B
RPA2501	217.0	15.5	14.0	poly(R)-hydroxyalkanoic acid synthase
RPA0575	284.8	31.9	8.9	polyhydroxyalkanoate depolymerase
RPA3175	164.9	20.8	7.9	carbamoyl-phosphate synthase L chain ATP-binding
RPA3448	90.5	12.3	7.4	acyl-CoA dehydrogenase
RPA2916	321.6	46.3	6.9	1-deoxy-D-xylulose 5-phosphate reductoisomerase
RPA2435	141.1	20.9	6.8	acetyl-CoA carboxylase biotin carboxylase subunit
RPA0264	46.0	7.2	6.4	O-antigen acetylase
RPA2020	147.0	23.5	6.3	3-oxoacyl-ACP synthase
RPA2918	339.1	57.5	5.9	undecaprenyl pyrophosphate synthase
RPA2741	255.6	44.0	5.8	3-oxoacyl-ACP synthase
RPA3203	419.2	73.4	5.7	carboxyl transferase
RPA2912	189.0	33.4	5.7	(3R)-hydroxymyristoyl-ACP dehydratase
RPA4504	163.5	29.2	5.6	AMP-dependent synthetase/ligase
RPA4568	103.2	19.1	5.4	enoyl-ACP reductase
RPA4352	325.9	63.2	5.2	acyl dehydratase
RPA2425	70.9	15.2	4.7	lipase/esterase
RPA2740	412.7	94.1	4.4	glycerol-3-phosphate acyltransferase PlsX
RPA0425	621.3	142.1	4.4	3-hydroxydecanoyl-ACP dehydratase
RPA3893	38.6	9.2	4.2	carboxylesterase
RPA0426	667.2	173.3	3.8	3-oxoacyl-ACP synthase
RPA4156	310.4	81.5	3.8	3-oxoacid CoA-transferase subunit A
RPA4799	44.2	12.0	3.7	acyl-CoA dehydrogenase
RPA0669	31.9	8.7	3.7	4-hydroxybenzoate-CoA ligase
RPA4002	178.0	51.0	3.5	acyl-CoA dehydrogenase
RPA0531	82.9	24.1	3.4	acetyl-CoA acetyltransferase
RPA4346	72.2	22.6	3.2	acetyl-CoA acetyltransferase
RPA2317	58.3	18.3	3.2	CoA transferase small subunit B
RPA3289	73.4	23.0	3.2	acyl-CoA dehydrogenase

RPA1170	266.2	94.2	2.8	CDP-diacylglycerol--glycerol-3-phosphate3-phosphatidyltransferase
RPA0335	1092.7	391.0	2.8	phospholipid N-methyltransferase
RPA2021	269.4	96.8	2.8	beta-hydroxyacyl-(acyl-carrier-protein)dehydratase FabA/FabZ
RPA3072	217.1	78.3	2.8	3-oxoacyl-ACP synthase
RPA3464	44.0	16.3	2.7	hypothetical protein
RPA3447	35.9	13.3	2.7	3-hydroxyisobutyryl-CoA hydrolase
RPA0393	50.3	18.6	2.7	PA-phosphatase-like phosphoesterase
RPA3194	33.4	12.4	2.7	acetyl-CoA acetyltransferase
RPA0958	40.5	15.7	2.6	acyl-CoA dehydrogenase
RPA4243	368.0	142.6	2.6	poly-beta-hydroxyalkanoate synthase
RPA3115	63.3	25.0	2.5	acyl-CoA dehydrogenase
RPA4351	315.9	125.5	2.5	acyl dehydratase
RPA2304	29.4	12.0	2.5	acyl-CoA thiolase
RPA1094	255.3	105.1	2.4	glutaryl-CoA dehydrogenase
RPA3075	69.8	29.9	2.3	malonyl CoA-ACP transacylase
RPA0211	130.0	57.2	2.3	acetyl-CoA synthetase
RPA0676	36.6	16.5	2.2	benzoyl-CoA-dihydrodiol lyase
RPA2019	122.2	55.3	2.2	3-oxoacyl-ACP synthase
RPA2917	259.2	119.1	2.2	phosphatidate cytidyltransferase
RPA2765	562.4	262.1	2.1	enoyl-CoA hydratase
RPA0427	114.3	53.7	2.1	enoyl-ACP reductase
RPA4595	42.7	20.5	2.1	hypothetical protein
RPA4592	47.1	23.0	2.0	long-chain-acyl-CoA synthetase
RPA4267	150.6	74.6	2.0	long-chain-fatty-acid--CoA ligase
RPA3714	23.4	11.9	2.0	pimeloyl-CoA dehydrogenase large subunit
RPA1614	23.8	12.1	2.0	isovaleryl-CoA dehydrogenase
RPA3050	339.6	187.4	1.8	CDP-alcohol phosphatidyltransferase
RPA4748	293.1	163.4	1.8	3-hydroxybutyryl-CoA dehydrogenase
RPA1612	607.0	351.2	1.7	acyl-CoA dehydrogenase
RPA2022	502.2	292.0	1.7	acyl carrier protein
RPA2316	31.8	18.5	1.7	enoyl-CoA hydratase
RPA3462	20.1	11.7	1.7	thiolase
RPA4334	29.3	17.0	1.7	phospholipid/glycerol acyltransferase
RPA0071	293.3	174.7	1.7	acetyl-CoA carboxylase subunit beta
RPA2318	45.9	28.8	1.6	glutaconate CoA transferase subunit A
RPA1003	27.4	17.2	1.6	acyl-CoA synthase
RPA3743	76.6	49.2	1.6	squalene/phytoene synthase
RPA3740	87.4	56.9	1.5	squalene cyclase
RPA3734	268.5	187.3	1.4	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
RPA1973	44.8	33.2	1.4	3-hydroxyisobutyrate dehydrogenase
RPA1683	688.0	519.3	1.3	short-chain dehydrogenase/reductase SDR
RPA0816	92.2	70.3	1.3	acyl-CoA dehydrogenase
RPA3073	840.1	641.2	1.3	acyl carrier protein
RPA2302	32.6	24.9	1.3	acyl-CoA synthetase
RPA0743	47.5	36.8	1.3	acid-CoA ligase
RPA4652	97.7	75.7	1.3	CoA transferase subunit B
RPA0818	55.5	44.0	1.3	3-hydroxyacyl-CoA dehydrogenase

RPA1237	15.4	12.5	1.2	acyl-CoA dehydrogenase
RPA0727	28.5	23.2	1.2	3-amino-3-carboxypropyltransferase
RPA3716	10.4	8.5	1.2	AMP-dependent synthetase/ligase
RPA1837	133.9	112.0	1.2	methylmalonyl-CoA mutase
RPA1039	55.0	48.0	1.1	4-diphosphocytidyl-2-C-methyl-D-erythritolkinase
RPA1835	221.8	199.9	1.1	methylmalonyl-CoA mutase
RPA0221	20.6	18.6	1.1	malonyl-CoA synthase
RPA0519	620.7	565.0	1.1	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase 2-C-methyl-D-erythritol 4-phosphate
RPA2590	72.8	71.2	1.0	cytidyltransferase
RPA0462	15.6	15.9	1.0	enoyl-CoA hydratase
RPA1242	18.3	18.6	1.0	AMP-dependent synthetase/ligase
RPA1603	13.1	13.4	1.0	fatty acid desaturase
RPA4347	34.7	35.3	1.0	hypothetical protein
RPA2143	8.5	8.7	1.0	propionyl-CoA carboxylase
RPA1763	17.5	17.8	1.0	long-chain-fatty-acid--CoA ligase
RPA2305	12.4	12.6	1.0	acyl-CoA dehydrogenase
RPA4042	7.1	7.2	1.0	AMP-dependent synthetase/ligase
RPA4443	148.2	155.1	1.0	acyl-CoA synthetase
RPA4829	23.9	26.6	0.9	crotonobetaine/carnitine-CoA ligase
RPA0888	73.7	81.8	0.9	alpha/beta hydrolase
RPA0459	27.7	30.7	0.9	fatty acid--CoA ligase
RPA2538	14.6	16.9	0.9	acyl-CoA carboxylase subunit beta
RPA1449	14.0	16.3	0.9	acyl-CoA synthetase
RPA3466	20.9	24.3	0.9	acetyl-CoA acetyltransferase
RPA1236	20.4	23.7	0.9	acyl-CoA dehydrogenase
RPA4733	114.3	143.1	0.8	phospholipid/glycerol acyltransferase
RPA2539	22.1	27.7	0.8	acyl-CoA carboxylase biotin-carrying subunit
RPA2006	463.1	586.4	0.8	phosphatidylserine decarboxylase
RPA1707	5.5	7.5	0.7	acyl-CoA synthetase
RPA1729	32.9	44.7	0.7	fatty acid desaturase
RPA0589	18.6	25.2	0.7	acyl-CoA dehydrogenase
RPA2672	53.6	79.4	0.7	phosphatidylcholine synthase
RPA3455	17.7	27.0	0.7	methylmalonyl-CoA decarboxylase subunit alpha acetyl-CoA carboxylase carboxyltransferase subunit
RPA0508	193.9	312.8	0.6	alpha
RPA0661	11.0	17.8	0.6	benzoate-CoA ligase
RPA3465	11.3	18.4	0.6	long-chain-fatty-acid CoA ligase
RPA1765	30.6	53.5	0.6	enoyl-CoA hydratase
RPA0659	10.6	21.5	0.5	benzoyl-CoA reductase subunit
RPA2142	4.3	8.8	0.5	fatty-acid--CoA ligase
RPA1702	8.9	18.1	0.5	long-chain-fatty-acid--CoA ligase
RPA4271	270.8	551.1	0.5	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
RPA1703	11.9	24.2	0.5	acetyl-CoA acetyltransferase
RPA0513	11.5	23.5	0.5	beta-ketoadipyl CoA thiolase
RPA4558	16.6	33.7	0.5	acyl dehydratase
RPA0383	57.8	126.7	0.5	enoyl-CoA hydratase
RPA1412	19.9	45.1	0.4	fatty-acid--CoA ligase
RPA2512	79.0	189.9	0.4	lipid kinase

RPA2137	9.2	25.0	0.4	acyl-CoA dehydrogenase
RPA4553	9.0	24.4	0.4	acyl-CoA dehydrogenase
RPA0817	28.8	82.2	0.4	acetyl-CoA acetyltransferase
RPA1199	47.6	140.9	0.3	Catalyzes a key regulatory step in fatty acid biosynthesis enoyl-ACP reductase
RPA0660	25.6	86.7	0.3	benzoyl-CoA reductase subunit
RPA2007	776.2	2939.5	0.3	CDP-diacylglycerol--serineO-phosphatidyltransferase
RPA1414	33.0	134.1	0.2	acyl dehydratase
RPA4651	93.0	378.6	0.2	glutaconate CoA transferase subunit A
RPA0484	4.5	18.4	0.2	enoyl-CoA hydratase
RPA1766	17.9	81.9	0.2	long-chain-fatty-acid--CoA ligase
RPA1811	160.1	763.5	0.2	enoyl-CoA hydratase
RPA3305	0.0	18.9	0.0	enoyl-CoA hydratase
<i>[P]Inorganic ion transport and metabolism</i>				
RPA0100	20.1	0.0	-	ABC transporter permease
RPA0101	17.9	0.0	-	dipeptide transport system permease 1
RPA0128	11.0	0.0	-	sugar ABC transporter permease
RPA0131	25.8	0.0	-	sulfate ABC transporter ATP-binding protein
RPA0132	41.5	0.0	-	sulfonate ABC transporter permease
RPA0149	22.4	0.0	-	iron ABC transporter ATP-binding protein
RPA0151	16.7	0.0	-	iron ABC transporter periplasmic binding protein
RPA0359	121.9	0.0	-	hypothetical protein
RPA0407	24.0	0.0	-	outer membrane siderophore receptor
RPA0445	189.0	0.0	-	HlyC/CorC family transporter
RPA0514	13.3	0.0	-	efflux pump protein FarB
RPA0688	49.9	0.0	-	phosphonate metabolism 1,5-bisphosphokinase (PRPP-forming) PhnN
RPA0689	24.6	0.0	-	phosphonate utilization (C-P lyase) component
RPA0698	8.0	0.0	-	phosphonate ABC transporter permease
RPA0699	31.1	0.0	-	phosphonate ABC transporter periplasmic phosphonate-binding protein
RPA0700	4.4	0.0	-	phosphonate ABC transporter ATP-binding protein
RPA0707	0.0	0.0	-	periplasmic divalent cation resistance protein CutA
RPA0720	39.4	0.0	-	cobalamin/Fe3+-siderophores ABC transporter periplasmic protein
RPA0722	13.7	0.0	-	iron(III) dicitrate ABC transporter ATP-binding protein FecE
RPA0723	27.4	0.0	-	hemin ABC transporter permease
RPA0725	60.8	0.0	-	chaperone
RPA0759	0.0	0.0	-	oligopeptide ABC transporter permease
RPA0760	11.1	0.0	-	oligopeptide ABC transporter permease
RPA0882	16.7	0.0	-	ABC transporter permease
RPA0883	9.0	0.0	-	ABC transporter ATP-binding protein
RPA0974	0.0	0.0	-	hydrogenase expression/formation protein HypB
RPA0983	38.3	0.0	-	phthalate 4,5-dioxygenase
RPA1059	24.7	0.0	-	outer membrane protein TonB-dependent receptor
RPA1215	103.3	0.0	-	ABC transporter permease
RPA1216	15.0	0.0	-	ABC transporter permease
RPA1333	11.0	0.0	-	hypothetical protein
RPA1362	0.0	0.0	-	sulfate ester transport system substrate-binding protein
RPA1363	4.3	0.0	-	sulfate ester transport system permease

RPA1365	8.3	0.0	- sulfatase
RPA1385	35.8	0.0	- phosphonate transport system substrate-binding protein
RPA1386	0.0	0.0	- phosphonate ABC transporter ATP-binding protein
RPA1389	9.6	0.0	- hypothetical protein
RPA1396	26.6	0.0	- sugar ABC transporter permease
RPA1408	4.5	0.0	- ABC transporter
RPA1409	26.1	0.0	- taurine transport system permease
RPA1421	2.5	0.0	- efflux protein
RPA1428	27.7	0.0	- lipoprotein
RPA1430	13.6	0.0	- outer membrane protein
RPA1438	12.9	0.0	- nitrogenase iron protein
RPA1447	0.0	0.0	- oligopeptide ABC transporter permease
RPA1448	0.0	0.0	- oligopeptide ABC transporter permease
RPA1458	5.4	0.0	- dinitrification protein NorD
RPA1477	30.5	0.0	- permease
RPA1478	4.5	0.0	- permease
RPA1795	101.6	0.0	- potassium-efflux system protein
RPA1830	0.0	0.0	- hypothetical protein
RPA1831	14.9	0.0	- hypothetical protein
RPA1876	41.5	0.0	- TonB-dependent iron siderophore receptor
RPA1961	32.3	0.0	- RND efflux transporter
RPA1990	17.3	0.0	- sulfatase
RPA2026	15.9	0.0	- ferric siderophore receptor
RPA2041	53.5	0.0	- ABC-transport protein ATP-binding protein
RPA2042	13.5	0.0	- ABC transporter permease
RPA2043	109.9	0.0	- ABC transporter periplasmic substrate-binding protein
RPA2056	12.7	0.0	- RND divalent metal cation efflux transporter CzcA
RPA2113	12.7	0.0	- nitrate transport system permease
RPA2114	35.9	0.0	- nitrate transport system ATP-binding protein
RPA2118	79.4	0.0	- hemin importer ATP-binding subunit
RPA2120	90.6	0.0	- periplasmic binding protein
RPA2307	4.4	0.0	- tonB-dependent receptor
RPA2310	9.3	0.0	- iron ABC transporter ATP-binding protein
RPA2329	4.2	0.0	- ABC transporter permease
RPA2353	16.2	0.0	- nitrogenase NifH subunit
RPA2360	3.1	0.0	- ABC transporter ATP-binding protein
RPA2361	0.0	0.0	- ABC transporter permease
RPA2372	5.4	0.0	- RND multidrug efflux transporter MexB
RPA2382	14.1	0.0	- iron(III) ABC transporter ATP-binding protein
RPA2383	3.7	0.0	- iron(III) ABC transporter permease
RPA2403	22.7	0.0	- iron-chelator utilization protein
RPA2497	13.6	0.0	- anion ABC transporter ATP-binding protein
RPA2608	22.2	0.0	- sulfonate binding protein
RPA2610	16.8	0.0	- aliphatic sulfonate ABC transporter ATP-binding protein
RPA2611	8.8	0.0	- aliphatic sulfonate ABC transporter permease
RPA2615	24.4	0.0	- nitrogenase iron protein
RPA2618	64.2	0.0	- sulfonate transport system substrate-binding protein

RPA2622	68.6	0.0	-	sulfonate ABC transporter ATP-binding protein
RPA2633	17.5	0.0	-	nitrate ABC transporter ATP-binding protein
RPA2635	4.1	0.0	-	nitrogenase reductase NifH subunit
RPA2644	34.2	0.0	-	ABC transporter permease
RPA2645	21.6	0.0	-	ABC transporter permease
RPA2673	54.6	0.0	-	hypothetical protein
RPA2785	20.8	0.0	-	flavodoxin oxidoreductase
RPA2792	30.4	0.0	-	monovalent cation/H ⁺ antiporter subunit E
RPA2793	43.1	0.0	-	monovalent cation/H ⁺ antiporter subunit F
RPA2794	0.0	0.0	-	pH adaptation potassium efflux system component phaG
RPA3003	8.0	0.0	-	potassium-transporting ATPase subunit B
RPA3004	0.0	0.0	-	potassium-transporting ATPase subunit A
RPA3027	123.5	0.0	-	K ⁺ potassium transporter
RPA3201	25.4	0.0	-	formate/nitrate transporter
RPA3279	10.5	0.0	-	iron-starvation protein
RPA3291	6.2	0.0	-	membrane transporter
RPA3310	24.3	0.0	-	catalase
RPA3497	27.8	0.0	-	chromate transporter
RPA3516	51.3	0.0	-	MFS family efflux pump
RPA3614	72.7	0.0	-	rhodanese-like protein
RPA3689	47.4	0.0	-	binding-protein-dependent transport system inner membrane protein
RPA3690	91.4	0.0	-	binding-protein-dependent transport systems inner membrane component
RPA3699	19.7	0.0	-	iron-chelator utilization protein
RPA3707	27.1	0.0	-	nitrate transporter component nrtA
RPA3711	2.1	0.0	-	sulfite reductase
RPA3736	10.5	0.0	-	phosphate transport system substrate-binding protein
RPA3840	23.7	0.0	-	hydroxamate-type ferrisiderophore receptor
RPA3843	6.0	0.0	-	potassium efflux system protein
RPA4038	0.0	0.0	-	ABC transporter ATP-binding protein
RPA4153	16.1	0.0	-	binding-protein-dependent transport system inner membrane protein
RPA4159	7.7	0.0	-	binding-protein-dependent transport systems inner membrane component
RPA4162	4.3	0.0	-	taurine ABC transporter permease
RPA4164	28.3	0.0	-	aliphatic sulfonate ABC transporter substrate-binding protein
RPA4385	33.4	0.0	-	TonB-dependent receptor
RPA4510	44.6	0.0	-	TRAP transporter solute receptor TAXI family protein
RPA4715	28.1	0.0	-	molybdate ABC transporter ATP-binding protein
RPA4716	21.0	0.0	-	molybdate ABC transporter permease
RPA0765	215.3	5.9	36.6	outer membrane receptor for iron transport
RPA2876	514.2	28.0	18.3	glucan biosynthesis protein G
RPA0220	250.1	15.2	16.5	cation diffusion facilitator family transporter
RPA1024	162.5	11.2	14.5	oxidoreductase
RPA0811	136.6	10.5	13.0	manganese transp
RPA0860	188.6	15.7	12.0	ABC transporter periplasmic binding protein
RPA0133	141.7	14.4	9.8	sulfate ABC transporter periplasmic binding protein
RPA1522	1432.8	157.6	9.1	chlorophyllide reductase iron protein subunit X
RPA1526	14443.9	1712.4	8.4	light-harvesting complex 1 subunit alpha

RPA4636	455.9	56.2	8.1	FeoA family protein
RPA4635	626.0	81.5	7.7	ferrous iron transport protein B
RPA1274	4930.3	653.7	7.5	Dps protein family starvation-inducible DNA-binding protein
RPA0123	121.9	17.1	7.1	KTN NAD-binding domain-containing protein
RPA0502	73.9	10.4	7.1	HlyC/CorC family transporter
RPA1472	190.6	28.2	6.8	dipeptide transport permease
RPA4620	1805.9	272.1	6.6	nitrogenase iron protein
RPA0013	351.6	57.0	6.2	cation (heavy metal) transporting ATPase
RPA2956	77.9	13.2	5.9	RND multidrug efflux transporter MexF
RPA1754	25.5	4.3	5.9	cation efflux system protein
RPA4430	35.2	6.0	5.9	TonB-dependent receptor
RPA0724	139.1	27.0	5.2	high-affinity nickel-transport protein
RPA3480	62.1	12.7	4.9	outer membrane receptor for iron transport
RPA1939	195.6	40.8	4.8	cation diffusion facilitator family transporter
RPA4292	813.6	179.0	4.5	light harvesting protein B-800-850 alpha subunit B
RPA4407	70.5	15.9	4.4	binding-protein-dependent transport systems inner membrane component
RPA0014	249.9	59.8	4.2	FixH family protein
RPA2011	29.8	7.1	4.2	potassium uptake protein Kup
RPA4606	1107.2	270.4	4.1	nitrogenase stabilizing/protective protein
RPA4480	50.6	12.9	3.9	RND divalent metal cation efflux membrane fusion protein CzcB
RPA3542	34.7	8.8	3.9	EmrB/QacA subfamily drug resistance transporter
RPA1845	23.8	6.1	3.9	tonB-dependent receptor protein
RPA2062	38.1	10.3	3.7	periplasmic ABC transport copper binding protein
RPA2706	75.7	20.6	3.7	manganese transport protein MntH
RPA0748	54.1	15.7	3.4	sulfate ABC transporter permease
RPA3055	60.6	17.6	3.4	TerC family integral membrane protein
RPA0056	58.5	17.0	3.4	binding-protein-dependent transport system inner membrane protein
RPA1968	74.3	21.9	3.4	RND efflux transporter
RPA3048	284.8	85.0	3.4	polyphosphate kinase
RPA0388	51.8	15.6	3.3	a potassium/proton antiporter
RPA2115	98.8	30.9	3.2	cyanate hydratase
RPA4479	54.9	17.9	3.1	heavy metal cation efflux system protein
RPA3953	33.1	11.2	2.9	chloride channel protein
RPA0794	226.1	76.7	2.9	carbonate dehydratase
RPA4387	18.0	6.1	2.9	ferrichrome-iron receptor
RPA1486	34.1	11.6	2.9	heme-Cu protein NnrS
RPA3483	154.8	52.9	2.9	RND efflux transporter
RPA3008	82.9	29.3	2.8	potassium uptake protein Kup
RPA1410	36.0	13.3	2.7	taurine transport system protein
RPA4803	158.9	59.4	2.7	outer membrane hemin/siderophore receptor protein
RPA1345	200.4	77.7	2.6	hypothetical protein
RPA1693	2239.6	869.4	2.6	superoxide dismutase
RPA2333	49.0	19.9	2.5	cation transport ATPase copper transporter
RPA2755	762.8	313.6	2.4	DNA-binding stress protein
RPA0405	419.0	175.4	2.4	hypothetical protein
RPA4186	103.0	44.1	2.3	TerC family integral membrane protein

RPA3844	54.1	23.6	2.3	sodium/hydrogen exchanger
RPA1376	218.9	97.9	2.2	nitrogenase iron protein
RPA1384	38.9	17.6	2.2	phosphate ABC transporter permease
RPA1823	56.9	26.3	2.2	TonB dependent receptor
RPA4757	94.1	44.0	2.1	outer membrane receptor for iron transport
RPA0429	13295.1	6643.1	2.0	catalase/oxidase HPI
RPA2258	21.7	11.0	2.0	arsenical pump membrane protein
RPA4501	157.3	80.0	2.0	PhnA protein
RPA2632	28.2	14.3	2.0	ABC transporter permease
RPA1420	8.6	4.4	2.0	heavy metal efflux pump CzcA
RPA4777	99.5	52.8	1.9	ATP-binding protein
RPA0859	186.7	99.1	1.9	hypothetical protein
RPA2272	167.7	91.0	1.8	hypothetical protein
RPA0470	174.3	99.6	1.8	ABC transporter periplasmic binding protein
RPA0702	20.5	11.9	1.7	phosphonate ABC transporter permease
RPA4152	48.7	28.3	1.7	family 1 extracellular solute-binding protein
RPA4326	55.0	32.0	1.7	sulfate transporter family protein
RPA0096	50.7	30.7	1.6	multidrug-efflux transport protein
RPA0225	142.3	86.9	1.6	superoxide dismutase binding-protein-dependent transport system inner membrane protein
RPA0468	86.2	52.6	1.6	tonB-dependent receptor protein
RPA2378	19.2	12.0	1.6	thiosulfate sulfurtransferase
RPA3613	53.5	33.5	1.6	hypothetical protein
RPA1881	202.9	130.9	1.5	sulfonate transport system substrate-binding protein
RPA2624	21.7	14.7	1.5	protein associated with Co ²⁺ and Mg ²⁺ efflux
RPA4770	408.3	277.0	1.5	Fe ABC transporter
RPA2081	22.6	15.4	1.5	RND divalent metal cation efflux transporter CzcA
RPA1808	18.5	13.3	1.4	acriflavin resistance protein
RPA2574	23.9	17.7	1.4	alkaline phosphatase
RPA0391	21.5	15.9	1.4	transport system permease binding-protein-dependent transport systems inner membrane component
RPA0057	44.2	32.7	1.4	Na ⁺ antiporter
RPA2704	26.6	19.7	1.4	hypothetical protein
RPA2280	151.5	114.2	1.3	outer membrane receptor for iron transport
RPA1816	24.6	18.7	1.3	ion transporter
RPA2821	73.5	56.1	1.3	sulfonate ABC transporter permease
RPA2623	67.4	51.4	1.3	heme receptor
RPA3280	15.2	11.6	1.3	monooxygenase
RPA1496	58.2	45.6	1.3	ABC transporter ATP-binding protein
RPA0858	195.6	155.4	1.2	bacterioferritin protochlorophyllide reductase iron-sulfur ATP-binding protein
RPA3600	1047.9	846.8	1.2	cation-transporting P-type ATPase
RPA1545	1589.5	1284.8	1.2	cation-transporting P-type ATPase
RPA3260	8.0	6.5	1.2	sulfate ester transporter ATP-binding protein
RPA1259	12.4	10.1	1.2	phosphonate metabolism PhnJ
RPA0469	42.8	34.8	1.2	ABC transporter permease
RPA0692	20.2	16.4	1.2	
RPA2498	20.2	16.4	1.2	

RPA4732	63.1	51.4	1.2	ChaC-like protein
RPA4779	52.6	42.9	1.2	phosphate ABC transporter permease
RPA1625	42.9	35.0	1.2	CysQ protein
RPA0884	35.2	28.7	1.2	ABC transporter substrate-binding protein
RPA4776	25.0	20.4	1.2	phosphate uptake regulator PhoU
RPA3384	17.0	13.9	1.2	nitrate/sulfonate/bicarbonate ABC transporter periplasmic-binding protein
RPA1498	52.6	43.7	1.2	RND multidrug efflux transporter MexD
RPA1660	33.8	28.4	1.2	cation transporting P-type ATPase
RPA1426	43.6	38.1	1.1	part of the metNIQ transport system for methionine
RPA2012	108.2	97.9	1.1	rhodanese-like protein
RPA4095	29.1	26.3	1.1	multidrug-efflux transport protein mexF
RPA0275	31.2	29.3	1.1	ammonium transporter
RPA1021	26.3	25.7	1.0	AcrB/AcrD/AcrF family transporter
RPA2126	27.4	27.9	1.0	hypothetical protein
RPA3553	12.5	12.7	1.0	bile acid:sodium symporter
RPA2359	16.3	16.6	1.0	hypothetical protein
RPA2398	65.6	69.7	0.9	iron siderophore uptake receptor family protein
RPA0753	19.8	21.9	0.9	bifunctional ATP-sulfurylase large subunit/adenylyl sulfate kinase CysN/CysC
RPA0232	61.7	68.5	0.9	carbonate dehydratase
RPA0012	439.1	496.5	0.9	cytochrome oxidase maturation protein
RPA3414	10.6	12.3	0.9	hydroxamate-type ferrisiderophore receptor
RPA4457	137.3	159.7	0.9	sulfide dehydrogenase with CysN catalyzes the formation of adenylylsulfate from sulfate and ATP
RPA0752	28.5	33.2	0.9	molybdenum ABC transporter periplasmic molybdate-binding protein
RPA4717	106.1	131.4	0.8	
RPA2745	19.4	24.3	0.8	ferric siderophore receptor protein
RPA2385	11.7	15.9	0.7	ABC transporter periplasmic Fe+3 siderophore binding protein
RPA2309	9.6	13.1	0.7	iron chelatin ABC transporter permease subunit
RPA1456	30.9	41.9	0.7	nitric-oxide reductase subunit B
RPA1773	35.7	48.4	0.7	DMT family permease
RPA3385	12.7	17.3	0.7	nitrate transport system ATP-binding protein
RPA0747	30.2	40.9	0.7	sulfate transport system permease 1
RPA2380	13.8	18.7	0.7	tonB dependent iron siderophore receptor
RPA2124	150.4	213.9	0.7	tonB dependent iron siderophore receptor
RPA2499	61.2	87.9	0.7	ABC transporter periplasmic protein
RPA0799	59.6	88.3	0.7	carbonic anhydrase
RPA2112	47.4	71.1	0.7	nitrate transporter component nrtA
RPA0874	119.0	182.9	0.7	high-affinity nickel-transporter
RPA0749	55.2	86.4	0.6	sulfate ABC transporter permease
RPA4158	22.2	36.2	0.6	binding-protein-dependent transport systems inner membrane component
RPA2678	20.0	32.6	0.6	ABC transporter permease
RPA2281	293.7	492.2	0.6	phosphate transporter
RPA4441	61.0	102.2	0.6	Mg2+ transporter
RPA2631	23.9	41.7	0.6	ABC transporter substrate-binding periplasmic protein
RPA3002	96.1	171.1	0.6	potassium-transporting ATPase subunit C
RPA1539	136.2	266.0	0.5	heme oxygenase
RPA0697	8.2	16.6	0.5	phosphonate ABC transporter permease

RPA0690	10.2	20.8	0.5	phosphonate ABC transporter ATP-binding protein
RPA0094	38.5	78.3	0.5	hypothetical protein
RPA0693	25.5	51.9	0.5	phosphonate metabolism protein
RPA2613	7.4	15.1	0.5	ABC transporter aliphatic sulfonate binding protein
RPA3554	17.7	36.1	0.5	arsenate reductase
RPA3619	26.9	54.7	0.5	vanillate O-demethylase oxygenase iron-sulfur subunit
RPA4778	32.8	66.8	0.5	phosphate ABC transporter permease
RPA0585	24.2	49.2	0.5	chromate transporter
RPA4514	52.6	107.1	0.5	alkaline phosphatase
RPA2679	26.1	53.0	0.5	transport system ATP-binding protein
RPA3163	21.9	49.6	0.4	magnesium transporter
RPA2269	22.9	54.3	0.4	hypothetical protein
RPA0153	25.1	63.8	0.4	outer membrane receptor for Fe transport
RPA4649	30.7	78.0	0.4	ABC transporter permease
RPA1643	36.8	99.9	0.4	high affinity iron permease FTR1
RPA0520	41.7	118.8	0.4	Na ⁺ /H ⁺ antiporter
RPA2677	14.7	45.0	0.3	ABC transporter substrate-binding protein
RPA0150	13.7	41.9	0.3	iron ABC transporter permease
RPA3698	11.3	36.7	0.3	MFS family drug efflux pump
RPA3498	36.6	124.2	0.3	chromate transporter
RPA2812	182.4	622.3	0.3	HlyC/CorC family transporter
RPA2440	55.4	195.5	0.3	cation efflux protein
RPA0750	85.3	347.2	0.2	thiosulfate-binding protein
RPA0998	10.1	41.0	0.2	rhodanese-like protein
RPA2790	22.8	92.9	0.2	monovalent cation/H ⁺ antiporter subunit C
RPA2328	3.4	14.0	0.2	ABC transporter permease
RPA3727	37.1	164.7	0.2	magnesium/cobalt transporter CorA
RPA2308	14.8	72.3	0.2	ABC transporter periplasmic iron siderophore binding protein
RPA0273	46.9	233.4	0.2	ammonium transporter AmtB catalyzes the reduction of 3'-phosphoadenylyl sulfate into sulfite
RPA0751	14.5	78.7	0.2	
RPA1364	13.2	71.6	0.2	sulfate ester transport system ATP-binding protein
RPA1476	52.3	312.3	0.2	periplasmic solute-binding protein
RPA2929	4.4	26.7	0.2	hypothetical protein
RPA2284	25.1	153.5	0.2	drug efflux protein
RPA2893	22.7	151.9	0.1	ATPase E1-E2 type
RPA3383	4.5	36.9	0.1	taurine transport system permease
RPA2384	3.6	29.3	0.1	ironIII transport permease
RPA0695	23.6	192.2	0.1	phosphonate metabolism PhnG
RPA1471	3.9	31.4	0.1	dipeptide ABC transporter permease
RPA4780	194.7	1740.5	0.1	periplasmic phosphate binding protein
RPA3398	46.7	604.0	0.1	arsenite permease
RPA0694	12.1	172.0	0.1	carbon-phosphorus lyase complex subunit