Faculty of Science and Engineering
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Enhanced biohydrogen production by integrating wastewater and photo-fermentation

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: …….

Date:.……...

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Abstract

Hydrogen is one of clean and future source of renewable energy. Different techniques for the production of H_2 have been implemented. The biological methods such as dark or photo fermentation can be one of the promising sustainable method to produce hydrogen. However, the biological methods are limited by the low hydrogen production yield and the expensive cost of synthetic medium utilized for growing and maintaining the hydrogen-producing bacteria. Therefore, the broader aim of this thesis is that investigation different strategies to enhance biohydrogen such as optimization using response surface methodology (RSM).

The synergistic effects and optimization of pH, carbon-to-nitrogen ratio (C/N), and light intensity (*I*) on the photo-fermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM and light conversion efficiency have been investigated under different conditions of pH (6.5 - 8); C/N (15 - 35); and light intensity (35 – 185 Wm^{-2}). Response surface methodology (RSM) and Box-Behnken experimental design (BBD) were used to identify the optimum values of the three key parameters of pH, C/N, and *I*, based on the impact on hydrogen production potential (HPP), hydrogen production rate (HPR), and light conversion efficiency η. With desirability value of 0.91, the optimum values of 7.4, 27.5, and 126 Wm^{-2} were identified for pH, C/N, and *I* respectively, with HPP, HPR and η reaching 960 mL L^{-1}, 41.74 mL L^{-1} h^{-1}, and 0.31 respectively. Regression analysis indicated a good fit between experimental and model data. The study showed that both C/N ratio and *I* have crucial and significant effect on the HPP, HPR and η followed by pH, the synergistic effect of pH–*I* and C/N–*I* on the light conversion efficiency (η) was significant while pH –C/N was insignificant. Thus, the optimal conditions achieved by this study can be a base for the next investigation.

The impacts of banana peels pre-treatment stage on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM using brewery effluent (BE) were also investigated in a batch bioreactor. The experimental results indicate that banana peels pre-treatments can significantly enhance the cumulative hydrogen production. The maximum hydrogen production yield (408.33 mL H_2 $L^{-1}_{wastewater}$) was achieved from the substrate, which was composed of 50 % BE pre-treated with 1 g L^{-1} of banana peels for 2 h and 50 % standard medium. This highest amount of hydrogen production was 2.7-folds higher than those that applied the same percentage of raw BE as the substrate source. Further experiments can be conducted to investigate the impact of

metal ions supplementation using combination of the brewery wastewater with another real wastewater to complement each other.

The influence of different concentrations of iron, Fe (30 -110 μ M), molybdenum, Mo (8-20 μ M), and ethylenediaminetetraacetic acid, EDTA (0.1-0.5 g L^{-1}) on the photofermentative hydrogen production and bacterial growth by *Rhodobacter sphaeroides* 158 DSM was investigated and discussed. A blend of pre-treated brewery (30%) and restaurant (70%) effluents was used successfully as a sole medium (without using the standard medium) for the photofermentative hydrogen production, producing a cumulative biohydrogen of 83 mL. The results show that sole-additions of Fe at 70 µM, Mo at 14 µM, and co-addition of Fe:Mo at 70 µM:8 µM to the mixture of pre-treated brewery and restaurant effluents, could enhance the cumulative biohydrogen production to 140 mL (69% increased), 105 mL (27% increased), and 160 mL (93% increased), respectively. The results also revealed that the addition of EDTA should be optimized to avoid the chelation of the added metal ions (Fe, Mo). The biohydrogen production was further enhanced to 192 mL, which represent 131% increase compared to control, when the optimized EDTA of 0.2 g L^{-1} was added the blended effluents at Fe:Mo concentrations of 70 µM:8 µM,. Furthermore, the study shows that the addition of Fe, Mo and EDTA to the blended effluent enhances the biomass growth as well. Utilizing the wastewater for biohydrogen production as a sole medium could open a new area for renewable energy production. Metal ions supplementation, feedstock pre-treatment, and combination of two types of wastewaters were applied to enhance photofermentative hydrogen production. Thus, it can be very useful in next investigation to focus on one of these techniques for such as feedstock pre-treatment and try to improve its performance.

The feasibility of integrated banana peels waste and ultra-sonication technology as feedstock pre-treatment to enhance photo fermentative hydrogen production has been investigated. With compared to the control (no pre-treatment), the experimental results demonstrate that integrated approach of banana peels waste and ultra-sonication using the combined effluents of 70% restaurants effluent (RE) and 30% brewery effluent (BE) treated with 2 g L^{-1} of banana peels waste and A60:T35 (amplitude=60% and ultra-sonication time=35) led to increase the cumulative biohydrogen production to 110 mL (144% increased), while pre-treating the same mixture with only banana peels pre-treatment enhanced the cumulative biohydrogen production to 83 mL (84%

increased) with compared to the control (no pre-treatment). In addition, simultaneous about 46% of soluble chemical oxygen demand (SCOD) removal was achieved during photo-fermentation. Based on these results, integrated agricultural waste and ultra-sonication was concluded to be a promising technique for enhancing photo fermentative hydrogen production processes by rising the bioavailability of organic content and decreasing the toxic properties of wastewater.

In conclusion, identification of the synergistic influences of significant factors and the optimal conditions achieved in this study will serve as a base for the future research that will use the same experimental set up and microorganism. In addition, banana peels can be considered as a promising pre-treatment agent to treat wastewater samples, which can be further used as a sole substrate for biohydrogen production. Moreover, integrated ultrasonic with banana peels retreatment was concluded to be a promising technique for photofermentative hydrogen production processes. In the presence of metal ions (Fe and Mo), only the optimum concentration of EDTA could enhance the biohydrogen production. However, the brewery wastewater cannot be applied alone as a single medium to produce biohydrogen and it has to supplement with standard medium or another real wastewater. Therefore, it will be very useful to apply wastewater as a sole feedstock for hydrogen production with efficient strategies to enhance biohydrogen yield. Based on this approach, double benefits of wastewater pre-treatment and hydrogen production are gained.

Publications

Journal Papers;

- Al-Mohammedawi HH, Znad H, Eroglu E. Synergistic effects and optimization of photo-fermentative hydrogen production of Rhodobacter sphaeroides DSM 158. **International Journal of Hydrogen Energy**. 2018;43:15823-34.

- Al-Mohammedawi HH, Znad H, Eroglu E. Improvement of photofermentative biohydrogen production using pre-treated brewery wastewater with banana peels waste. **International Journal of Hydrogen Energy**. 2019;44:2560-8.

- Al-Mohammedawi HH, Znad H. Impact of metal ions and EDTA on photofermentative hydrogen production by Rhodobacter sphaeroides using a mixture of pre-treated brewery and restaurant effluents. **Biomass and Bioenergy**. 2020;134:105482.

- Hassan H. Al-Mohammedawi, Hussein Znad. Integrated banana peels waste and ultra-sonication technology as feedstock pre-treatment to enhanced photo fermentative hydrogen production. (submitted to **Bioresource Technology** 2020).

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List of Symbols and Abbreviations

Symbol	***Description***
C/N	Carbon-to-Nitrogen ratio
I	Light intensity, W m^{-2}
Fe	Iron
Mo	Molybdenum
EDTA	Ethylenediaminetetraacetic acid
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
H_2	Hydrogen gas
V_{H_2}	Volume of the produced hydrogen gas, L
A	Irradiated area, m^2
Δt	Duration of hydrogen production, h
f	Number of factors
N	Number of tests
cp	Number of central points
D	Desirability function
d_i	Individual desirability function
w/w	Weight to weight ratio
OD_{680}	Optical density at 680 nm
X	Dry weight biomass
Ar	Argon gas
x_i	Coded value of the actual value for a certain factor
Fd	Oxidized ferredoxin
R^2	Determination coefficients

Greek characters	***Description***
η	Light conversion efficiency, %
ρ_{H_2}	Density of produced hydrogen gas, g L^{-1}

Abbreviations	***Description***
RSM	Surface methodology
DSM,	German Collection of Microorganism and Cell Cultures
BBD	Box-Behnken experimental design
HPP	Hydrogen production potential, mL L^{-1}
HPR	Hydrogen production rate, mL L^{-1} h^{-1}
BE	Brewery effluent
RE	Restaurants effluent
PNS	Purple non sulfur bacteria
PHB	Poly-β-hydroxybutyrate
CCD	Central composite design
VS	Volatile solids
TVS	Total volatile solids
OMW	Olive mill wastewater
DF	Dark fermentation
PF	Photo fermentation
NPs	Nanoparticles
SCOD	Soluble chemical oxygen demand, mg L^{-1}
TCOD	Total chemical oxygen demand, mg L^{-1}
TOC	Total organic carbon, mg L^{-1}
TN	Total Nitrogen, mg L^{-1}
SEM	Scanning Electron Microscope
FTIR	Fourier-transformed infrared spectroscopy
TCD	Thermal conductivity detector
Bt	Treated brewery wastewater
SM	Standard medium
Bu	Untreated brewery wastewater
A	Amplitudes, %
T	Ultra-sonication duration, min

Chapter 1
Introduction

Chapter 1 Introduction

1.1 Background and motivation

Currently, most countries use fossil fuels as a main energy source to supply their energy demands [1, 2]. It is estimated that fossil fuels supply about 80% of world's energy demands (Figure 1.1) [1].

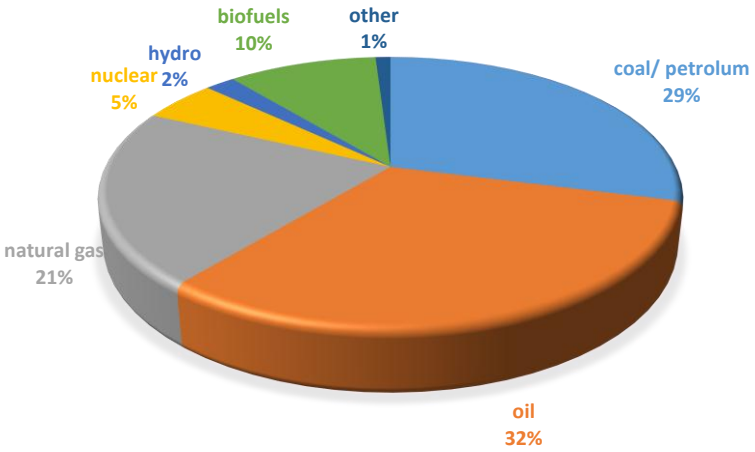

Figure 1. 1 Fuel shares of global energy supply in 2011

However, on combustion, fossil fuels generate harmful by-products such as CO_2, CO, and sulfur dioxide which lead to adversely impact the environment and increase the global warming [3] . It can be seen from Figure 1.2 that fossil fuel is responsible for the most CO_2 emissions. Petroleum and natural gas with current consumption rates will be depleted within 50 and 65 years, respectively [4].

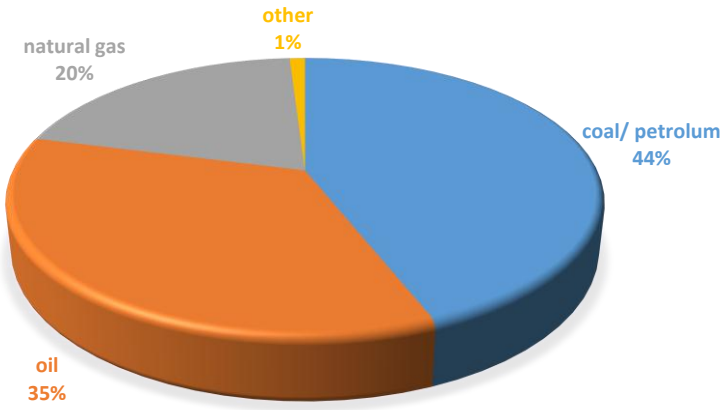

Figure 1. 2 CO_2 emissions from fuel globally consumed in 2011 (adapted from [1])

Therefore, there is a growing need to explore alternative fuels that are environmentally friendly [5]. Among various fuels, hydrogen (H_2) has potential advantages. It is an environmental-friendly fuel that is regarded as one of the alternatives to overcome carbon emissions and global warming. Also, the specific energy content for H_2 is close to 140 MJ kg^{-1}, while it is only 48.6 MJ kg^{-1} for gasoline [6]. Furthermore, H_2 can be generated by both sustainable and non-sustainable recourses [7].

Hydrogen can be produced by physical-chemical processes such as steam reforming of methane or by biological methods including photo or dark fermentation processes [8]. In steam reforming of methane, two stages are required to produce hydrogen. In the first stage, high temperatures (700- 1100℃) are applied to produce syngas ($CO+H_2$) by reaction of methane with steam. After that, additional hydrogen is produced by the reaction of carbon monoxide with steam. However, 1 ton of hydrogen produced by this method lead to release 2.5 ton of CO_2 into the atmosphere which can be considered as a main reason for global warming [7, 9].

Generally the biohydrogen can be produced biologically by dark fermentation, photo fermentation or water bio photolysis processes [2]. During dark-fermentation process, microorganisms convert substrates into soluble materials such as alcohols and volatile fatty acids (i.e. acetic acid, butyric acid). These materials are converted to glucose, and then glucose generates pyruvate. After that, pyruvate involves in complex reactions that lead to produce hydrogen [2]. In the presence of light, purple non sulfur (PNS) bacteria can generate hydrogen as a metabolic by-product using the light energy and the energy from the oxidation of the substrate in order to reduce H^+ ions into hydrogen gas. In this process, nitrogenase enzyme catalyzes the reaction of hydrogen production, while hydrogenase enzyme can be active for both the consumption and production of H_2 [10]. Nitrogenase is an enzyme that reduces protons to H_2 gas. It catalyses the conversion of N_2 into NH_3 and hydrogen [11]. As cofactors, molybdenum, iron and vanadium are present in the catalytic positions of nitrogenase enzyme [11, 12]. In the absence of N_2, this enzyme catalyses the following reaction;

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP \quad (1.1)$$

Oxygen has a negative impact on nitrogenase because it could deactivate the enzyme irreversibly. Also, ammonium is able to inhibit the activity of nitrogenase enzyme [10,

13]. Hydrogenase is an enzyme that is responsible for catalyzing the reaction of hydrogen consumption or formulation by the following reaction [2, 11];

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (1.2)$$

In the presence of electrons acceptor and hydrogen gas, the consumption reaction will be catalysed by hydrogenase. While in the presence of protons and electrons donor, hydrogenase will catalyse the reaction of hydrogen formulation.

The main criteria to select the suitable substrate for biohydrogen production are availability with low cost, biodegradability, and carbohydrate content [2, 14]. Pure sugars such as glucose and sucrose are easily biodegradable and the most suitable as carbon sources for biohydrogen production. However, these sugars cannot economically be viable as carbon source for biohydrogen production [2]. Wastewater such as food industries wastewater can supply the necessary nutrients for biological fermentation and reduce treatment cost [14]. Waste and wastewater are renewable resources which can be applied as substrates to produce bio hydrogen by biological fermentation methods in addition to treat wastes in environmentally friendly methods [15]. In this regard, biological methods help to reduce the greenhouse emissions by 57-73% [16]. However, the yield and production rate of biological methods are still lower than those produced by chemical methods. Several wastewaters have been applied as substrate for photo fermentative hydrogen production such as olive mill wastewater [17], dairy wastewater [18] , brewery wastewater [19], and sugar refinery wastewater [20]. Hence, biohydrogen production could be a promising technique for both biohydrogen production and treatment of the wastewater utilized as a medium.

Literature reviews have indicated that there were considerable efforts have been performed to enhance the performance and yield of biohydrogen production based on novel strategies developed over the last years (Figure 1.3).

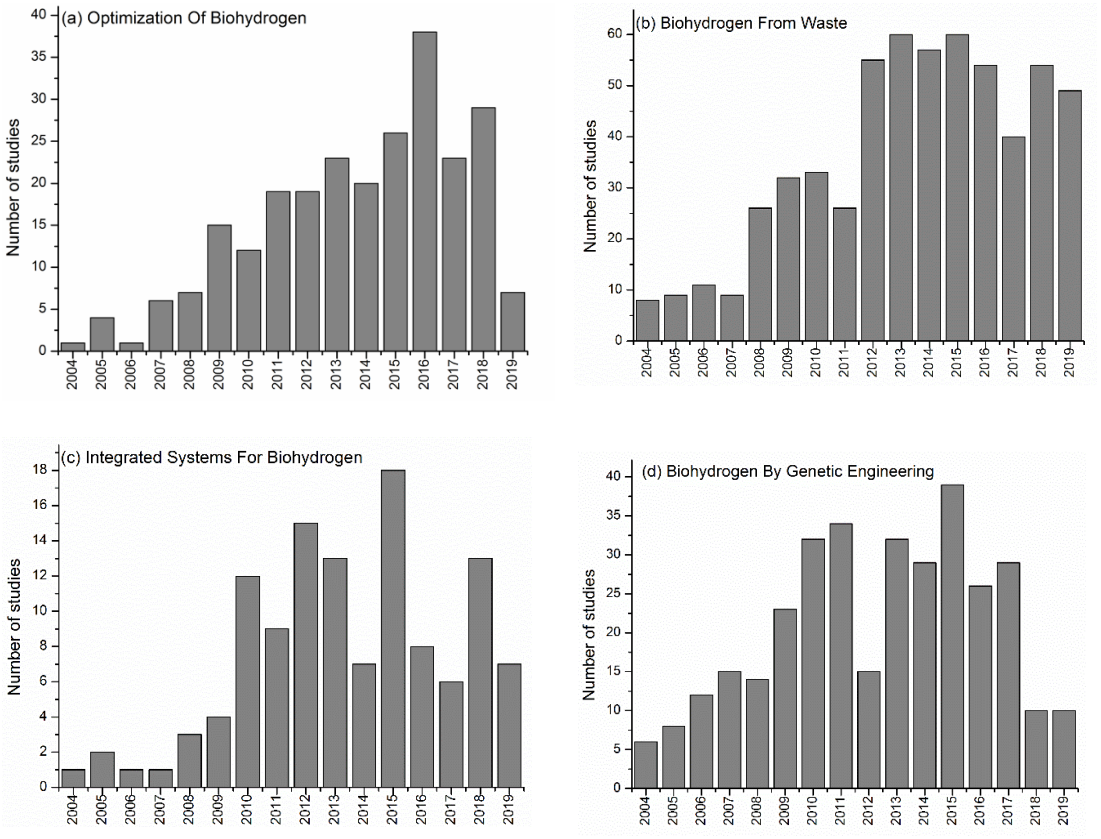

Figure 1.3 Number of published studies on biohydrogen with emphasis on (a) Optimization of biohydrogen; (b) Biohydrogen From Waste; (c) Integrated systems For Biohydrogen; (d) Biohydrogen by Genetic Engineering. Source SciFinder, August 26 2019

Several strategies have been applied to enhance yields of photo fermentation processes. The main strategies for enhancing biohydrogen are shown in Figure 1.4. Optimization of influencing variables of biohydrogen production can be applied to enhanced the yield [21]. The fermentative hydrogen production process can be affected by different parameters including pH, temperature, inoculum, reactor type, substrate type, organic loading rates, metal ions, C/N ratio , light source and light intensity (for photofermentative hydrogen process) [5, 22]. If aforementioned factors are not set at optimum ranges, the fermentative hydrogen process may be inhibited. In photo-fermentative hydrogen production processes, several studies have been reported that the carbon to nitrogen (C/N) ratio of the medium can be a crucial variable influencing the yield of hydrogen production and the bacterial growth [23, 24].

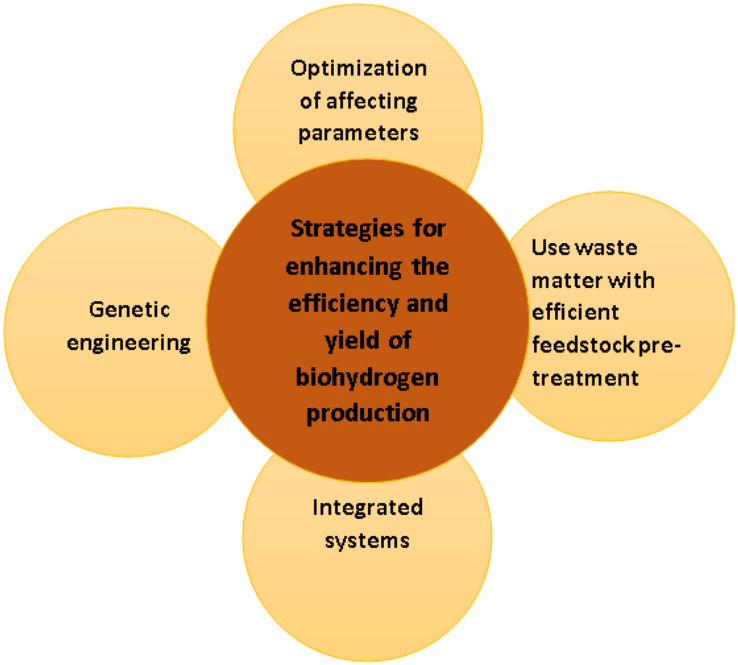

Figure 1. 4 Main strategies for enhancing the efficiency and yield of biohydrogen production

A high C/N ratio led to increase a bacterial growth and decrease hydrogen production yield. In the same way, a high bacterial growth with low biohydrogen yield was also observed at a low C/N ratio, so it is necessary to find the optimum C/N ratio [25]. In addition, previous studies revealed that the light intensity can play a significant role in biohydrogen production processes [26-28]. However, it has been found that the optimum value of the light intensity depends on the used light source and also on the type of strain [23, 29]. Furthermore, biohydrogen was reported to enhance photo fermentative hydrogen production [30-32]. However, previous studies have shown that the achieved results and optimal concentration of metals in the biohydrogen production medium depend on type of substrates and microorganisms [31-34]. Extensive literature reviews have indicated that the studies of metals supplementation have tended to focus on using defined mediums rather than actual wastewater as a substrate to produce fermentative hydrogen production [32-37].

The second approach applied by researchers to enhance biohydrogen yields was feedstock pre-treatment. Different wastewater qualities have been applied as feedstock for biohydrogen production by dark fermentation [38-41]. It was reported that photofermentative hydrogen could also be produced from wastewater [17, 19, 31, 42, 43]. However, this approach has some disadvantages such as lower yields and rates of

biohydrogen production compared to standard medium. To overcome these hurdles, feedstock pre-treatment techniques have been applied. The feedstock pre-treatment is one of the best strategies to enhance the nutritional value of the feedstock [21]. There have been several studies in the literature focused on the role of wastewater pre-treatment stages prior photo fermentation process [44-51].

Several studies have reported the use of integrated two-stage technique to enhance the overall biohydrogen production efficiency. In the first stage of this system, substrate with high carbohydrate content is partially consumed to produce biohydrogen, CO_2 and organic acid in anaerobic dark fermentation. The organic acid produced from the first stage as by-products are then utilized in a second stage as a substrate to generate additional biohydrogen using photo fermentation process. The integrated system could theoretically lead to produce 12 mol of biohydrogen per 1 mol of glucose. Equations 1.3 and 1.4 show the overall reactions of integrated dark fermentation followed by photo fermentation.

$$C_6H_{12}O_6+2H_2O \rightarrow 2CH_3COOH+2CO_2+4H_2 \quad (1.3)$$

$$2CH_3COOH+4H_2O \rightarrow 4CO_2+8H_2 \quad (1.4)$$

Several researchers have investigated the integration of these two systems [52-60]. For example, Eroğlu et al. [55] applied olive mill wastewater as a substrate for dark fermentation using activated sludge, and then the effluent of this stage was used as photofermentative hydrogen production medium using *Rhodobacter sphaeroides O.U.001*. Rai et al. [52] achieved enhanced biohydrogen by the integration of dark fermentation using *Enterobacteraerogenes* MTCC 2822 and photo-fermentation using *Rhodopseudomonas BHU 01* from sugarcane bagasse. Zagrodnik and Laniecki [53] also applied such integrated technique by integrating dark fermentation by *Clostridium acetobutylicum* and photofermentation by *Rhodobacter sphaeroides*. Chandra and Mohan [54] observed 40% enhancement in biohydrogen production with simultaneous 40% decrease in volatile fatty acid accumulation compared to dark fermentation when integrated system was applied.

Genetic engineering can be used as an alternative technique to increase biohydrogen production [61]. It allows to overcome limitations of biohydrogen production in different ways Figure 1.5 , including deactivation of uptake hydrogenase enzymes [62-64], repression biosynthesis pathways competing biohydrogen pathway [63, 65],

truncation the bacteriochlorophyll antenna size of BNS bacteria [66, 67] and increasing the ammonia tolerance of BNS bacteria [68, 69]

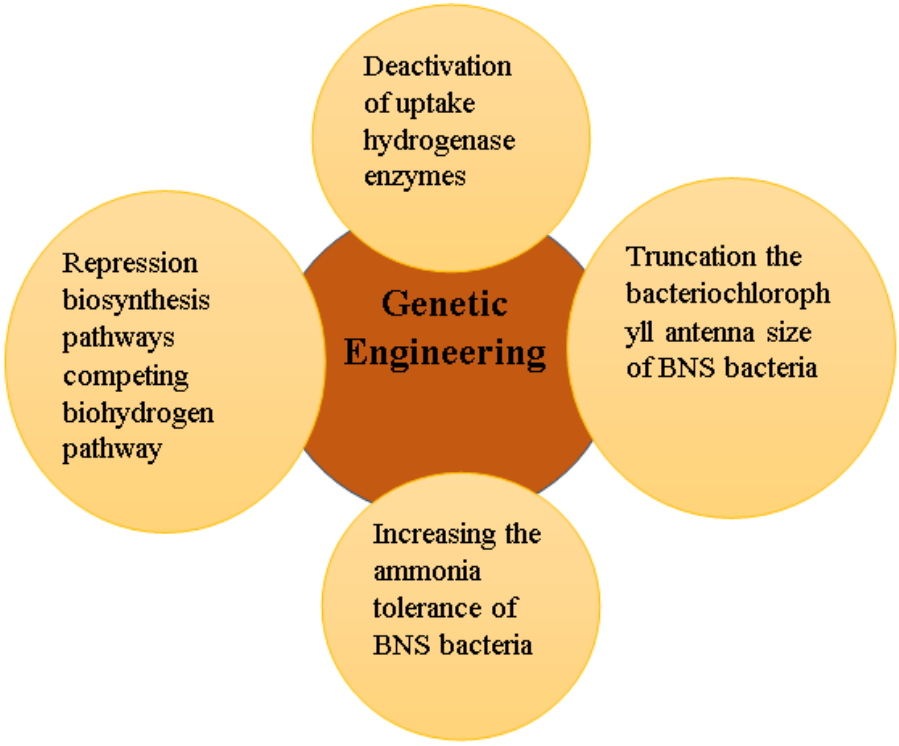

Figure 1. 5 Various way of genetic engineering applied for biohydrogen production

1.2 Thesis objectives

The major objective of this PhD thesis to enhance the biohydrogen production utilizing different wastewater qualities. Therefore, the following aims will be thoroughly addressed:

1. Optimize the most influencing factors on biohydrogen production rate using Response Surface Methodology (RSM).
2. Investigate the pre-treatment stage on the biohydrogen production.
3. Investigate the feasibility of different wastewater qualities as a viable medium for biohydrogen production.
4. Investigate the influence of different enhancers such as iron, molybdenum and EDTA on the biohydrogen production

1.3 Significance

Industrially, photofermentative biohydrogen production process cannot be feasible, due to the low biohydrogen yield and the expensive cost of synthetic hydrogen production medium. In this investigation, wastewater was successfully used as a sole feedstock for hydrogen production. Efficient strategies to enhance biohydrogen yield such as such as optimization with response surface methodology (RSM), feedstock pre-treatment techniques and supplementation of growth media with trace of metals were applied. Therefore, applying wastewater as a sole feedstock for hydrogen production with efficient strategies simultaneously, can significantly improve the feasibility of biohydrogen production in large scale and open a new avenue for the utilization of clean and sustainable energy resources due to its dual benefits of waste treatment along with hydrogen production. Additionally, the applied banana peels and ultra-sonication techniques have a low environmental impact due to applying non-hazardous banana peels and safe and non-toxic waves of ultra-sonication.

1.4 Thesis structure

The current thesis consists of 8 chapters which are related to above objectives. A brief description for these chapters is explained in this section. The thesis structure is also presented in Figure 1.6.

- **Chapter 1** provides a background to the thesis, the thesis objectives and structure.
- **Chapter 2** reviews the relevant literature on biohydrogen production.
- **Chapter 3** shows a detailed description of the applied methodology, including used experimental setup and analytical methods.
- **Chapter 4** reports the result of identification the optimum values of the three key parameters of pH, C/N, and *I*, based on the impact on hydrogen production potential (HPP), hydrogen production rate (HPR), and light conversion efficiency η, using response surface methodology (RSM) and Box-Behnken experimental design (BBD).

- **Chapter 5** Reports the impacts of banana peels pre-treatment stage on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM in a batch photo bioreactor.
- **Chapter 6** Demonstrates and explains the influence of iron, molybdenum and EDTA on photofermentative hydrogen production by *Rohodobacter sphaeroides* 158 DSM using mixed wastewaters in a batch bioreactor.

- **Chapter 7** Reports the impacts of banana peels integrated with ultra-sonication as pre-treatment stage on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM using mixed wastewaters.
- **Chapter 8** provides the main conclusions of the current study and suggests some recommendations for the future work.

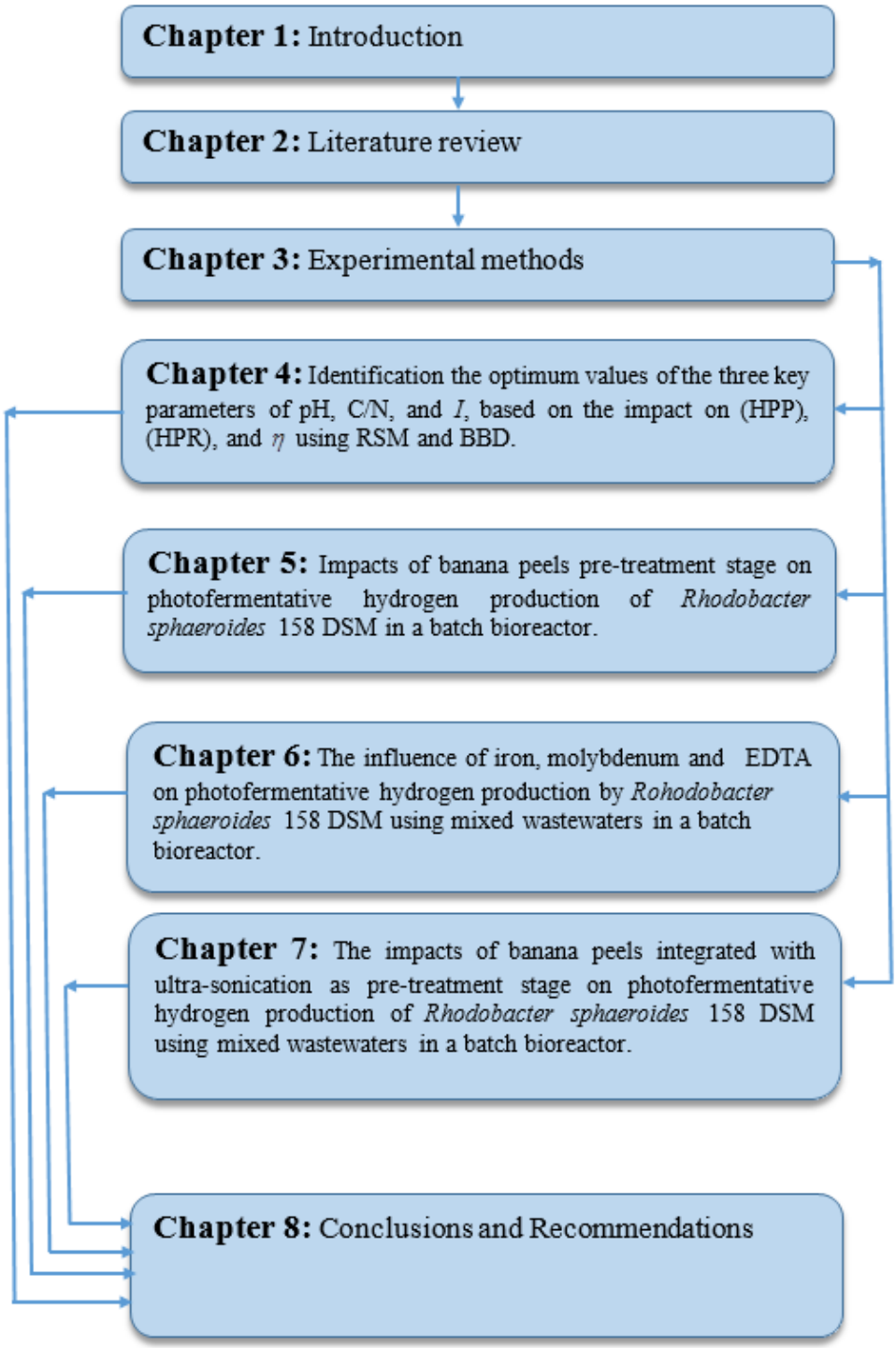

Figure 1. 6 Thesis structure

Chapter 2
Literature Review

Chapter 2 Literature Review

2.1 Introduction

The decrease in the amount of fossil fuel and its harmful impacts on the environment stimulate researchers to find alternative, clean and sustainable energy resources. Among different alternative, hydrogen (H_2) is considered as a significant and promising alternative fuel because of its high energy density and an environmental-friendly nature to overcome CO_2 emissions and global warming [23]. At present, H_2 can be produced by physicochemical processes and biological processes. Compared with other methods, biological H_2 production processes are more close to achieve the requirements of a sustainable development due to allowing the utilization of various sustainable resources such as energy crops, wastes and wastewaters at ambient temperature and atmospheric pressure by simply applying microorganisms [70].

Main biological processes for hydrogen production can be categorized into two basic types: (a) direct/indirect bio-photolysis, and (b) dark/ photo fermentation processes [71, 72]. Direct bio-photolysis refers to the process that directly produces hydrogen from H_2O by utilizing light energy via photosynthetic system of microorganisms such as algae. On the other hand, indirect bio-photolysis allows an H_2 production in two steps from water molecules via photosynthetic system of microorganism, such as cyanobacteria, under the presence of light energy. The first step is responsible for producing biomass that is used for the production of H_2 in the second step [70]. During dark-fermentation process in the absence of light, anaerobic microorganisms convert substrates into soluble materials such as alcohols and volatile fatty acids (i.e. acetic acid, butyric acid). These materials are converted to glucose, which later generates pyruvate. After that, pyruvate involves in the complex reactions that produce hydrogen[73]. Under the presence of light, purple non sulfur (PNS) bacteria can generate hydrogen as a metabolic by-product using the light energy and the energy from the oxidation of the substrate in order to reduce H^+ ions into hydrogen gas. In this process, nitrogenase enzyme catalyses the reaction of hydrogen production, while hydrogenase enzyme can be active for both the consumption and production of H_2 [23].

Recently, significant efforts have been accomplished to enhance fermentative hydrogen production. These efforts ranges from finding appropriate feedstocks with high productivity [15] to applying efficient pre-treatment processes for providing more suitable feedstocks for the fermentative H_2 production processes [45, 74]. As one of the key factors for improving the efficiency of fermentative H_2 production, pre-treatment processes have been largely investigated by various researchers [75]. The transformation of organic waste and wastewater into a valuable biohydrogen fuel can be considered as an integrated solution to both waste and energy managements [2, 76, 77]. However, utilization of feedstocks like lignocellulosic materials for fermentative hydrogen production is limited by the application of efficient pre-treatment techniques [15, 78].

2.2 Hydrogen production methods

Hydrogen can be generated by both renewable and non-renewable resources. The current technologies of H_2 production are physical-chemical methods such as the steam reforming of natural gas, and biological methods such as dark/photo fermentation production. Figure 2.1 illustrates the sources and methods of hydrogen production [8].

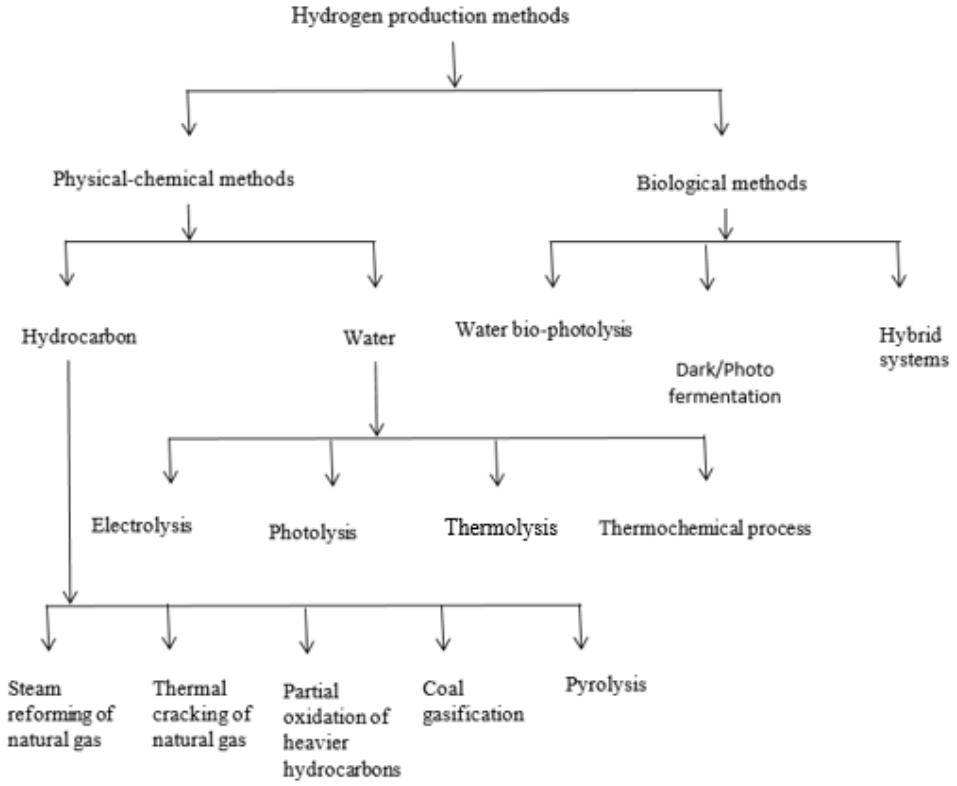

Figure 2. 1 Hydrogen production methods

Presently, steam reformation can be considered the most popular and least cost method to generate hydrogen. This method is a two-stage process. At first step, Syn gas (CO + H_2) is produced by reacting methane with steam at temperature 700–1100 °C and then, CO_2 reacts with steam to generate extra hydrogen [79]. However, Hydrogen generated by reforming of hydrocarbons produces CO_2 as a by-product. About 2.5 ton of CO_2 is released into the environment for each ton of H_2 generated by steam reforming of hydrocarbons [79]. Massive CO_2 emissions are also vented into the environment coal gasification process. In this technology, coal reacts with steam or O_2 to generate hydrogen. [7]. A huge CO_2 emission during hydrogen production decrease potential benefits. Therefore, it is necessary to improve technology with lower CO_2 emissions.

The focus is to modify or find new hydrogen production technologies which do not release high carbon emissions. Another approach is to capture the CO_2 generated by such processes to prevent its release to the environments. However, currently, the applied energy of CO_2 capture technologies is mainly supplied by burning fossil fuels. Therefore, CO_2 capture technologies can accelerate the depletion rate of the global fossil fuel reserves. Different technologies have been suggested to diminish carbon emission during hydrogen production. However, most of those suggested technologies are either costly compared to those applying fossil fuels, or they are in their early stages of improvement [7].

Hydrogen can also be generated from other resources such as water [80]. Unlike fossil fuels technologies, water does not release CO_2 during hydrogen production. However, the direct splitting of water needs excessive temperature (>2000 °C) [81]. Different methods have been proposed to directly split water such as electrolysis, photo electrochemical, photocatalytic, and thermal decomposition [82]. The huge endothermic water-splitting technology supplied by burning fossil fuels results in high CO_2 emissions. Therefore, sustainable energy sources such as wind or solar are required to be improved for the electrolysis of water [7]. Fortunately, solar energy can also be applied to decompose water into hydrogen and oxygen. However, the solar-based technologies need wide land area [83].

Hydrogen production applying nuclear energy can be considered as a possible approach for large-scale hydrogen production. Nuclear technology can produce hydrogen in different methods such as electrolysis of water, and nuclear heated steam reforming of natural gas [7]. Wind energy is plentiful and clean source of energy with

only a negligible environmental effect [84]. Therefore, great attentions have been achieved for the electrolytic hydrogen production technology applying large-scale wind installations. However, wind energy technology cannot generate electrical power at a very high percentage. Therefore, such system can be useful only for sites with high wind. The electricity produced by wind energy can be connected with the hydrogen generating stacks of electrolyzers and which would decrease energy conversion losses [7].

2.3 Purple non-sulfur (PNS) bacteria

Purple non-sulfur (PNS) bacteria can be considered as the first phototrophic organisms on the earth. The common features of these types of organisms are their ability to achieve an anaerobic photosynthesis processes without the generation of oxygen. These bacteria are named purple nonsulfur bacteria because they do not apply hydrogen sulfide as electron donor whereas growing photo autotrophically. Under photoheterotrophic circumstances, they have ability to produce hydrogen and generate purple to deep red pigments [23]. *Rhodobaca, Rhodobacter*, *Rhodovulum, Rhodopseudomonas, Rhodoblastus, Blastochloris*, *Rhodomicrobium, Rhodobium, Rhodoplanes, Rhodocista*, *Rhodospirillum*, *Phaeospirillum, Rhodopila, Rhodospira, Rhodovibrio*, *Rhodothallasium, Roseospira*, *Roseospirillum*, *Rhodocyclus*, *Rhodoferax*, and *Rubrivivax* are documented genera of PNS bacteria [85]. Depend on the presence of light, O_2, and type of carbon sources (organic or inorganic), PNS bacteria can grow as photo-heterotrophs (energy from light and carbon from organic substrate), photoautotrophs (energy from light and carbon from carbon dioxide) or chemo-heterotrophs (carbon and energy from organic substrate). Inorganic substrate (CO_2) can be applied in autotrophic growth and organic substrate can be used in heterotrophic growth. Figure 2.2 shows the modes of PNSB for growing [12].

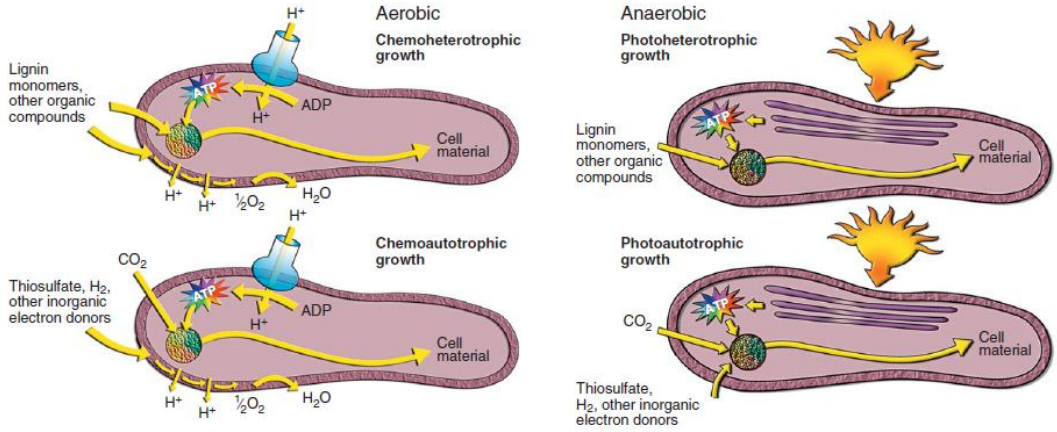

Figure 2. 2 Growth modes for PNS bacteria [12]

The species *Rhodobacter*, *Rhodopseudomonas* and *Rhodospirillum* have been mostly examined in laboratory studies [86]. Photofermentative hydrogen production is mainly linked with nitrogenase activities (Figure 2.3). PNS bacteria can apply nitrogenase enzymes to produce biohydrogen by catalyzing the reduction of molecular nitrogen (N_2) to ammonia (NH_3) [23] . Molybdenum, iron and vanadium are present in the catalytic positions of nitrogenase enzyme. Based on these metals, this enzyme is classified as Mo – nitrogenase, V – nitrogenase, Fe – nitrogenase [11]. Among three different types of nitrogenase, Mo-nitrogenase can be considered the most effective nitrogenase enzyme to convert N_2 to NH_3 equations (2.1, 2.2 and 2.3) [23];

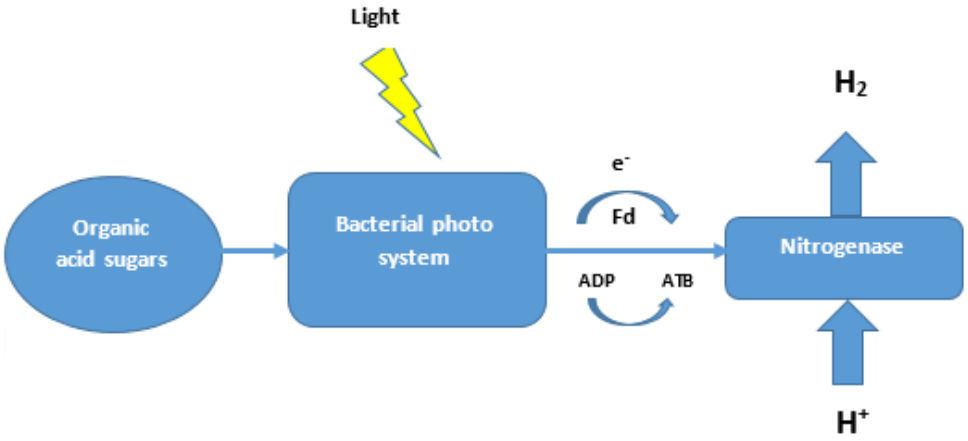

Figure 2. 3 The scheme of photofermentative hydrogen production

$$\text{Mo – nitrogenase: } N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP \qquad (2.1)$$

$$\text{V - nitrogenase: } N_2 + 12H^+ + 12e^- + 24ATP \rightarrow 2NH_3 + 3H_2 + 24ADP \quad (2.2)$$

$$\text{Fe - nitrogenase: } N_2 + 24H^+ + 24e^- + 48ATP \rightarrow 2NH_3 + 9H_2 + 48ADP \quad (2.3)$$

In the absence of N_2, this enzyme catalyses the reaction below to produce biohydrogen [87]:

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP \quad (2.4)$$

Large amounts of reducing power and ATP are required to achieve an efficient performance for nitrogenase. Oxygen has a negative impact on nitrogenase because it could deactivate the enzyme irreversibly [23]. Also, a high concentration ammonium can suppress the synthesis nitrogenase enzyme and inhibit the enzyme activity. This inhibition can be reversible since nitrogenase can recuperate after eliminating ammonium [88].

Hydrogenase is an enzyme that is responsible for catalyzing the reaction of consumption or formulation by the following reaction [11].

$$H_2 \leftrightarrow 2H^+ + 2e^- \quad (2.5)$$

In the presence of electrons acceptor and hydrogen gas, the consumption reaction will be catalyzed by hydrogenase. While, in the presence of protons and electrons donor, hydrogenase will catalyze the reaction of hydrogen formulation. This enzyme can be classified based on metals of active positions into three types: (Ni Fe)-hydrogenase, (Fe Fe)-hydrogenase and (Fe)-hydrogenase [89]. Hydrogenase can be considered as a metabolic oppositionist for nitrogenase [23]. In biohydrogen production processes, activation of uptake hydrogenase enzyme is undesired, since it affects the hydrogen generation. Therefore, an inactivation of such enzyme commonly lead to increase biohydrogen production [11].

2.4 Factors affecting photo fermentative hydrogen production process

The process factors which can impact hydrogen production of photo fermentation are explained as below.

2.4.1 C/N ratio

Photofermentative hydrogen production yields greatly depends on C/N. Several studies have shown that the C/N ratio of the hydrogen production medium can be a significant factor affecting biohydrogen production yield and the growth of microorganisms [23, 24]. A high bacterial growth with low hydrogen yield was noticed at a low C/N ratio. In the same way, a high C/N ratio also led to improve a microbial growth and reduce the hydrogen production yield, which makes it necessary to find the optimum C/N ratio [25]. Also, the source of nitrogen can strongly influence the photofermentative hydrogen production yield, due to the fact that NH^{+}_{4} is known to inhibit the activity of nitrogenase enzyme [90].

The hydrogen production from purple non sulfur (PNS) bacterium has been impacted by the combinations of carbon and nitrogen sources. For example, when proline, alanine or tyrosine were used as the nitrogen source, *R. sphaeroides* A-10 was only able to generate biohydrogen using the medium with succinate as a carbon source [91]. Glutamic acid has been one of the widely used nitrogen source for the production of hydrogen by photo-fermentation process [26, 92]. The concentration of the nitrogen source in the medium can also affect the amount of hydrogen production of *R. sphaeroides*. It was reported that biohydrogen generation was higher for the nitrogen-limited conditions (i.e., 2 g yeast extract L^{-1}) than those applied under nitrogen-excess conditions (i.e., 5 g yeast extract L^{-1}) [93].

As observed for the hydrogen production, generation of by-products such as poly-β-hydroxybutyrate (PHB) was also reported to be enhanced under nitrogen-limited conditions [17, 94] As carbon and energy source, malate is favored source for biohydrogen production by (PNS) bacterium [95]. A paper by Han et al. [96] investigated the impact of the different carbon sources -such as malate and succinate- on the photo fermentative hydrogen production by *Rhodobacter sphaeroides* RV. The maximum bio hydrogen was 1340 mmol H_2 mol^{-1} substrate achieved by 1 g L^{-1} malate, while it was 300 mmol H_2 mol^{-1} substrate achieved by 3 g L^{-1} succinate.

2.4.2 Light source and light intensity

various studies reported that the light intensity of the illumination source also plays a significant role in the hydrogen production processes [26-28].The optimum intensity of light depends on the type of strain and also on the light source applied [23, 29].

Florescent lamps and halogen lamps cab be artificial illumination sources for photo fermentation process. One of the most significant factors determining process productivity is the light conversion efficiency (η). It was calculated was calculated according to the following equation [17, 27, 97, 98];

$$\eta(\%) = \frac{33.61 \rho_{H_2} V_{H_2}}{I A \Delta t} \times 100 \quad (2.6)$$

Where ρ_{H_2} is the density of produced hydrogen gas (g L^{-1}), V_{H_2} is the volume of the produced hydrogen gas (L), I is the light intensity (W m^{-2}), A is the irradiated area (m^2), and Δt is the duration of hydrogen production (h). The light conversion efficiency relies on the type species and the substrate applied. Mostly it was varied between 1 and 6%. However, light conversion efficiency of 9.23% was obtained by *Rhodobacter sphaeroides* with a light intensity of 200 Wm^{-2} of a tungsten illumination using lactate as carbon source [23, 99].The photo-fermentation process can use combined of sunlight with artificial lights or it can apply the sunlight alone. Generally, light intensity can be measured by two units either Wm^{-2} or lux. The conversion factor between these two units relies on the wavelength. However, it can be estimated as 1 Wm^{-2} is equal to 30–100 lux [100].

2.4.3 pH

Essentially, initial pH can be considered as a key factor in photo-fermentation processes since it has a crucial impact on the metabolic pathways of the microorganisms[101]. Several studies have examined the impact of pH on photo-fermentative hydrogen production. The results indicated that suitable pH values depend on the type of strains used for the process [63, 102, 103]**.** For instance, the reported optimum pH varied among species, pH 7.2 for *R. sphaeroides* VM81[102], pH 7.0–7.5 for *R. sphaeroides* O.U.001[103] and pH 7.4–7.6 for *R. sphaeroides* KD131 [63]. Kim et al. [104] also reported that the increase in pH of the medium above the optimum-range (7.4 - 7.6) led to dissipate the membrane of the cells which would cause cell clustering. These clustered cells might reduce the light conversion efficiency of microorganisms into biohydrogen.

2.4.4 Inoculum age and temperature

To enhance hydrogen productivity, it is very important to apply microorganism at the early stationary phase [103]. After long retention period in the growth media, the

metabolic pathways of PNS bacteria are shifted toward producing PHB [23, 105]. The optimum temperature values are varied between 30 and 40 ^{o}C depending on the strain [100].

2.5 Optimization of influential factors for enhanced biohydrogen production

The term optimization refers to enhance the performance of process that leads to the best response [106]. Consequently, many studies of optimization for biohydrogen production have been performed to maximize the production using response surface methodology (RSM) [107-110] . In conventional methods, experiments are usually carried out by varying a single factor while the other factors are set at constant values. This approach would lead to missing interactive effects among all factors [111, 112]. In addition, these methods are costly due to the long-time of the large number of experiments that might be required [106]. Therefore, RSM has been applied as alternative technique to design the experiments that investigates the interaction among all factors and determines optimum experimental conditions.

RSM is a collection of mathematical and statistical techniques that was introduced by Box and Wilson in 1951 [113]. This term was derived from the graphical perspective of the response (dependent variables) and factors (independent variables) created after fitness of the polynomial equation to the experimental results [106]. The necessary steps of application of RSM for optimization systems are as follows; (1) the choice of independent factors that have significant impacts on the system; (2) the selection of the experimental designs such as Box-Behnken design (BBD) or central composite design (CCD) ; (3) based on selected matrix, the experiments are performed; (4) fitness of the achieved experimental results via a polynomial model; (5) the assessment the fitness models and determination the optimum response [106, 112]. The number of experiments (N) of BBD is calculated as $N=2f\,(f-1)+cp$, (where f is number of factors and cp is number of central points), while the number of experiments required for CCD is $N=2^{f}+2f+cp$. It was found that BBD matrix are more effective than the three-level full factorial designs and slightly more effective than CCD [114].

The desirability function approach is one of the most significant methods to optimize of multiple response problems (D) which was proposed by Derringer and Suich in 1980 [115]. In this approach, each response is transformed into individual desirability function (d_i). The scale of this function is varied from $d_i=0$ representing a completely

unacceptable response and d_i=1 representing a completely desirable response value. Individual desirability functions are then combined using the geometric mean[114].

$$D= (d_1 \times d_2 \times \ldots. d_n)^{1/n} \quad (2.7)$$

Several studies have been published on the application of RSM to optimize operational conditions of bio hydrogen production; Assawamongkholsiri and Reungsang [108] investigated the optimization of pH , temperature, and light intensity to maximize hydrogen production by RSM with CCD. They found the optimal operational conditions for bio hydrogen from malic acid as carbon source and Na-glutamate as nitrogen source of *Rhodobacter* sp. KKU-PS1were 7.0, 25.6℃, and 2500 lux of pH, temperature and light intensity, respectively. In a different study, Sangyoka et al. [109] studied the interactive influence among substrate concentration, substrate buffer: ratio, and inoculum: substrate ratio by RSM with CCD. The obtained results indicated that these factors had a significant impact on the biohydrogen production from sugarcane bagasse. The optimum conditions were 22.8 g sugar L^{-1}, 4.31, and 0.31 of substrate concentration, substrate buffer: ratio, and inoculum: substrate ratio. In another study, optimization of biohydrogen production from de-oiled jatropha waste was achieved by CCD and the optimum substrate concentration, pH, and temperature were 211 g L^{-1} 6.5 and 55.4℃ , respectively [110]. Kieu et al. [116] reported that RSM with CCD was adopted to enhance biohydrogen and they showed that soluble starch concentration, ferrous iron concentration, and L-cysteine can significantly affect the hydrogen production of an anaerobic bacterial strain from soluble starch.

Karthic et al. [117] researched the interactive influence among xylose concentration, pH, and peptone concentration for biohydrogen of *Enterobacter* (MTCC7104) by RSM with BBD. A study by Sekoai [118] demonstrated the RSM with BBD can be applied to optimize operational conditions of fermentative hydrogen production. The optimized variables were 39.56 g L^{-1}, 82.58 h, 5.56, and 37.87℃ of substrate concentration, fermentation time, pH, and temperature, respectively. Al-Mohammedawi et al. [111] applied RSM with BBD to determine the optimum values of of pH, C/N, and *I*, based on their influence on hydrogen production potential, hydrogen production rate, and light conversion efficiency η. They found that the optimum values were 7.4, 27.5, and 126 Wm^{-2} for pH, C/N, and *I* respectively,

2.6 Pre-treatment methods of wastes and wastewater

The major aim of pre-treatment methods is to improve the waste conversion to hydrogen conversion efficiency of fermentative H_2 production processes. This aim can be achieved by converting the complex materials such as lignocellulosic biomass into its simple forms [119], recovering of the fermentable sugars of wastewater[120] and removing inhibitors from wastewater such phenols [44]. Pre-treatment methods prior to fermentation processes can be accomplished by chemical (alkali hydrolysis, ozonolysis, and acid hydrolysis), biological (enzymatic hydrolysis), physical (mechanical distraction, ultra-sonication) or physicochemical methods (steam explosion, ammonia fibre explosion) [121]. In this section, brief details of the pre-treatment processes applied to organic wastes and wastewater have been explained. Table 2.1 summarizes the current research studies conducted to produce fermentative hydrogen from solid wastes and wastewater by employing various types of pre-treatment techniques.

2.6.1 Chemical pre-treatment methods

The purpose of chemical pre-treatment is to enhance the biodegradability of cellulosic biomass by removing lignin and lignocellulosic compounds and reducing the crystallinity of cellulose. Acid pre-treatment processes are one of the most applied chemical pre-treatment methods to enhance biohydrogen production from organic wastes, as shown in Table 2.1. These methods can employ diluted or concentrated acids to hydrolyse hemicelluloses. Generally, diluted acids used by hydrolysis methods generate lower quantity of inhibitors with lower loss of sugars. On the other hand, the high concentration of acids used during hydrolysis methods has the problem of causing equipment corrosions. Therefore, these methods require costly corrosion-resistance equipment.

Diluted acid hydrolysis methods can be performed either by continuous processes at high temperatures ($T>160^oC$) or under batch conditions at temperatures less than 160^oC. Continuous modes are suitable for low the wastes with low solid contents (5-10% w/w), while batch modes are suitable for high solid contents (10-40 % w/w) [122, 123]. Alternative to the acidic hydrolysis, alkaline pre-treatment methods have also been applied to remove lignin content from lignocellulosic biomass under lower temperature and pressure conditions than the ones applied for the acidic hydrolysis method. Sodium hydroxide and ammonium hydroxide are the agents that are mostly

used for the alkaline pre-treatment methods, as also shown in Table 2.1. Compared to the acid hydrolysis methods, alkaline methods requires more time to accomplish the treatment process [122, 123]. Ozonolysis can also be considered as a chemical pre-treatment method that is used to enhance the digestibility of cellulose by degrading lignin and hemicellulose. These methods generate low inhibitors such as furfural. Also they can operate at ambient temperature and pressure. Although ozonolysis processes can be considered as fairly an environmental-friendly, they are very costly due to their requirements for large amounts of ozone [123].

Cui and Shen [74] investigated the impacts of different concentrations (0.5, 1, 2, 4 and 8 % (w/v)) of HCl (acid hydrolysis) and NaOH (alkaline hydrolysis) on fermentative hydrogen production from grass at an initial pH of 7. Their results showed that the maximum volume of H_2 was 72.2 mL g^{-1}dry grass at 4% HCl, which was approximately 16.5-folds higher compared to the value obtained by the raw feedstock. It also showed that the maximum H_2 production was 19.3 mL at 0.5% NaOH, which was 4.4-folds greater than that the results of raw substrate. Han et al. [39] reported the effects of acid hydrolysis (HCl), alkaline hydrolysis (NaOH) and hydrogen peroxide pre-treatment on biohydrogen production from soybean straw by anaerobic mixed bacteria at an initial pH 7. Similarly, they found that maximum hydrogen yield was around 47.7, 10.5 and 23 mL H_2 g^{-1}substrate at 4% HCl, 0.5% NaOH and 16% H_2O_2 respectively. They all showed improvements with respect to untreated raw material, as the hydrogen production yield of raw soybean straw was only 5.5 mL H_2 g^{-1}substrate. Xing et al. [38] reported that acid hydrolysis (0.2 HCl) was more efficient for producing biohydrogen from dairy manure than infrared radiation and alkaline hydrolysis (0.2% NaOH). The maximum yield by employing these pre-treatment methods was 18.1, 14.2 and 13.9 mL g^{-1}TVS (total volatile solids) for acid hydrolysis, infrared radiation and alkaline hydrolysis respectively, while the yield was 13.3 mL g^{-1} TVS for untreated substrate.

Carrillo-Reyes and Buitrón [124] noted that thermal-acidic pre-treatment of the native microalgae consortium was capable of enhancing hydrogen yield to about 45.4 mL H_2 g^{-1} VS at 1% of HCl and 90 ^oC for 2 hr. Nguyen et al. [125] observed that the combined pre-treatment (10% ammonia +10%H_2SO_4) slightly enhanced the hydrogen yield of rice to 2.7 mmole H_2 g^{-1}straw, when compared with the untreated straw that yielded a hydrogen production of 2.3 mmole H_2 g^{-1}straw. Asadi and Zilouei [126] concluded the

concentration of ethanol used in ethanol organosolv of rice straw had considerable impact on biohydrogen yield. Their results indicated that at 45 % (v/v) ethanol had the maximum H_2 yield of 19.73 mL g^{-1}straw.

2.6.2 Biological pre-treatment methods

Cellulolytic microorganisms can play an important role in the degradation of lignocellulosic materials by producing cellulolytic enzymes, such as cellulose and xylanase that hydrolyse lignocellulosic biomass. The main advantages of this method include low energy requirements and moderate operating conditions. However, this method suffers from the high-cost of the enzyme as well as the low hydrolysis rate, when compared with chemical processes [76].

Zhao et al. [127] investigated the influence of fungal pre-treatment method on cornstalk to produce biohydrogen by applying *Thermoanaerobacterium thermosaccharolyticum* W16. The maximum hydrogen yield of 89.3 mL g^{-1}cornstalk was achieved at 60 ^{o}C, pH 6.5, 75% feedstock. In another study, Cui et al. [128] investigated the effects of acidic and enzymatic hydrolysis as pre-treatment steps for hydrogen production from poplar leaves. They found that enzymatic pre-treatment at 35 ^{o}C with an initial pH 7 was an efficient method to enhance biohydrogen production yield from poplar leaves. In this method, the maximum hydrogen yield was 44.9 mL g^{-1}dry poplar leaves, which was approximately 3-folds higher than that from raw feedstock and around 1.3-folds higher than the yield obtained from the pre-treated feedstock with 4% HCl. Ramprakash and Muthukumar [129] have demonstrated the influence of acidic, enzymatic and combined (acid + enzymatic) hydrolysis as pre-treatment stages for enhancing the biohydrogen yield from rice mill wastewater. The result indicated that the hydrogen yield was 1.97, 1.78 and 1.63 mol H_2 mol^{-1} sugar from combined, enzymatic and acid pre-treatment methods, respectively.

2.6.3 Physicochemical pre-treatment methods

Steam explosion is the most commonly applied pre-treatment method to break down the structure of lignocellulosic materials [76]. In this method, a high-pressure saturated steam is injected into reactor with or without chemicals for a few minutes to heat biomass. Then, the pressure is rapidly reduced to make the lignocellulosic materials undergo on an explosive decompression. This action removes hemicelluloses materials and enhances the penetration of enzymes by increasing surface area of the feedstock

[130]. The main advantage of this method its low environmental impact due to applying less hazardous chemicals, whereas the disruption of lignin remains incomplete [76, 123].

Ultra-sonication methods have also been widely applied to improve the solubilisation of organic materials. The ultrasonic waves that are generated in liquid phase cause the generation and implosion of microbubbles. Generally, the major advantages of this technique are their lower operation conditions in a short time at ambient temperatures, and its requirement for a low amount of catalyst [122]. Additionally, ultra-sonication techniques can be combined with other methods. These methods can be considered as environmentally friendly as their waves are safe and non-toxic. However, their main drawback comes from the requirement of high-energy; and possible generation of free radicals [131].

Budiman et al. [51] demonstrated the impacts of various ultra-sonication amplitudes (10, 20 and 30%) and the duration of ultra-sonication (5, 10, 15 min) on hydrogen yield from combined pulp and paper mill effluents (25% pulp+75% paper) by photo-fermentation process. These authors found that 14.4 mL H_2 mL^{-1} medium was the maximum hydrogen yield at 20% ultra-sonication for 10 minutes, whereas the hydrogen yield without ultra-sonication was 9.9 mL H_2 mL^{-1} medium. Gadhe et al. [132] applied the ultra-sonication as a pre-treatment method to enhance fermentative H_2 yield from distillery wastewater. They found that the maximum hydrogen production yield (10.95 mmol H_2 g^{-1} COD) was achieved by applying an ultra-sonication pre-treatment at 0.12 W mL^{-1} ultrasonic density.

Eroğlu et al. [45] found that clay pre-treatment of olive mill wastewater (OMW) was the optimum pre-treatment method to enhance the yield of photofermentative hydrogen production when compared with other techniques including zeolite, ozone, UV radiation and Fenton's reagent pre-treatments. The hydrogen yields were 31.5, 17.6, 4.7, 3 and 2.1 L H_2 L^{-1} for clay, zeolite, ozone, UV radiation and Fenton's reagent pre-treatment methods respectively. It should be also noted that the hydrogen yield produced by diluted OMW (4% v/v) without any pre-treatment stage was 16 L H_2 L^{-1}. According to an investigation by Jafari and Zilouei [133], applying nano-particle sized titanium dioxide under UV irradiation before acidic pre-treatment effectively enhanced hydrogen production yield of sugarcane bagasse. They observed that the

highest H_2 yield (101.5 ml H_2 g^{-1}VS) was achieved when 1 g TiO_2 L^{-1} was used for 120 min UV irradiation and 30 min acidic pre-treatment. The hydrogen production yield was enhanced by 127% when compared with the results obtained from only acidic hydrolysis process without Nano titanium dioxide.

Zhang and Zang [134] indicated in their recent studies that the biohydrogen production yield of brewers' spent grain could be enhanced up to 67.7% by applying calcined-red mud as pre-treatment method, with a maximum hydrogen yield of 198.7 mL g^{-1}VS at 10 g calcined-red mud. Xu et al. [135] reported that the steam explosion of acetic acid can be applied as a pre-treatment method to produce biohydrogen from corn straw. They found that the hydrogen yield of acetic acid steam exploded corn straw was 1.3 times higher than the yield obtained by the steam exploded corn straw.

Table 2. 1 Comparison of various pre-treatment methods used for enhancing the fermentative biohydrogen production

Pre-treatment	**Substrate**	**Process/ Reactor**	**Inoculum**	**H_2 yield**	**Reference**
Acid hydrolysis (HCl)	Dairy manure	DF/ Batch	Mixed cultures	18.1 mL g^{-1} TVS	[38]
Acid hydrolysis (HCl)	Soybean straw	DF/ Batch	Anaerobic mixed culture	47.7 mL g^{-1} substrate	[39]
Acid hydrolysis (H_2SO_4)	De-oiled rice bran	DF/ Batch	*Clostridium acetobutylicum* YM1	117.2 mL g^{-1} sugar	[40]
Acid hydrolysis (HCl)	Poplar leaves	DF/ Batch	Mixed cultures	33.5 mL g^{-1} dry poplar leaves	[128]
Acid hydrolysis (HCl)	Cornstalk wastes	DF/ Batch	Anaerobic sludge	126.2 mL g^{-1} Cornstalk	[136]
Acid hydrolysis (HCl)	Grass	DF/ Batch	Mixed cultures dominated by *Clostridium Pasteurianum*	72.2 mL g^{-1} dry grass	[74]

Table 2.1 (Continued)

Pre-treatment	Substrate	Process/ Reactor	Inoculum	H_2 yield	Reference
Acid hydrolysis (H_2SO_4)	Oil palm waste	PF/ Batch	*R. sphaeroides* S10	2019 mL L^{-1} medium	[31]
Acid hydrolysis	Beer lees	DF/ Batch	Anaerobic mixed bacteria	53 mL g^{-1} Beer lees	[137]
Acid hydrolysis (H_2SO_4)	Rice mill wastewater	DF/ Batch	*Enterobacter aerogenes* RM 08	1.6 mol mol^{-1} sugar	[129]
Alkaline hydrolysis (NaOH)	Dairy manure	DF/ Batch	Mixed cultures	14.2 mL g^{-1} TVS	[38]
Alkaline hydrolysis (ammonia solution)	Cotton stalk	DF/ Batch	Mixed culture	15.2 mL g^{-1} volatile solids	[138]
Alkaline hydrolysis (NaOH)	Cotton boll	DF/ Batch	Mixed culture	17 mL g^{-1} volatile solids	[138]
Alkaline hydrolysis (NaOH)	Grass	DF/ Batch	Mixed cultures dominated by *Clostridium Pasteurianum*	19.3 mL g^{-1} dry grass	[74]
Alkaline hydrolysis (NaOH)	Soybean straw	DF/ Batch	Anaerobic mixed culture	10.5 mL g^{-1} substrate	[39]
Alkaline hydrolysis (NaOH)	Sweet sorghum bagasse	DF/ Batch	*C. saccharo-lyticus*	73.6 mL mol^{-1} sugars	[139]
Hydrogen peroxide	Soybean straw	DF/ Batch	Anaerobic mixed culture	23 mL g^{-1} substrate	[39]

Table 2.1 (Continued)

Substrate	Substrate	Process/ Reactor	Inoculum	H_2 yield	Reference
Chemical oxidation with ozone	Olive mill wastewater (OMW)	PF/ Batch	*Rhodobacter sphaeroides O.U.001*	4.7 L L^{-1} OMW	[45]
Oxidation with Fenton's reagent	Olive mill wastewater (OMW)	PF/ Batch	*Rhodobacter sphaeroides O.U.001*	2.1 L L^{-1} OMW	[45]
Ethanol organosolv	Rice straw	DF/ Batch	*Enterobacter aerogenes*	19.7 mL g^{-1} straw	[126]
Enzymatic	Poplar leaves	DF/ Batch	Mixed cultures dominated by *ClostridiumPa steurianum*	44.9 mL g^{-1} dry poplar leaves	[128]
Fungal pre-treatment	Cornstalk	DF/ Batch	*T. thermosacchar -olyticum W16*	89.3 mL g^{-1} cornstalk	[127]
Enzymatic hydrolysis	Rice mill wastewater	DF/ Batch	*Enterobacter aerogenes* RM 08	1.78 mol mol^{-1} sugar	[129]
Ultra-sonication	Palm oil & pulp and paper mill effluents	PF/ Batch	*Rhodobacter sphaeroides*	14.5 mL m L^{-1} medium	[51]
Calcined-red mud	Brewers' spent grain	DF/ Batch	Anaerobic sludge	198.6 mL g^{-1} volatile solids	[134]
Adsorption with clay	Olive mill wastewater (OMW)	PF/ Batch	*Rhodobacter sphaeroides O.U.001*	31.5 L L^{-1} OMW	[44]
Photo degradation by UV radiation	Olive mill wastewater (OMW)	PF/ Batch	*Rhodobacter sphaeroides O.U.001*	3 L L^{-1} OMW	[45]

Table 2.1 (Continued)

Substrate	Substrate	Process/ Reactor	Inoculum	H_2 yield	Reference
Adsorption zeolite-4A	Olive mill wastewater (OMW)	PF/ Batch	*Rhodobacter sphaeroides O.U.001*	17.6 L L^{-1} OMW	[45]
Adsorption by Dry-Azolla and granular active carbon	Olive mill wastewater	PF/ Batch	*Rhodopseud-omonas palustris* 6A	682 mL L^{-1} culture	[47]
Thermal sterilization	Dairy waste	PF/ Batch	*Rhodobacter sphaeroides O.U. 001*	3.2 L L^{-1} medium	[43]
Infrared radiation	Dairy manure	DF/ Batch	Mixed cultures	13.9 mL g^{-1} TVS	[38]
Heat/130 ºC	Wheat straw	DF/ Batch	*C. saccharolytic-us*	3.8 mol mol^{-1} glucose	[140]
Steam-exploded	Corn straw	DF/ Batch	*Clostridium butyricum* AS1.209	68 mL g^{-1} corn straw	[141]
Acetic acid steam-exploded	Corn straw	DF/ Batch	*Ethanoligenes harbinense* B49	72 mL g^{-1} corn straw	[135]
Adsorption by Dry-Azolla and granular active carbon	Olive mill waste	PF/ Batch	*Rhodopseudo monas palustris* 42OL	1030 mL L^{-1} - broth	[46]
Ultrasonic pre-treatment	Distillery wastewater	DF/ Batch	Anaerobic sludge	11 mmol g^{-1} COD	[132]
Combination of ammonia and dilute sulfuric acid	Rice straw	DF/ Batch	*Thermotoga-neapolitana*	2.7 mmol g^{-1} straw	[125]
NanoTiO_2 under (UV) followed by acid hydrolysis	Sugarcane bagasse	DF/ Batch	Anaerobic sludge	101.5 mL g^{-1} volatile solids	[133]

Table 2.1 (Continued)

Substrate	Substrate	Process/ Reactor	Inoculum	H_2 yield	Reference
Combined acid and enzymatic hydrolysis	Rice mill wastewater	DF/ Batch	*Enterobacter aerogenes* RM 08	2 mol mol^{-1} sugar	[129]
Thermal-acidic hydrolysis	Microalgae biomass	DF/ Batch	Granular anaerobic sludge	45 mL/ g volatile solids	[124]

DF: dark fermentation; PF: photo fermentation; TVS: total volatile solid

2.7 Sustainable resources for fermentative hydrogen production

The feedstocks applied for the fermentative H_2 production processes can be classified into the following groups: pure carbohydrates, energy crops, solid wastes and wastewaters. Selection of the optimum feedstock for fermentative hydrogen production process requires some criteria such as its availability, sustainability, biodegradability, low cost and high organic content with less requirement to the pre-treatment stage [142]. In this section, various types of substrates are discussed to explain their operational feasibility as well as availability.

2.7.1 Pure carbohydrates

Sugars are carbohydrates that contain carbon, hydrogen and oxygen in their structure. Simple sugars such as glucose, sucrose, lactose and maltose are mostly applied as standard substrates for fermentation processes. Among these sugars, glucose is the simplest sugar that is present in all types of plant; while sucrose is a disaccharide sugar which can be produced from sugarcane and sugar beets [143]. In biohydrogen production researches, glucose [144-148] and sucrose [149-153] are the mostly used sugars for the production of fermentative H_2 production due to their biodegradable nature and availability. On the other hand, these sugars cannot be listed as economically viable feedstocks for biohydrogen production as they are expensive raw materials [2, 154].

2.7.2 Energy crops

Energy crops are plants that are grown with low cost and maintenance requirements to generate bioenergy. Exploitation of these crops as power resources has launched since 1980s. Remarkable criteria are required for these crops to serve as energy feedstocks,

which include high growth rates and minimal requirement for water and nutrients. Also, harvest of these crops can be achieved along sessions to gain double aims firestone is that the supply of these biomass can be constant and the second aim is that the cost of storage can be cancelled[76]. The crops that were used for the production of bioenergy can be classified into: sugar crops (sugarcane, sugar beet and sweet sorghum), cereal crops (barley, corn, oats and wheat), oil crops (sunflower, olive and palm) and cellulose and woody crops (switch grass, miscanthus and poplar) [155, 156]. Deferent crops, including sweet sorghum[139, 140], Ryegrass[157], sugar beet, barley and corn grains [158] and miscanthus[159]. However, some of these crops are also utilized as food sources for human and animals, which means that using them as feedstocks for bioenergy production can led to a rise in food prices [76].

2.7.3 Solid wastes and wastewater

The fast growth of industrial activities and rapid increase of population led to the generation of huge amounts of solid wastes and wastewater that have negative impacts on the environment. To eliminate these impacts, effective management of wastewater must be applied [2]. In this context, fermentative hydrogen production processes from solid wastes and wastewater can be a solution to a sustainable waste management by generating clean fuels [15].

2.7.3.1 Food waste

These wastes are derived from different sources such as food manufacturing processes, domestic or restaurant kitchens[160]. They can be considered as potential substrates for fermentative H_2 production due to their high carbohydrate content[161]. Annually, 33% of global food production is converted into food wastes[162]. The yield of H_2 produced by utilizing these types of waste varies between 0.68 mol H_2 mol^{-1} hexose to 2.7 mol H_2 mol mol^{-1} hexose, which is approximately equal to the yield achieved by pure carbohydrate [163].

2.7.3.2 Crop wastes

Preparation crop to consumption is one of the most important sources of lignocellulosic wastes. These wastes such as corn stover [164-168], wheat straw [169-172], barely straw [173-175] and rice straw [176-178] are lignocellulosic wastes that can serve as feedstocks to produce biohydrogen by fermentation. However, these are

complex materials that con not be directly used by microorganisms. Therefore, pre-treatment processes are required to convert cellulose and hemicellulose compounds into their simple forms that can be utilized by microorganisms[76].

2.7.3.3 Livestock wastes

Livestock wastes are basically protein rich wastes that are discharged in different forms. These forms can be as wastewater, slurry or solid wastes[123]. Compared to food wastes or lignocellulosic wastes, livestock wastes are not very suitable for fermentative hydrogen production due to their high protein and low carbohydrate content[76]. Therefore, employing these wastes as a supplement to high carbohydrate containing wastes can be a good alternative for their utilization. Since livestock wastewaters are alkaline materials with pH above 7.5, it might be a useful idea to add these wastes to low pH wastes -such as food wastes- in order to keep pH of the fermentation process within optimum ranges [179, 180].

2.7.3.4 Wastewater

Main types of wastewater that can be utilized as feedstock for dark fermentation processes include brewery wastewater [181], dairy wastewater [182], olive mill wastewater [55], cattle wastewater [183], cheese processing wastewater [184]. On the other hand, the major wastewater types for photo-fermentation processes include olive mill wastewater [45, 47], brewery wastewater [19], dairy wastewater [43] and palm oil mill effluent[185].

2.8 The influence of metal ions on fermentative hydrogen production

Supplementation of growth media with trace of metals is essential for the cell growth of many microorganisms and many enzyme and coenzymes involved metabolism of fermentation process [186]. Several researchers have investigated in the influence of several of supplementation of trace metal ions (Table 2.2) on bio hydrogen production. Iron is a significant metal for activity of hydrogenase and it also plays as an active site for the ferredoxin protein, which acts as electrons carrier to the hydrogenase enzymes. Therefore, several studies reported that reported that supplementation of fermentative hydrogen production media with iron ions helps to enhance hydrogen production [41, 187-189]. The previous studies indicated that the optimal iron concentration ranged from 25 to 200 mg L^{-1} (Table 2.2).

Nickel is a significant metal for many microorganisms that utilize hydrogenase enzyme to interconvert the hydrogen into protons and electrons ($H_2 \leftrightarrow 2H^+ + 2e^-$) [187, 189, 190]. This significance attributes to presence of nickel as active site of [Ni Fe]-hydrogenase. The optimum concentration of nickel lead to enhance the biohydrogen production by increasing [Ni Fe]-hydrogenase activity, while higher concentrations result in shift the metabolic pathway from biohydrogen production [191]. The optimal concentration of added nickel varied from 1 to 25 mg L^{-1} (Table 2.2).

Table 2. 2 Comparison among several metal supplemented studies.

Additive	**Process**	**Carbon source**	**Optimum concentration of additive**	**Maximum HY** [a]	**% E**	**Reference**
Fe^0 NPs	DF	Glucose	25 mg L^{-1}	338 mL g^{-1}	37%	[188]
Ni^0 NPs	DF	Glucose	2.5 mg L^{-1}	249 mL g^{-1}	0.9%	[188]
$FeSO_4$	DF	Glucose	10 mg L^{-1}	284 mL g^{-1}	15%	[188]
$NiSO_4$	DF	Glucose	25 mg L^{-1}	383 mL g^{-1}	55%	[188]
Fe^0 NPs plus Ni^0 NPs	DF	Starch	37 mg L^{-1} Fe^0 + 37 mg L^{-1} Ni^0 NPs	149.8 mL g^{-1}	200%	[187]
NiO NPs	DF	Dairy wastewater	10 mg L^{-1}	15.7 mmol g^{-1}	16%	[189]
NiO NPs	DF	Distillery wastewater	5 mg L^{-1}	6.73mmol g^{-1}	24%	[41]
Fe_2O_3 NPs	DF	Dairy wastewater	50 mg L^{-1}	16.75mmolg^{-1}	24%	[189]
Fe_2O_3 NPs	DF	Distillery wastewater	200 mg L^{-1}	7.85 mmol g^{-1}	44%	[41]
Fe_2O_3 NPs plus NiO NPs	DF	Dairy wastewater	50 mg Fe_2O_3 L^{-1} + 10 mg NiO L^{-1} NPs	17.2 mmol g^{-1}	27%	[189]
Fe_2O_3 NPs plus NiO NPs	DF	Distillery wastewater	200 mg Fe_2O_3 L^{-1} + 5 mg NiO L^{-1} NPs	8.83 mmol g^{-1}	62%	[41]

Table 2. 3 (Continued)

Additive	Process	Carbon source	Optimum concentration of additive	Maximum HY [a]	% E	Reference
$FeSO_4$	DF	Glucose	25 mg L^{-1}	1.45 mol mol^{-1}	52.6%	[192]
Fe NPs	DF	Glucose	100 mg L^{-1}	1.9 mol mol^{-1}	100 %	[192]
$FeSO_4$	DF	Glucose	25 mg L^{-1}	1.7 mol mol^{-1}	11%	[193]
Iron oxide NPs	DF	Glucose	125 mg L^{-1}	2.07 mol mol^{-1}	18%	[193]
$FeSO_4$	DF	Sucrose	25 mg L^{-1}	5.19 mol mol^{-1}	12%	[193]
Iron oxide NPs	DF	Sucrose	200 mg L^{-1}	5.44 mol mol^{-1}	19%	[193]
Nickel NPs	DF	Glucose	5.67 mg L^{-1}	2.54 mol mol^{-1}	22.7%	[194]
$Na_2MoO_4.H_2O$	PF	Olive mill wastewater	16.5 µM	0.31 mol L^{-1}	55%	[30]
$Fe(C_6H_5O_7)$	PF	Olive mill wastewater	0.1 mM	0.63 mol L^{-1}	215%	[30]
$NiCl_2$	PF	Acetate	4 µmol L^{-1}	2.87 mol mol^{-1}	28 %	[33]
$FeSO_4$.7H2O	PF	Acetate	80 µmol L^{-1}	2.78 mol mol^{-1}	16 %	[33]
$MgSO_4$	PF	Acetate		2.45 mol mol^{-1}	No impact	[33]
Ni^{+2}	DF	Glucose	1 mg L^{-1}	2.38 mol mol^{-1}	55 %	[190]
$FeSO_4$	DF	Starch	150 mg L^{-1}	279.9 ml g^{-1}	163%	[195]
$FeCl_2$	DF	Sucrose	80 mg L^{-1}	131.9mL g^{-1}	40%	[196]
Silver NPs	DF	Glucose	20 nmol L^{-1}	2.48 mol mol^{-1}	67.5%	[197]
EDTA	PF	Acetate	0.3 g L^{-1}	2.97 mol mol^{-1}	40%	[36]

Table 2. 4 (Continued)

Additive	Process	Carbon source	Optimum concentration of additive	Maximum HY [a]	% E [b]	Reference
Anhydrous calcium chloride	PF	Acetate	4 mmol L^{-1}	2.66 mol mol^{-1}	11.7 %	[198]
Ferric oxide NPs	DF	Glucose	200 mg L^{-1}	192.4 mL g^{-1}	17 %	[199]
Ferric oxide NPs	DF	Pre-treated cassava starch	200 mg L^{-1}	124.3 mL g^{-1}	63 %	[199]
$FeSO_4.7H2O$	DF	Glucose	0.2 g L^{-1}	1.5 mol mol^{-1}	17%	[200]
$MgCl_2.6H2O$	DF	Glucose	0.1 g L^{-1}	1.51 mol mol^{-1}	52%	[200]
L-cysteine	DF	Glucose	0 g L^{-1}	1.73 mol mol^{-1}	Less than control	[200]
$FeSO_4.7H2O$	DF	Starch flour	0.02 g L^{-1}	1.94 mol mol^{-1}	105%	[201]
$MgSO_4$	DF	Starch flour	0.02 g L^{-1}	0.73 mol mol^{-1} glucose	Less than control	[201]
L-cysteine	DF	Starch flour	0.3 g L^{-1}	1.53 mol mol^{-1}	60%	[201]
L-cysteine + $FeSO_4.7H_2O$	DF	Starch flour	0.3 g L^{-1}+ 0.02 g L^{-1}	1.22 mol mol^{-1}	28.5%	[201]
L-cysteine + $MgSO_4$	DF	Starch flour	0.3 g L^{-1}+ 0.02 g L^{-1}	1.15 mol mol^{-1}	21%	[201]
$NiCl_2$	PF	Succinate	4 µM	9.80 mmol L^{-1}	170 %	[202]
$MgSO_4.7H_2O$	PF	Succinate	5 mM	8.05 mmol L^{-1}	122%	[202]

% E: enhancement percentage; DF: dark fermentation; PF: photo fermentation

In photo-fermentative processes, metals additives are essential for hydrogen production [191]. Molybdenum and iron are the main active centre of nitrogenase and hydrogenase that play a critical role in photo fermentative process of hydrogen

production. It is well known that the nitrogenase enzyme plays a crucial role in photo-fermentative hydrogen production pathways by catalysing the reduction of protons to hydrogen [10, 203]. The active sites of Mo-nitrogenase are Fe protein and Mo-Fe protein [10]. The presence of iron and molybdenum in the structure of nitrogenase indicates that these metals have significant influences on enzyme activities that catalyse bio reactions [30]. It was reported that the nitrogenase activity reduced in the absence of iron and molybdenum [35, 204] ,while it was enhanced in the presence of aforementioned metals. It has conclusively been confirmed that the addition of iron and molybdenum metals led to enhance photofermentative hydrogen production [30-32]. However, the optimum concentrations for these metals showed significant variations in the literature due to using various substrates and microorganisms [31-34].

2.9 Scale-up and economic feasibility of biohydrogen of biohydrogen production Processes

Currently, it is well known that the biohydrogen is environmentally safe and its production processes are renewable [205, 206]. However, its production still requires moving up from a laboratory-scale to a commercial-scale. In fact, only several biohydrogen pilot-scale systems have been examined so far while its industrial production, storage and transportation have not fulfilled desired levels [207, 208]. a commercial biohydrogen production might be limited by the availability of a suitable feedstock and biohydrogen yield. Therefore, utilizing waste from different streams as a sole feedstock for biohydrogen production with efficient strategies simultaneously, can significantly enhance the feasibility of biohydrogen production in a commercial-scale [207, 209]. It is expected that efficient investments in infrastructure and technology will lead to a transform from a petroleum-based economy to a hydrogen-based economy by 2050 [210]. To achieve a developed commercial-scale technology, essential kinetics investigation in a batch reactor could be conducted first. After that, continuous reactors system should be carried out. Then, the efficiency of the biohydrogen production process should be examined in a pilot-scale. After that, a commercial-scale could be designed from the achieved data of the pilot system including kinetics and hydrodynamics [207, 211].

Cost can be considered significant factor for sustainability of fuel. the cost of biohydrogen production in commercial-scale could mainly depend on the cost of the

bioreactor and storage system [212]. The cost 1kg of biohydrogen is about $2.80 and daily 80 kg of biohydrogen per acre could be generated [213]. [210] explained the evolution of the biohydrogen sector in USA, Japan, China and India. They showed that China as the largest biohydrogen market followed by the USA, Japan and India by 2050. High investments promote the fast evolution of the biohydrogen sector in all the four economies. In addition, the study reported that investments in the evolution of biohydrogen technologies could lead to more advantages than investments in hydrogen infrastructure [210]. Therefore, biohydrogen can be commercialized successfully and economically feasible.

2.10 Summary

In this chapter, different methods of hydrogen production and their advantages and disadvantages have been firstly summarized. Thereafter, details about purple non-sulfur (PNS) bacteria have been explained with reference to different genera of PNS bacteria and the important enzymes involved photo-fermentation. Factors which can impact hydrogen production of photo-fermentation have been also explained. Optimization of influential factors for enhanced biohydrogen production with reference to the application of response surface methodology (RSM) such as Box-Behnken design (BBD) or central composite design (CCD) to optimize operational conditions and enhance biohydrogen production has been discussed. Pre-treatment methods to improve conversion efficiency of the wastes to hydrogen the feedstocks applied for the fermentative H_2 production processes have been summarized. This chapter finished by appraising the role of several of supplementation of trace metal ions on fermentative of hydrogen production.

(H_2) can be considered as a promising alternative fuel due to its high energy density and an environmental-friendly nature to overcome CO_2 emissions and global warming. Hydrogen can be produced by renewable or non-renewable resources. The current technologies of H_2 production are physical-chemical methods such as the steam reforming of natural gas, and biological methods such as dark/photo fermentation production. Compared with other methods, biological H_2 production processes are more close to achieve the requirements of a sustainable development due to allowing the utilization of various sustainable resources such as wastes and wastewaters at ambient temperature and atmospheric pressure by simply applying microorganisms. As one of the key factors for improving the efficiency of fermentative H_2 production,

pre-treatment processes have been largely investigated by various researchers. The transformation of organic waste and wastewater into a valuable biohydrogen fuel can be considered as an integrated solution to both waste and energy managements.

Under the presence of light, purple non sulfur (PNS) bacteria can produce hydrogen as a metabolic by-product using the light energy and the energy from the oxidation of the substrate in order to reduce H^+ ions into hydrogen gas. In this process, nitrogenase enzyme catalyses the reaction of hydrogen production.

C/N ratio and pH of the hydrogen production medium can be a significant factor affecting biohydrogen production yield and the growth of microorganisms. Inoculum age, temperature of bioreactor, light intensity and light source also play a significant role in the hydrogen production processes. In addition, Supplementation of growth media with trace of metals is essential for the cell growth of many microorganisms and many enzyme and coenzymes involved metabolism of fermentation process.

Chapter 3
Experimental Methods

Chapter 3 Experimental Methods

3.1 Introduction

This chapter describes the overall research methodology applied to achieve the thesis objectives mentioned in Chapter 1. The experimental setup and analytical techniques are described in detail in this chapter. In this study, medium preparation and rehydration the dried microorganisms were conducted. The batch experiments of biohydrogen production of *Rhodobacter sphaeroides* 158 DSM were conducted in clear glass photo-bioreactor. Several strategies such as optimization of influencing variables for biohydrogen production, feedstock pre-treatment, and metals supplementation have been applied to enhance yields of photo fermentation processes. Box-Behnken design (BBD), regression analysis, 3D response surface graphs and 2D contour plots were performed by JMP statistical discovery ^{TM} software.

3.2 The growth medium

The standard medium (Table 3.1) used for inoculation and cultivation is Biebl and Pfennig medium with some modifications. DL_malic acid (1 g L^{-1}) was used as the carbon source, while L_ glutamic acid (1.5 g L^{-1}) was the nitrogen source. Chemicals used for preparation the medium were analytical high grad chemicals and purchased from (Sigma-Aldrich Pty Ltd, Australia).

Table 3. 1 The components of growth medium

Component	Amount in 1 L	
KH_2PO_4	0.50	g
$CaCl_2$. 2 H_2O	0.15	g
$MgSO_4$. 7 H_2O	2.00	g
NH4Cl	0.34	g
NaCl	20.00	g
Yeast extract	0.40	g
Ferric citrate (0.1% w/v)	5.00	mL
Trace element solution (SL-7)*	1.00	mL

* See Table 3.2

Table 3. 2 Trace element solution

Component	Amount in 1 L	
HCl (25%)	1.0	mL
$ZnCl_2$	70.0	mg
$MnCl_2$. 4 H_2O	100.0	mg
H_3BO_3	60.0	mg
$CoCl_2$. 6 H_2O	200.0	mg
$CuCl_2$. 2 H_2O	20.0	mg
$NiCl_2$. 6 H_2O	20.0	mg
Na_2MoO_4. 2 H_2O	40.0	mg

The used glassware items such as bakers, flasks, cylinders, funnels, and burets were from Chemical Engineering laboratories, Curtin University, Australia. The initial pH was adjusted to 7.2 with 1 N NaOH. The medium was boiled under a stream of argon gas for few minutes and then 10 mL transferred into 20 mL pre-flushed with argon gas Hungate tubes with a screw cap and a butyl rubber septum (Figure 3.1). Each tube was bubbled with argon gas for about 1 min and closed immediately with a rubber septum and screw cap. Autoclave (Floor Autoclave, LABEC, Australia) was used to autoclave the medium under the conditions of 120 ℃ for 15 minutes.

Figure 3. 1 Hungate tubes with a screw cap and a butyl rubber septum

3.3 The applied bacteria

The strain *Rhodobacter sphaeroides* DSM 158 was supplied by German Collection of Microorganism and Cell Cultures (DSMZ). The freeze-dried of anaerobic microorganisms supplied by DSMZ are exclusively in double-vial preparations, heat-sealed under vacuum. Double-vial preparations have the feature that a contamination of the atmosphere generated by sudden release of the vacuum in single-vial preparations is effectively prevented (Figure 3.2).

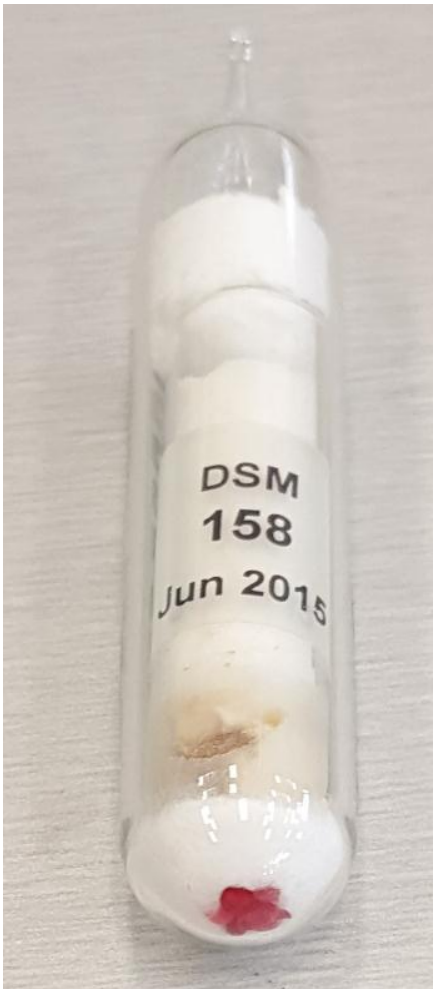

Figure 3. 2 The freeze-dried strain *Rhodobacter sphaeroides* DSM 158

To open the ampoules and rehydrate the dried microorganisms, the tip of the ampoule was heated in a flame. Then, three drops of water were placed onto the hot tip to crack the glass. The glass tip was carefully struck off using forceps. The insulation material was removed and the inner vial was taken out. Under sterile conditions and flame the top of the inner vial, the cotton plug was lifted and 0.5 mL of growth medium was added. The plug was replaced and the pellet was allowed to rehydrate for up to 30 minutes. The cell suspension was transferred using 5 mL sterile syringe with

hypodermic needle which was made anoxic by flushing with argon gas to Hungate tube containing the appropriate cultivation medium (Figure 3.3).

Figure 3. 3 Hungate tube containing growth medium of *Rhodobacter sphaeroides* DSM 158

The above steps were conducted in Laminar Flow cabinet (HWS Series, CLYDE-APAC, Australia) which was sterilized with 70% ethanol and UV-light. The liquid culture was incubated under continuous illumination (20 W m^{-2}) in an incubator at 30℃ (Temperature Cycling Chamber, LABEC, Australia) (Figure 3.4).

Figure 3. 4 Incubator refrigerator

3.4 Hydrogen production media

Hydrogen production medium was established by the addition of DL_ malic acid (2.68 g L^{-1}) and certain concentrations of L_glutamic acid to the aforementioned medium (Table 3.1). The initial pH was adjusted to certain values with 1 N NaOH. After that, the medium was boiled under a stream of argon gas for few minutes and then specific volume transferred into clear glass photo-bioreactor (Figure 3.5). Each bioreactor was bubbled with argon gas for about 5 min. The autoclave (Floor Autoclave, LABEC, Australia) was applied to autoclave the medium under the conditions of 120 ℃ for 15 minutes. 10% of pre-activated culture was transferred to the photo-bioreactor using sterile syringe with hypodermic needle which was made anoxic by flushing with argon gas. The inoculation was conducted in Laminar Flow cabinet.

Figure 3. 5 Photo-bioreactor applied in this study

3.5 Wastewater characterization

Brewery effluent (BE) was collected from a local brewery production plant in Perth, Western Australia, Australia, while, Restaurant effluent (RE) was collected from a Chinese Restaurant in Perth, Western Australia. Both BE and RE samples were filtered

using 0.2 μm nylon microfilters and then autoclaved at 120 °C for 15 min by floor autoclave (LABEC, Australia) to ensure sterile conditions. Sterilized wastewater effluent samples were stored in the fridge. Table 3.3 shows the characteristics of the BE and RE used in this study.

Table 3. 3 Characteristics of the BE and RE used in this study

Parameter	Unit	Brewery effluent (BE)	Restaurant effluent (RE)
pH	-	7.2	7.6
Soluble COD (SCOD)	mg L^{-1}	2130	3100
Total COD (TCOD)	mg L^{-1}	3710	5950
C/N	-	7.4	24.5
TOC	mg L^{-1}	350	1621
N-NH_4^+	mg L^{-1}	36.4	15.2
Fe	mg L^{-1}	1.1	2.3
Mo	mg L^{-1}	Undetectable	Undetectable
Ca	mg L^{-1}	15.4	41.2
Mg	mg L^{-1}	66.7	59.5

3.6 Experimental setup and procedure for biohydrogen production

The batch experiments of hydrogen production were conducted in 120 mL clear glass photo-bioreactor at 30 ∓ 2 ℃, which was stirred at 300 rpm with a magnetic stirrer (RCT basic, IKA, Germany). The illumination was supplied by two 50 W halogen lamp. The temperature of the bioreactor was frequently monitored to maintain a constant value around 30 ∓ 2 ℃ using two external thermostatic air fans. The produced gas mixture was determined volumetrically by water displacement at atmospheric

pressure using 200 mL graduated gas cylinder filled with water and partially submerged in the 1 L beaker with enough water (Figure 3.6).

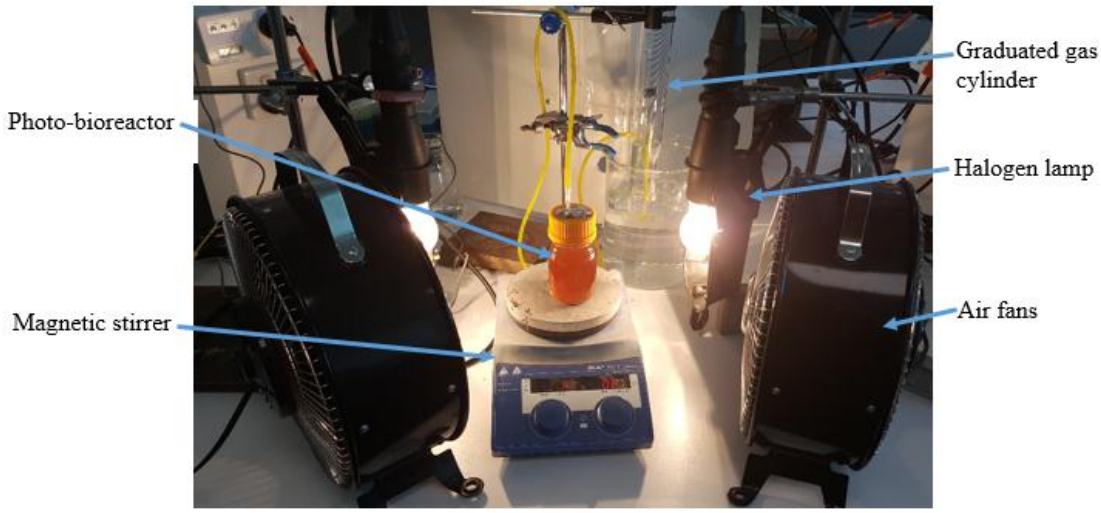

Figure 3. 6 Experimental set up of hydrogen production experiments

3.7 pre-treatment stages

In this work different pre-treatment stages have been applied to qualify the effluents for photofermentative hydrogen production;

3.7.1 Pre-treatment stage using banana peels waste

Collected banana peels were cut into small pieces (< 5 mm), washed with distilled water to remove external dirt. Wetted banana peels were kept under air for removing the free water from their surface and dried in an oven for 24 h at 90°C. Dried samples were then finely grounded using a mortar and pestle. The ground banana peels (Figure 3.7) were sieved and graded into small particle sizes of less than 250 µm. The samples were then stored in an air tied bottle prior to the experiments. Wastewater samples were treated with different concentrations of banana peels particles. Banana peels were thoroughly mixed at an agitation speed of 250 rpm for various time intervals. This process was carried out at a pH value of around 7.2. At the end, the mixture was rested for 2 h to settle down. After settlement, the above liquid was pre-filtered by Whatman^{TM} filter paper (0.45 µ) to separate liquid from the solids.

Figure 3. 7 The ground banana peels

3.7.2 Pre-treatment stage using ultra-sonication

The treated combined effluents of 70% RE and 30% BE samples resulted from the first stage (banana peels pre-treatment) were subjected to different amplitudes (A) of 30, 60 and 90 and ultra-sonication duration (T) of 15, 25, 35 and 45 min. Ultra-sonication was conducted using Q sonica Q700 Sonicator (QSonica LLC., Connecticut, USA) with transducer probe of 12.5 mm dimeter.

3.8 Instrument and analytical techniques

3.8.1 Optical density measurement

The biomass of *Rhodobacter sphaeroides* DSM 158 was measured using the optical density which was determined by UV-Vis spectrophotometry (Jasco V-670, JASCO Corporation, Japan) at 680 nm (Figure 3.8). To ensure the OD_{680} measurements were less than 1. Samples of culture were diluted with distilled water prior to OD_{680} measurements. The final value of each sample was achieved from three independent measurements. To achieve the correlation between the optical density (OD) and dry weight biomass, 10 mL of the culture was centrifuged at 5,000 g. Then supernatant was removed and the sample was dried at 90 ℃ for 24 hours in an oven. After that, reweighed to measure the dry weight of the dried biomass. Thus, the following linear equation (3.1) was achieved;

$$X\ (g\ L^{-1}) = 0.49 \times OD_{680} + 0.05 \quad , \quad (R^2=0.98) \qquad (3.1)$$

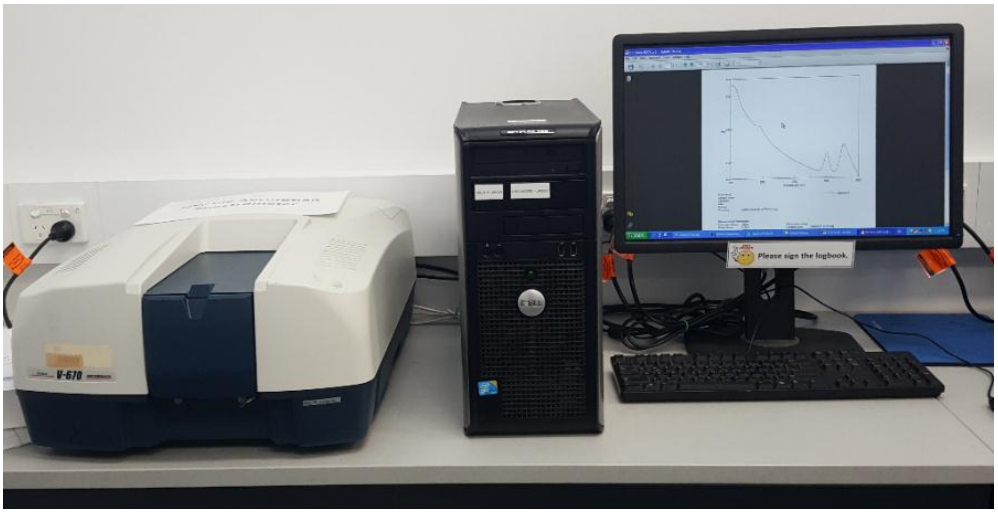

Figure 3. 8 UV-Vis spectrophotometer

3.8.2 Chemical oxygen demand (COD) analysis

DR/890 HACH spectrophotometer was used to measure COD applying reactor digestion method (8000) [214]. In this method, samples are heated with strong oxidizing agent of potassium dichromate for 2 h. Oxidisable organic materials react, reducing the dichromate ion ($Cr_2O_7^{2-}$) to green chromic ion (Cr^{3+}). Then, the amount of generated Cr^{3+} is measured. The COD reagent also contains silver and mercury ions. Silver is a catalyst, and mercury is used to complex the chloride interference (Figure 3.9). The obtained measurements (mg L^{-1}) are defined as the mg of O_2 consumed per litter of sample under conditions of this method.

Figure 3. 9 DR/2400 HACH Spectrophotometer

3.8.3 Total nitrogen (TN) analysis

Total Nitrogen (*TN*) concentration in the hydrogen production medium was measured calorimetrically by HACH test kits (DR/890 Colorimeter, HACH, USA) (Figure 3.9). In this procedure, an alkaline persulfate digestion transforms all form of nitrogen to nitrate. After the digestion, sodium metabisulfite is added to eliminate halogen oxide interference. Under strongly acidic conditions, nitrate then reacts with chromotropic acid to from a yellow complex with an absorbance peak near 420 nm. This measurement procedure was adopted from standard persulfate digestion method (Hach DR/890 colorimeter procedure manual, 2013, method 10072).

3.8.4 Total organic carbon (TOC) analysis

A Shimadzu TOC analyser (TOC-VCPH, Shimadzu, Japan) (Figure 3.10) was applied to determine the total organic carbon (TOC) in the samples. Prior to sample injection into the TOC analyser, 5 mL of the sample was pre-filtered by Whatman^{TM} filter paper (0.45 µ) to separate the solid materials.

Figure 3. 10 Total organic carbon (TOC) analyser

3.8.5 Metals analysis

Plasma optical emission spectrometry (ICP – OES) (PerkinElmer, USA) (Figure 3.11) was used to measure the initial concentrations of Fe, Mo, Ca and Mg. The auxiliary gas (Ar) flow rate, carrier gas (Ar) flow rate and plasma gas flow rate were 1, 1, and 12 L min^{-1}, respectively. 5 mL of the sample was pre-filtered by Whatman^{TM} filter paper (0.45 µ) to separate the solid materials and then the sample was acidified with nitric acid (1% HNO_3) to ensure that its elemental components remain in solution.

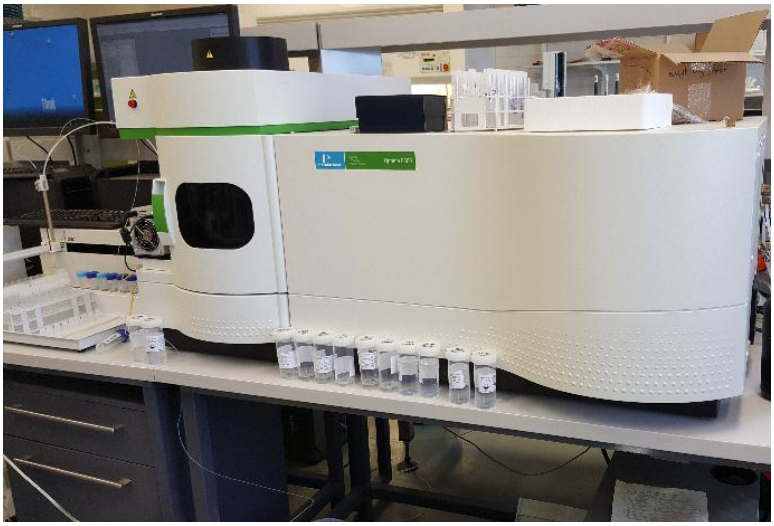

Figure 3. 11 Plasma optical emission spectrometry (ICP – OES)

3.8.6 Morphology banana peels

A Scanning Electron Microscope (SEM) analyses of banana peels samples were performed using Tescan Mira3 FESEM with Oxford Instruments X-Max SDD X-ray detector to investigate the banana peels morphology before and after pre-treatment. A small portion of banana peels was placed on a 10 mm diameter of aluminium stub. Each sample was laid on a double side carbon adhesive placed on the stub and it was coated with platinum (Pt). Finally, the sample was analysed by SEM machine.

3.8.7 Functional groups of the banana peels

The functional groups of the banana peels particles were determined by Fourier-transformed infrared (FTIR) spectroscopy (Perkin Elmer spectrum 100) (Figure 3.12). A small amount of banana peels particles was place on the hydraulic press which was cleaned by ethanol to identify the presence of functional groups.

Figure 3. 12 Fourier transform infrared (FTIR) spectroscopy

3.8.8 Gas composition analysis (GC)

A gas-tight syringe (SGE, Supelco, Sigma-Aldrich) was employed to collect the gas samples and the compositions of these samples was analysed by a gas chromatograph (Agilent 7890B GC) (Figure 3.13) equipped with thermal conductivity detector (TCD). Supelco carboxen 1010 PLOT capillary column, (30 m) L, (0.53 mm) I.D was used as a column. As a carrier gas, argon was applied with the flow rate of 15 mL min^{-1}. The temperature of oven, detector, and injector were 190 ^{o}C, 210 ^{o}C, and 160 ^{o}C, respectively.

Figure 3. 13 Gas chromatograph (Agilent 7890B GC)

3.8.9 pH of medium

The initial pH was adjusted to certain values with 1 N NaOH. pH meter (Model WP-Cond, TDS, Sal, pH, ORP, Temp. Meter) was used to measure the pH of culture medium. Prior to measurements, pH meter was calibrated with standard solutions of 4.0, 7.0 and 10 pH.

3.8.10 Light intensity

Continuous illumination at light intensity between (35 – 185 Wm^{-2}) was supplied by two 50 W halogen lamps. The distance from the surface of bioreactor to lamps can be adjustable, so different values of light intensity emitted to the bioreactor can be achieved. A light meter (LI-250A, LI-COR, US& Canada) equipped with a quantum Sensor (LI-192SA, LI-Core Inc.) was used to measure the light intensity (Figure 3.14). This sensor has been designed to measure photosynthetically active radiation (PAR,

400-700 nm) in aquatic environments. It is made of corrosion resistant metal with an acrylic diffuser (3.18 cm diameter × 4.62 cm height) and has a flat, high-stability, silicon photovoltaic detector. A light meter was applied which provided a direct digital readout. The digital LCD was updated every 0.5 seconds in instantaneous mode. Sensor output was collected and displayed as a 15 second average which represents approximately 60 readings. A typical accuracy of 0.4 % at 25 °C is specified for this device. Values of light intensity can be displayed in many units, including W m^{-2}, lux or μmol m^{-2} s^{-1}.In this study, light intensity was measured in W m^{-2}.

Figure 3. 14 Light meter

3.9 Experimental design and regression analysis for optimization

In Box Behnken Design (BBD) experimental design, the matrix design depends on the number of factors. The number of tests (N) required to apply BBD is calculated as $N=2f\,(f-1)+C_{p,}$ (where *f is* number of factors and $C_{p,}$ is the number of central points) [114]. After conducting tests, the impact of factors can be evaluated via a statistical model. The regression analysis and graphs were performed by JMP statistical discovery ™ software from SAS (version 13.1.0). The statistical model was then applied to identify the interaction among the independent variables. Response surface methodology (RSM) with BBD was applied to optimize hydrogen productivity and investigate interactive impacts of three independent variables (pH, C/N, and light intensity). To achieve these aims, 15 experiments were conducted. The hydrogen

production potential (HPP), hydrogen production rate (HPR) and light conversion efficiency (η) were selected as the response variables.

The hydrogen production potential (HPP) and hydrogen production rate (HPR) were calculated using Equations (3.2) and (3.3) respectively;

$$HPP(mL\ L^{-1}) = \frac{V_{H_2}}{V_{culture}} \tag{3.2}$$

$$HPR(mL\ L^{-1}h^{-1}) = \frac{V_{H_2}}{V_{\ culture}\ t} \tag{3.3}$$

Where V_{H_2} is total volume of the produced hydrogen (mL), $V_{culture}$ is the volume of the culture (L), *t* is time of hydrogen production (h). While the light conversion efficiency η was calculated according to the following equation (3.4) [111].

$$\eta(\%) = \frac{33.61\ \rho_{\ H_2} V_{H_2}}{I\ A\ \Delta t} \times 100 \tag{3.4}$$

Where ρ_{H_2} is the density of produced hydrogen gas (g L^{-1}), V_{H_2} is the volume of the produced hydrogen gas (L), *I* is the light intensity (W m^{-2}), *A* is the irradiated area (m^2), and Δt is the duration of hydrogen production (h).

Chapter 4

Synergistic effects and optimization of photo-fermentative hydrogen production of *Rhodobacter sphaeroides* DSM 158

Chapter 4 Synergistic effects and optimization of photo-fermentative hydrogen production of *Rhodobacter sphaeroides* DSM 158

4.1 Introduction

The environment has been continuously impacted using fossil fuels as the main energy sources [215]. Currently, fossil fuels supply about 80% of the world's energy demands. However, reserves of fossil fuels are non-renewable and can face depletion in the future [2]. Hence, development of clean and sustainable energy resources is an essential solution to mitigate environmental problems and depletion of the fossil fuel resources [216]. Hydrogen gas can be considered as a promising and environmentally friendly fuel due to its high specific heat content (122 kJ g^{-1}) [217, 218], and possibility of being generated from either sustainable or non-sustainable resources [7]. Among various alternative methods of hydrogen generation, the biological conversion of organic biomass into hydrogen fuel by light-dependent (photo-fermentation) or light-independent (dark fermentation) are promising methods, as these methods have the ability to produce hydrogen from renewable substrates at moderate operating conditions (ideal temperature and pH of photo-fermentation processes range between 30 - 40 ℃ and 6.8 - 9.0, respectively) [23, 96]. In photo-fermentative hydrogen production, purple non-sulfur (PNS) bacteria can generate hydrogen by applying the light as an energy source and organic compounds as the electron donors to catalyze nitrogen fixation via nitrogenase enzymes [219]. Generally, PNS bacteria can utilize a wide wavelength of light (520-860 nm) [220].

Several parameters influencing photo-fermentative hydrogen production include pH, C/N ratio and light intensity. Essentially, initial pH can be considered as a key factor in photo-fermentation processes since it has a crucial impact on the metabolic pathways of the microorganisms [101]. Several studies have examined the impact of pH on photo-fermentative hydrogen production. The results indicated that suitable pH values depend on the type of strains used for the process [63, 102, 103]**.** For instance, the reported optimum pH varied among species, pH 7.2 for *R. sphaeroides* VM81 [102], pH 7.0–7.5 for *R. sphaeroides* O.U.001 [103] and pH 7.4–7.6 for *R. sphaeroides* KD131 [63]. Kim et al. [104] also reported that the increase in pH of the medium above

the optimum-range (7.4 - 7.6) led to dissipate the membrane of the cells which would cause cell clustering. These clustered cells might reduce the light conversion efficiency of microorganisms into biohydrogen.

In addition, several studies have shown that the C/N ratio of the hydrogen production medium can be a significant factor affecting biohydrogen production yield and the growth of microorganisms [23, 24]. A high bacterial growth with low hydrogen yield was noticed at a low C/N ratio. In the same way, a high C/N ratio also led to improve a microbial growth and reduce the hydrogen production yield, which makes it necessary to find the optimum C/N ratio [25]. Also, the source of nitrogen can strongly influence the photofermentative hydrogen production yield, due to the fact that NH^{+}_{4} is known to inhibit the activity of nitrogenase enzyme [90]. The hydrogen production from purple non sulfur (PNS) bacterium has been impacted by the combinations of carbon and nitrogen sources. For example, when proline, alanine or tyrosine were used as the nitrogen source, *R. sphaeroides* A-10 was only able to generate biohydrogen using the medium with succinate as a carbon source [91]. Glutamic acid has been one of the widely used nitrogen source for the production of hydrogen by photo-fermentation process [26, 92]. The concentration of the nitrogen source in the medium can also affect the amount of hydrogen production by *R. sphaeroides*. It was reported that biohydrogen generation was higher for the nitrogen-limited conditions (i.e., 2 g yeast extract L^{-1}) than those applied under nitrogen-excess conditions (i.e., 5 g yeast extract L^{-1}) [93]. As observed for the hydrogen production, generation of by-products such as poly-β-hydroxybutyrate (PHB) was also reported to be enhanced under nitrogen-limited conditions [17, 94] As carbon and energy source, malate is favoured source for biohydrogen production by PNSB [95]. A paper by Han et al. [96] investigated the impact of the different carbon sources -such as malate and succinate- on the photo fermentative hydrogen production by *Rhodobacter sphaeroides* RV. The maximum bio hydrogen was 1340 mmol H_2 $mole^{-1}$-substrate achieved by 1 g L^{-1} malate, while it was 300 mmol H_2 $mole^{-1}$-substrate achieved by 3 g L^{-1} succinate.

Furthermore, various studies reported that the light intensity of the illumination source also plays a significant role in the hydrogen production processes [26-28].The optimum intensity of light depends on the type of strain and also on the light source applied [23, 29]. Therefore, optimization of the above-mentioned factors is essential to improve the hydrogen production yield.

In conventional methods, experiments are commonly performed by varying a single factor while setting all other factors at fixed values. This would result in missing the interactions among all factors. Thus, response surface methodology (RSM) has been employed to design experiments in order to investigate the interactive influences of variables and determine optimum experimental conditions [109]. Recent studies have applied different statistical techniques to determine the optimum conditions of biohydrogen production [109, 110, 221, 222]. Chong et al. [107] investigated the experimental optimum conditions for biohydrogen production from palm oil effluent by *Clostridium butyricum* EB6. They employed a central composite design (CCD) to optimize the important variables for this process including pH, temperature and chemical oxygen demand (COD). RSM with CCD was applied to investigate the impact of initial pH, initial glucose concentration and nickel nanoparticles concentration on the production of hydrogen using microbiota [194]. However, to the best of the authors' knowledge, identification of the synergistic influences of significant factors and determination of the optimum operating conditions for photo-fermentative hydrogen production by *Rhodobacter sphaeroides* DSM 158 still needs more research. In this study, three factors-three levels of a Box-Behnken design is applied to investigate the impacts of the significant factors including pH, C/N and light intensity on photo-fermentative hydrogen production by *Rhodobacter sphaeroides* DSM 158.

4.2 Material and methods

4.2.1 Photosynthetic bacteria and experimental procedure

Rhodobacter sphaeroides DSM 158 was anaerobically activated and grown in modified Biebl and Pfennig medium [223] under sterile conditions and light irradiated by the procedure described previously in sections 3.2 and 3.3. Hydrogen production medium was established by the addition of DL_ malic acid (2.68 g L^{-1}) and varied concentrations of L_glutamic acid (0.39, 0.59, and 1.17 g L^{-1}) to the aforementioned medium. The batch experiments of hydrogen production were accomplished in 120 mL clear glass photo-bioreactor at 30 ∓ 2 ℃ . The produced gas mixture was collected by water displacement method and gas compositions were analysed by a gas chromatograph (Agilent 7890B GC). The preparation hydrogen production medium,

experimental setup, hydrogen production procedure, gas compositions analyses and biomass analyses were detailed in Chapter 3.

4.2.2 Experimental design and regression analysis

RSM with BBD was applied to optimize hydrogen production process via *Rhodobacter sphaeroides* DSM 158 and investigate interactive impacts of three independent variables. Since pH(X_1), C/N(X_2), and light intensity(X_3) can significantly influence photo-fermentative hydrogen production process, they were selected as the critical factors. To achieve these aims, 15 experiments were accomplished by varying pH from 6.5 to 8.0 with a central point of 7.25, C/N from 15 to 35 with a central point of 25, and light intensity 35 to 185 W m^{-2} with a central point 110 W m^{-2}. The ranges of these factors were chosen based on a literature search [62, 92, 224, 225]. The hydrogen production potential (*HPP*), hydrogen production rate *(HPR*) and light conversion efficiency (η) were selected as the response variables. The actual values of independent factors (X_i) were coded as (x_i) by applying the following equation (4.1) [111];

$$x_1 = \frac{X_i - X_0}{\Delta X_i} \tag{4.1}$$

Where x_i is the coded value of the actual value for factor X_i, X_o is the actual value of the factor at the centre, and ΔX_i is the step change value.

The predicted responses were calculated according to a second-order polynomial equation, Equation (4.2);

$$Y = \beta_o + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} X_i X_j + \varepsilon \tag{4.2}$$

Where Y is the predicted response variable, β_o is the constant coefficient (i.e., intercept), i and j are the index number of patterns, k is the number of factors, and β_j, β_{jj}, β_{ij} are the estimated coefficients from regression of linear, quadratic, and interaction effects, respectively. The actual and coded levels of independent variables in box-Behnken design matrix with experimental and predicted responses were shown in Table 4.1.

Table 4. 1 Actual and coded levels of factors in the Box-Behnken design matrix with experimental and predicted results.

Run no.	Actual values [a]			Coded values			Response values [b]					
	X_1	X_2	X_3	x_1	x_2	x_3	HPP_e	HPP_p	HPR_e	HPR_p	η_e	η_p
1	6.5	15	110	-1	-1	0	170.50	166.03	6.09	6.74	0.06	0.06
2	6.5	35	110	-1	+1	0	457.25	426.41	15.24	14.64	0.17	0.18
3	8	15	110	+1	-1	0	178.25	209.09	5.94	6.54	0.07	0.06
4	8	35	110	+1	+1	0	640.00	644.47	25.60	24.95	0.24	0.24
5	7.25	15	35	0	-1	-1	162.75	79.66	5.81	2.01	0.19	0.18
6	7.25	15	185	0	-1	+1	200.00	256.72	9.09	11.65	0.04	0.05
7	7.25	35	35	0	+1	-1	317.75	261.03	13.24	10.68	0.37	0.36
8	7.25	35	185	0	+1	+1	688.00	771.09	25.48	29.28	0.15	0.16
9	6.5	25	35	-1	0	-1	286.75	374.31	11.95	15.11	0.33	0.33
10	8	25	35	+1	0	-1	258.50	310.75	9.57	12.77	0.30	0.31
11	6.5	25	185	-1	0	+1	576.00	523.75	25.04	21.84	0.13	0.12
12	8	25	185	+1	0	+1	936.00	848.44	37.44	34.28	0.20	0.20
13	7.25	25	110	0	0	0	928.00	932.00	42.18	41.13	0.34	0.34
14	7.25	25	110	0	0	0	932.00	932.00	40.52	41.13	0.34	0.34
15	7.25	25	110	0	0	0	936.00	932.00	40.70	41.13	0.34	0.34

[a] X_1, X_2, X_3 are actual values of pH, C/N, and light intensity (W m^{-2}), respectively

[b] HPP_e, HPR_e, and η_e respectively shows experimental values (means of three replicates) hydrogen production potential (mL L^{-1}), hydrogen production rate (mL $L^{-1}h^{-1}$), and light conversion efficiency, %. While, HPP_p, HPR_p, and η_p are predicted values of hydrogen production potential (mL L^{-1}), hydrogen production rate (mL L^{-1} h^{-1}), and light conversion efficiency, % respectively.

All runs were conducted in triplicate to reduce the impact of temporal related errors and the coefficients of Equation (4.2) were generated. In this study, BBD experimental design, regression analysis and 3D response surface graphs and 2D contour plots were performed by JMP statistical discovery ™ software from SAS (version 13.1.0).

4.3 Result and discussion

4.3.1 Multiple regression analyses

Multiple regression analysis for determining the relationships between the three response parameters of hydrogen production potential (HPP), hydrogen production

rate (HPR) and light conversion efficiency (η) with respect to pH, C/N, and light intensity generated second-order polynomial equations based on the BBD matrix of actual data (Table 4.1):

$$HPP = 932 + 65.281\ x_1 + 173.937\ x_2 + 171.7812\ x_3$$
$$-199.156\ x_1^2 \ -371.343\ x_2^2 \ -218.531\ x_3^2$$
$$+ 43.75\ x_1x_2 + 97.062\ x_1x_3 + 83.25\ x_2x_3 \quad (4.3)$$

$$HPR = 41.133 + 2.528\ x_1 + 6.578\ x_2 + 7.06\ x_3$$
$$-10.160\ x_1^2 \ -17.755\ x_2^2 \ -9.972\ x_3^2$$
$$+2.627\ x_1x_2 + 3.695\ x_1x_3 + 2.24\ x_2x_3 \quad (4.4)$$

$$\eta = 0.34 + 0.015\ x_1 + 0.071\ x_2 - 0.083\ x_3$$
$$-0.076\ x_1^2 \ -0.128\ x_2^2 \ -0.023\ x_3^2$$
$$+ 0.015\ x_1x_2 \ +0.025\ x_1x_3 - 0.017\ x_2x_3 \quad (4.5)$$

Where x_1, x_2 and x_3 are coded values (calculated based on equation (4.1)) of initial values of pH, C/N, and light intensity, respectively. The determination coefficients (R^2) of the regression models of HPP, HPR, and η were 0.97, 0.97, and 1, respectively, indicating that the regression models (Equations (4.3), (4.4) and (4.5)) can adequately explain the relationship between independent variables and responses. The predicted values from Equations (4.3), (4.4) and (4.5) are in acceptable agreement with those determined experimentally (Figure 4.1).

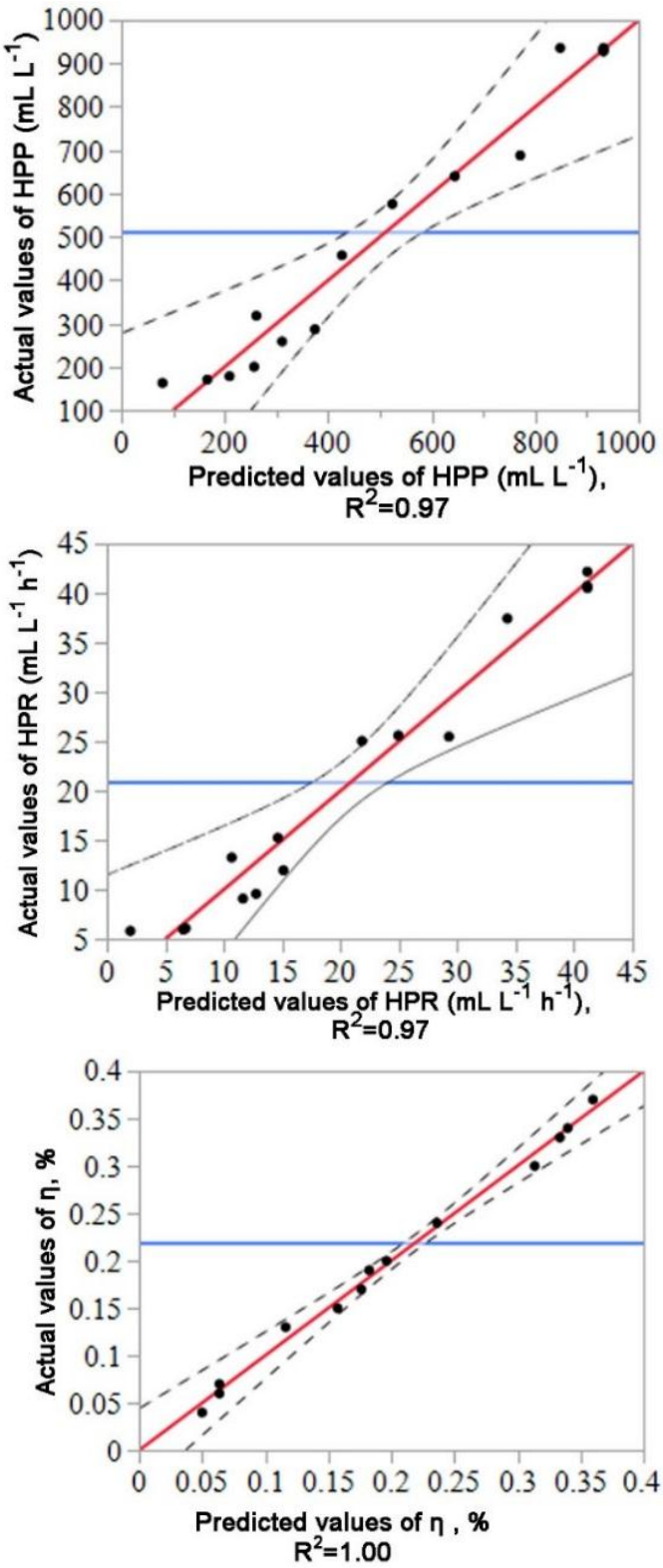

Figure 4. 1 Comparison between experimental and predicted values of *HPP*, *HPR* and η (.) experimental values, (---) confidence bands, (-) fit line

4.3.2. Analysis of variance (ANOVA)

ANOVA of the second-order polynomial models and significance of linear, interactive, and quadratic terms of models are shown in Table 4.2. The significance of

each term of fitting models was checked by *P*-value. Statistically, *P*-value less than 0.05 indicate that the term is significant. It can be noted from Table 4.2 that the linear term coefficients (x_2 and x_3) and quadratic term coefficients (x_1^2 , x_2^2 and x_3^2) significantly influenced the HPP, HPR, and η. Also, the linear term coefficient (x_1) and interactive term coefficients ($x_1 . x_3$ and $x_2 . x_3$) had significant impacts on η. In other words, *HPP*, *HPR*, and η were significantly impacted by linear terms of C/N and light intensity and the quadratic terms of pH, C/N and light intensity with *P*-value <0.05. The linear term of pH and interactive terms of pH, C/N and *I* were also influencing η with *P*-value <0.05. The other term coefficients did not significantly influence the HPP, HPR, and η. Therefore, all the three factors (*I*, C/N, pH) were found to have significant impacts on HPP, HPR, and η. However, for HPP and HPR, the most important factors were C/N and *I*, followed by pH. While for η, light intensity had a greater impact than other factors, followed by pH and C/N (Table 4.2). The synergistic effect of pH–*I* and C/N–*I* on the light conversion efficiency (η) was significant (*P-value*< 0.05) while pH –C/N was less significant (*P-value*=0.066) (Table 4.2). The little interaction between pH and C/N (P-value > 0.05) on light conversion efficiency suggests that *Rhodobacter sphaeroides* 158 does not favour different C/N when applied to produce biohydrogen at different initial pH.

Table 4. 2 ANOVA analysis for models using BBD

Model term	Estimate	Standard error	T-value	P-value
HPP			-	-
Intercept	932	53.5489		
x_1	65.281	32.7918	1.99	0.1031
x_2	173.937	32.7918	5.30	0.0032*
x_3	171.7812	32.7918	5.24	0.0034*
$x_1.x_2$	43.75	46.3747	0.94	0.3888
$x_1.x_3$	97.062	46.3747	2.09	0.0906
$x_2.x_3$	83.25	46.3747	1.80	0.1326
x_1^2	-199.156	48.2683	-4.13	0.0091*
x_2^2	-371.343	48.2683	-7.69	0.0006*
x_3^2	-218.531	48.2683	-4.53	0.0062*

HPR				
Intercept	41.133	2.3899	-	-
x_1	2.528	1.4635	1.73	0.1446
x_2	6.578	1.4635	4.50	0.0064*
x_3	7.06	1.4635	4.82	0.0048*
$x_1.x_2$	2.627	2.0697	1.27	0.2601
$x_1.x_3$	3.695	2.0697	1.79	0.1343
$x_2.x_3$	2.24	2.0697	1.08	0.3285
x_1^2	-10.160	2.1542	-4.72	0.0053*
x_2^2	-17.755	2.1542	-8.24	0.0004*
x_3^2	-9.972	2.1542	-4.63	0.0057*
$\boldsymbol{\eta}$				
Intercept	0.34	0.007	-	-
x_1	0.015	0.004	3.30	0.0214*
x_2	0.071	0.004	15.69	<.0001*
x_3	-0.083	0.004	-18.44	<.0001*
$x_1.x_2$	0.015	0.006	2.34	0.0668
$x_1.x_3$	0.025	0.006	3.89	0.0115*
$x_2.x_3$	-0.017	0.006	-2.72	0.0415*
x_1^2	-0.076	0.006	-11.41	<.0001*
x_2^2	-0.128	0.006	-19.26	<.0001*
x_3^2	-0.023	0.006	-3.55	0.0163*

*P-value indicates the term of responses statistically significant < 0.05

The 3D response surfaces and 2D contours plots in Figures (4.2),(4.3), and (4.4) are based on Equations (4.3), (4.4) and (4.5) ,respectively with one independent factors was kept at constant level (coded zero level), while the other two factors were changed within the experiential ranges. These figures show the impacts of pH and C/N, pH and the light intensity, and C/N and the light intensity. In Figures (4.2), (4.3), and (4.4), the responses enhanced as corresponding factors increased, until the peak reached, after that they decreased even when both factors continued to increase. For example,

Figure 4.2 indicated that HPP increased rapidly when pH extended from 6.5 to 7.7 and then slightly declined when pH continued to increase from 7.7 to 8. The possible reason for this behaviour is that the ionic form of different initial pH values can impact the activities of involved enzymes in photo fermentative hydrogen production such as nitrogenase and hydrogenase as well as metabolic pathways [101]. At photo-production of hydrogen, the alkaline pH (8.5-9) is favourable for the uptake hydrogenase activity (enzyme consumes hydrogen and produces protons), while it is unfavourable for nitrogenase function (enzyme catalyzes biohydrogen) [226, 227]. Peng et al. [228] reported that at pH 8, no nitrogenase activity for *Rhodobacter sulfidophilus* was observed. Similarly, *HPP* initially increased when C/N increased from 15 to 30 and then decreased with increase C/N. This might be caused by the insufficient nitrogen source that would preserve the growth and metabolism of microorganisms [25]. Figures (4.2) and (4.3) show that *HPP* and *HPR* were enhanced when light intensities increased from 35 Wm^{-2} to the optimum value and then decreased when light intensities continued to increase above the optimum value. The enhancement is possibly due to a higher light intensity providing more ATP and Fd_{red} [229]. ATP and Fd_{red} are essential for photo fermentative hydrogen production. However, the presence of excess ATP and Fd_{red} more than nitrogenase capacity might lead to the light saturation which can result in the reduction of nitrogenase activities [108, 229]. The present findings are in agreement with various earlier studies [26, 63, 108, 229, 230]. It should also be noted that some of the previous studies reported that the further increase in the light intensity above the optimum value had a little impact on biohydrogen production and no photo inhibition was observed [27, 231, 232].

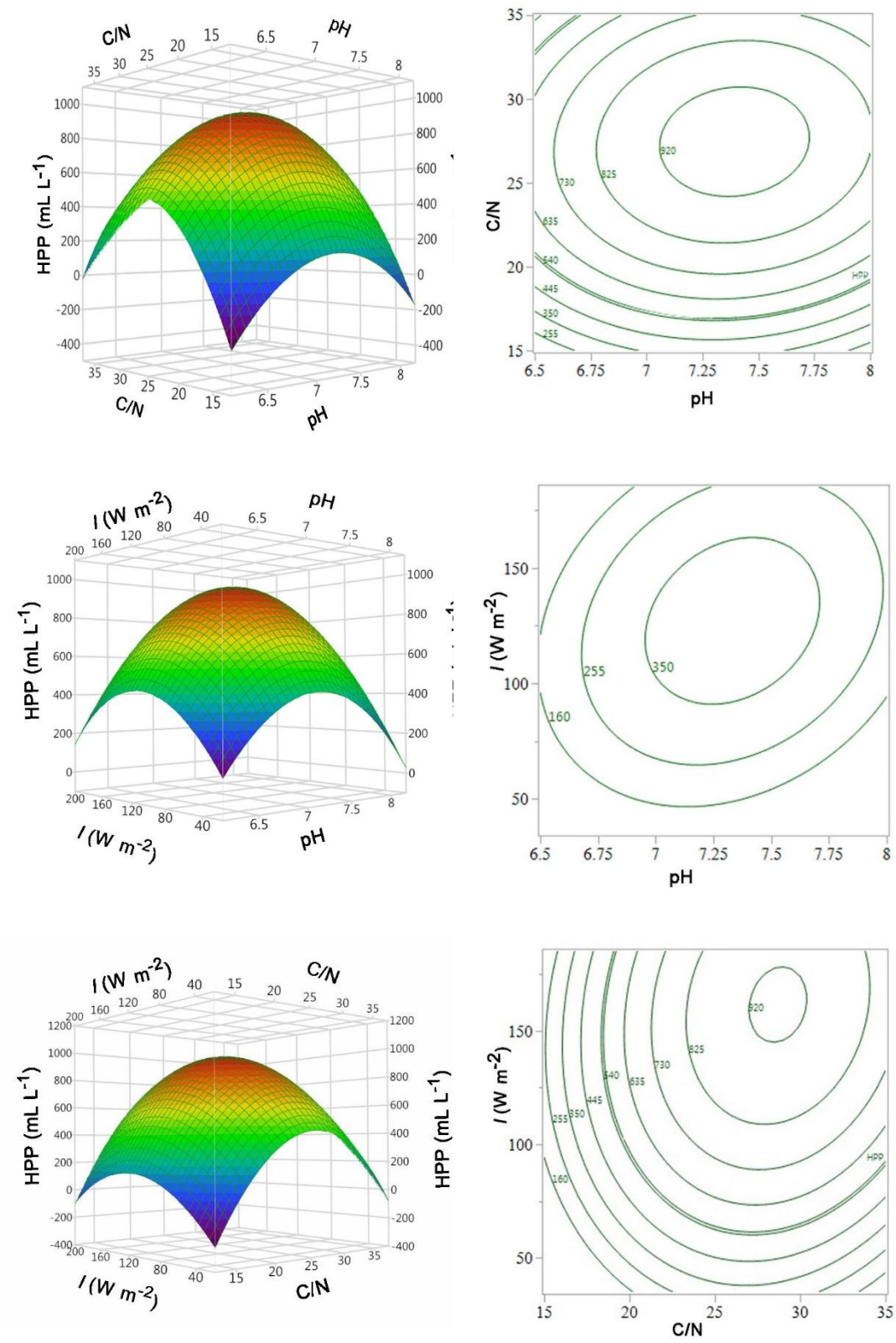

Figure 4. 2 Response surface plot and corresponding contour plot for hydrogen production potential (*HPP*).

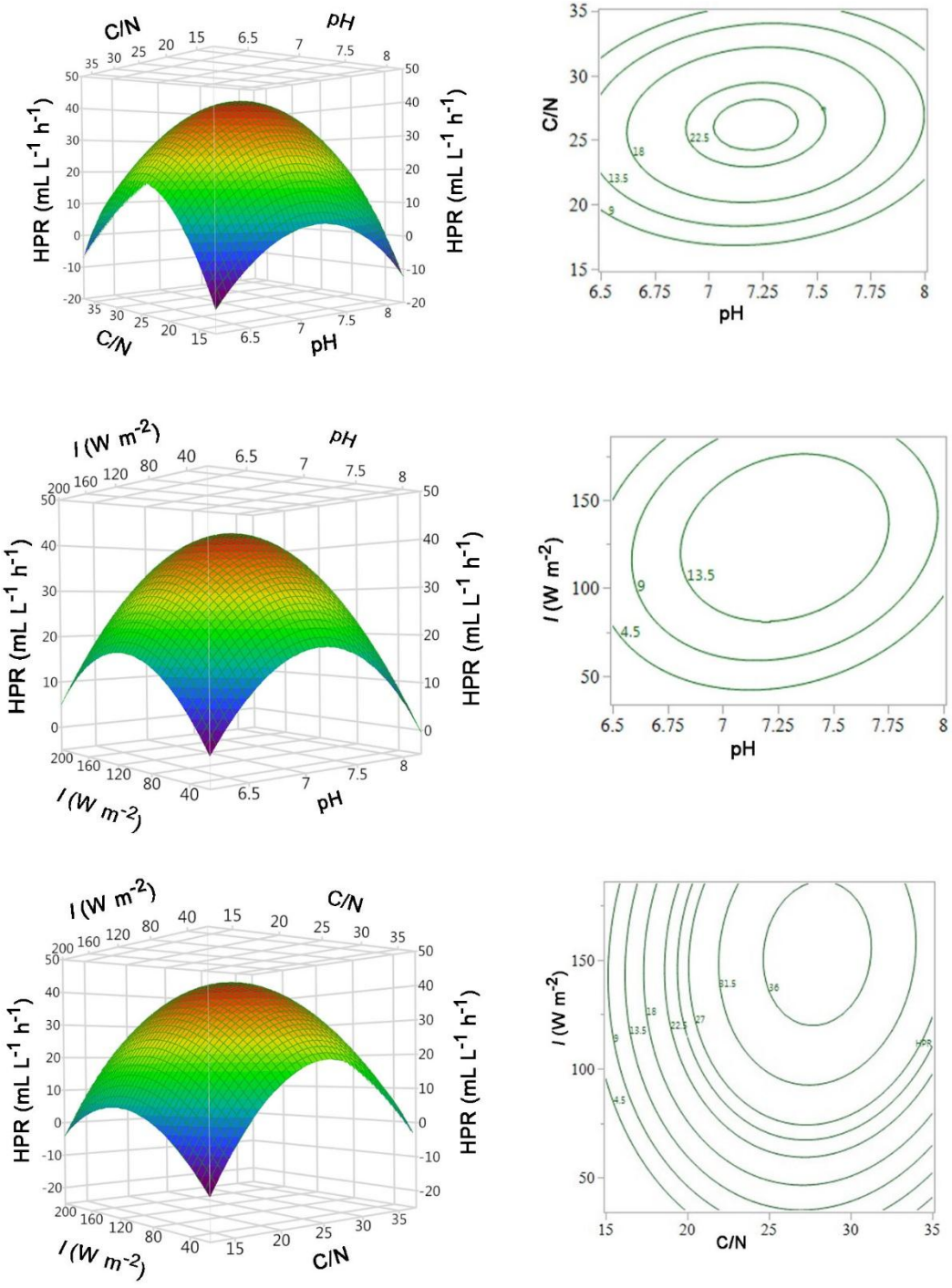

Figure 4. 3 Response surface plot and corresponding contour plot for hydrogen production rate (HPR).

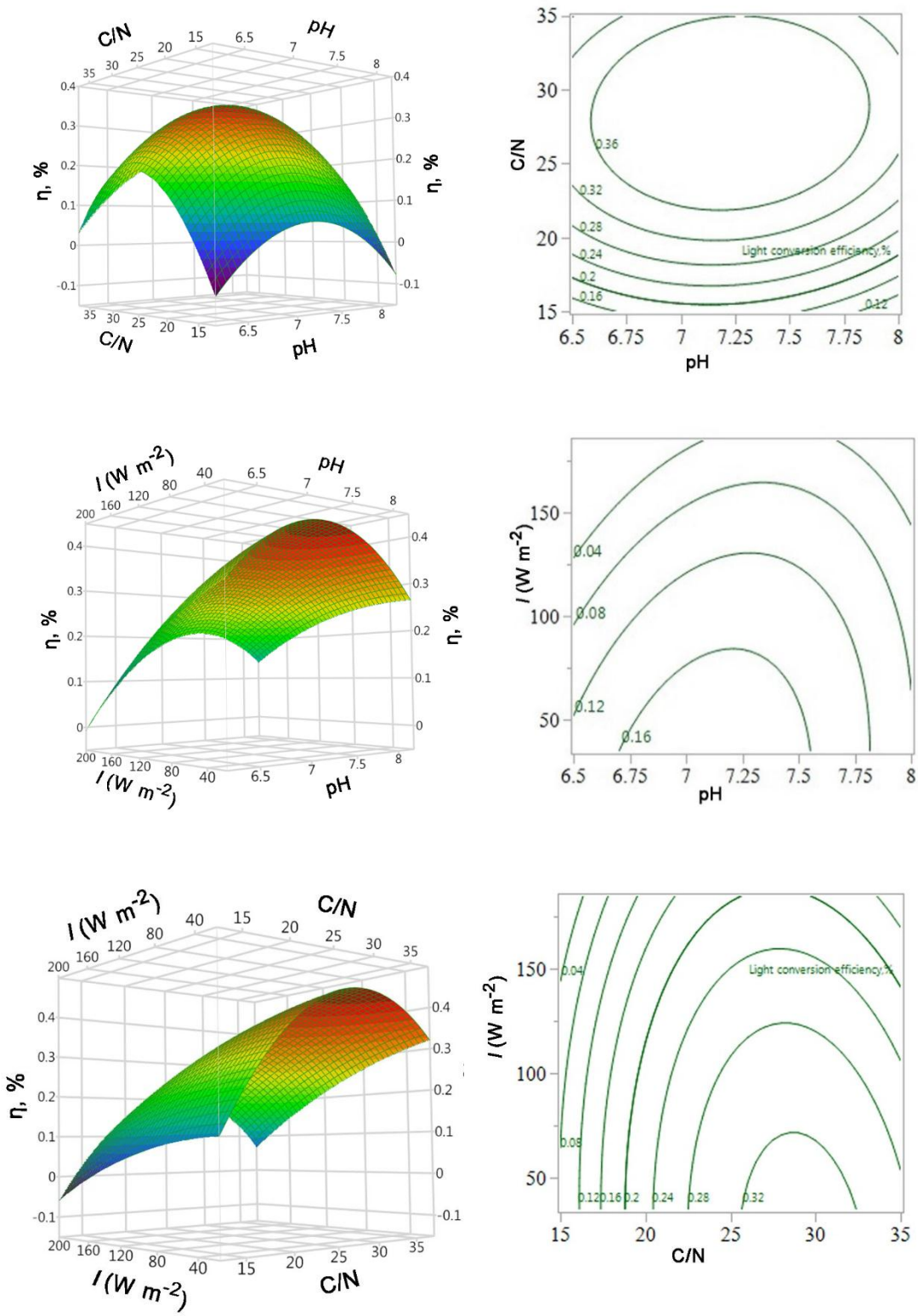

Figure 4. 4 Response surface plot and corresponding contour plot for Light conversion efficiency, % (η).

4.3.3 Optimization and validation of the models

Ultimately, the optimum value of independent factors for photofermentative hydrogen production process were calculated and the ability of the fitting models for predicting optimal values of responses were examined. The scale of desirability function ranges between zero for an unacceptable response values and one for a totally desirable one. At 0.91 desirability, the optimal conditions were found as 7.4, 27.5, and 126 W m^{-2} of pH, C/N, and light intensity, respectively. Under these conditions the predicted responses of HPP, HPR, and η were 994.6 mL L^{-1}, 43.2 mL L^{-1} h^{-1}, and 0.33 respectively. To validate the accuracy of the current optimization analysis, a separate experiment was conducted in triplicates under the optimum values of pH, C/N, and *I*. The measured response values were in the acceptable agreement with the predicted response values (Table 4.3), which confirms the validity of the second-order polynomial model equations applied in the current study. Higher agreements were observed for *HPP* and *HPR* values with an error of around 3.5%, compare to the η value with 6% error which is a parameter that is highly sensitive to the variations of various factors such as the irradiated area, intensity of the light, duration and the total amount of hydrogen production [97].

Table 4. 3 Comparison between experimental and predicted responses at optimum conditions.

Response	Optimum condition	Experimental	Predicted	% error
HPP	pH=7.4	960	994.57	3.48
HPR	C/N=27.5	41.74	43.225	3.44
η	I =126 Wm^{-2}	0.31	0.33	6

4.3.4 Investigating the performance of a typical experiment

A typical experiment for photo-fermentative hydrogen production was conducted under the optimum conditions (7.4, 27.5, and 126 W m^{-2} of pH, C/N and *I*), the bio-hydrogen produced, cell growth and the pH profiles are demonstrated in Figure 4.5.

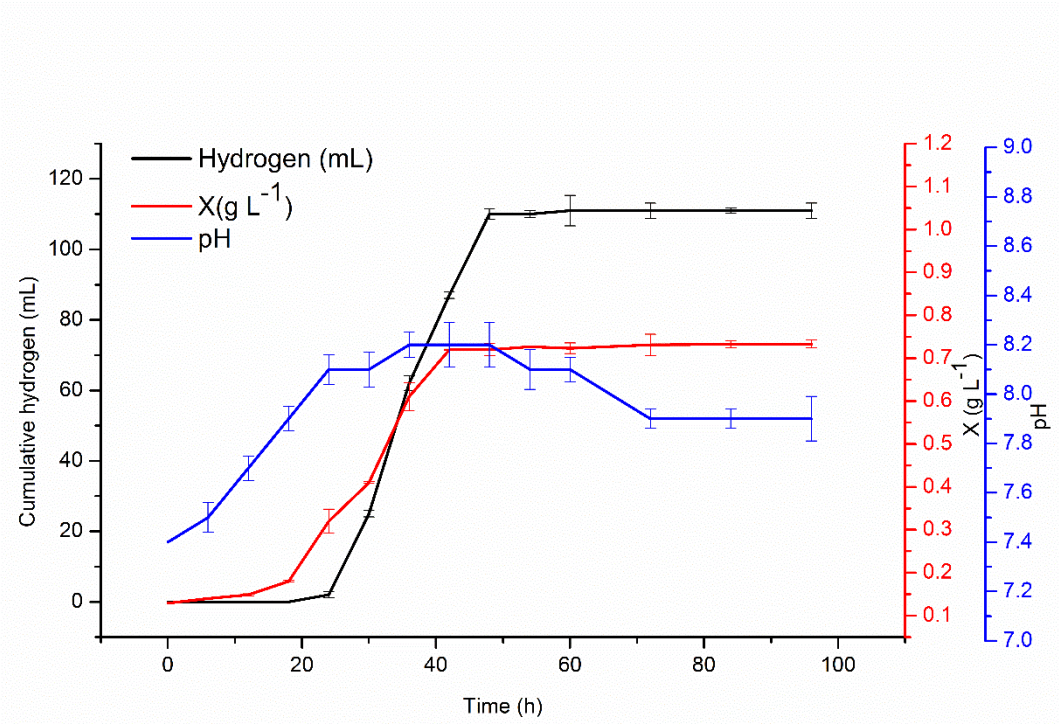

Figure 4. 5 Cumulative photofermentative hydrogen production, cell growth, and pH profiles of *R. sphaeroides* DSM 158 medium at optimum condition

The hydrogen yield increased after the characteristic lag period of about 25 h. The growth followed the expected pattern of exponential and stationary phases. Analysis of pH profile indicated that the initial pH of the medium increased from 7.4 to around 8.1 (0-24 h) until the initiation of the hydrogen production, and fairly stayed stable during hydrogen production time (24-48 h). Thereafter, it showed a slight drop from 8.2 to 7.9 for the period from 48 to 96 h, when hydrogen production was stopped, and the cellular growth was in its stationary phase. The noticed increase in pH during bacterial growth -before the initiation of the hydrogen production- can be attributed to the accumulation of reducing equivalents inside and outside the cells and the suppression in photo fermentative hydrogen production. The excess reducing equivalents used for the generation of by-products such as poly-β-hydroxybutyrate (PHB) can also lead to increase pH. When hydrogen starts to generate the co-evolved carbon dioxide (CO_2), it may moderate the pH variations [35, 37, 233], as also observed in the current study. These findings of this study are in agreement with the previous studies [63, 226].

The maximum HPP of 960 mL L^{-1} achieved in this study was highly comparable with those previously reported in the literature (as shown in Table 4.4). Uyar et al. [27] reported that HPP of 800 mL/L was produced from malate by *Rhodobacter*

sphaeroides O.U. 001 at pH, C/N, and light intensity values of 6.6, 35, and 277 W m^{-2}, respectively. In another study, HPP was 753.9 mL L^{-1} for malate by *Rhodobacter sphaeroides* O.U. 001 at pH, C/N, and light intensity of 6.8, 35, and 15 $W.m^{-2}$, respectively [220]. Han et al. [96] found that HPP of 800 mL L^{-1} was obtained from malate by *Rhodobacter sphaeroides* RV at pH, C/N, and light intensity of 7, 35, and 220 W m^{-2}, respectively.

Table 4. 4 Comparison of HPP, (HPR), and η using different strains of *Rhodobacter sphaeroides*.

Parameters	Microorganism	Carbon source	Nitrogen source	Conditions			Reference
				pH	C/N	*I* (light source)	
HPP (mL L^{-1})							
800	*R. sphaeroides* O.U. 001	Malate (15mM)	Glutamate (2 mM)	6.6	35	277 Wm^{-2} (tungsten)	[27]
754	*R. sphaeroides* O.U. 001	Malate (15 mM)	Glutamate (2 mM)	6.8	35	15 Wm^{-2} (tungsten)	[220]
284	*R. sphaeroides* RV	Malate (45 mM)	Glutamate (5.4 mM)	7	35	220 Wm^{-2} (tungsten)	[96]
960	*R. sphaeroides* DSM 158	Malate (20 mM)	Glutamate (3.5 mM)	7.4	27.5	126 Wm^{-2} (halogen)	This study
HPR (mL L^{-1} h^{-1})							
12	*R. sphaeroides* O.U. 001	Malate (15 mM)	Glutamate (2 mM)	7	35	200 Wm^{-2} (halogen)	[24]

33	*R. sphaeroides* O.U. 001	Malate (15mM)	Glutamate (2 mM)	6.6	35	277 Wm^{-2} (tungsten)	[27]
6.9	*R. sphaeroides* O.U. 001	Malate (15 mM)	Glutamate (2 mM)	7	35	940 µE	[62]
6.5	*R. sphaeroides* O.U. 001	Malate (15 mM)	Glutamate (2 mM)	6.8	35	15 Wm^{-2} (tungsten)	[220]
165.9	*R. sphaeroides* ZX-5	Malate (30 mM)	Glutamate (7 mM)	7	23	8000 lux (halogen)	[229]
102.3	*R. sphaeroides* ZX-5	Malate (30 mM)	Glutamate (7 mM)	7.1	23	5000 lux (halogen)	[225]
12.9	*R. sphaeroides* NMBL-01	-	-	8	13	1.8 klux (tungsten)	[92]
41.9	*R. sphaeroides* O.U. 001	Malate (15 mM)	Glutamate (2.5 mM)	6.5	30	64 Wm^{-2} (halogen)	[224]
195	*R. sphaeroides* DSM 158	Lactate (40 mM)	Glutamate (10 mM)	7	13	2250 Wm^{-2} (halogen)	[26]
41.7	*R. sphaeroides* DSM 158	Malate (20 mM)	Glutamate (3.5 mM)	7.4	27.5	126 Wm^{-2} (halogen)	This study

***η*, %**							
0.27-0.70	*R. sphaeroides* O.U. 001	OMWW*	-	6.8 -7	42.3	200 Wm^{-2} (tungsten)	[17]
0.5	*R. sphaeroides* O.U. 001	Malate (15mM)	Glutamate (2 Mm)	6.6	35	277 Wm^{-2} (tungsten)	[27]
0.12-0.23	*R. sphaeroides* O.U. 001	OMWW*	-	6.8	73.8	150 Wm^{-2} (tungsten)	[44]
0.19- 0.51	*R. sphaeroides* O.U. 001	Malate (30mM)	Glutamate (2 mM)	6.8	17	3.75-15 Wm^{-2} (tungsten)	[234]
0.31	*R. sphaeroides* DSM 158	Malate (20 mM)	Glutamate (3.5 mM)	7.4	27.5	126 Wm^{-2} (halogen)	This study

*OMWW: Olive mill wastewater

Another significant parameter of biohydrogen production studies is the hydrogen production rate (HPR). It is apparent from Table 4.4 that the HPR of 41.7 mL L^{-1} h^{-1} obtained in the current study lays on the higher range of the reported literature values [24, 27, 62, 92, 220, 224]. However, it is smaller than those by *Rhodobacter sphaeroides* ZX-5 [225, 229] and *Rhodobacter sphaeroide*s DSM 158 [26]. It is expected to get various results from different studies, due to the variations on the carbon source, nitrogen source and experimental conditions. For instance, Krujatz et al. [26] reported a HPR of 195 mL L^{-1} h^{-1} when lactate was used as a carbon source at pH, C/N and, light intensity of 7, 13, and 2250 W m^{-2} respectively, while the present study yielded 41.7 mL L^{-1} h^{-1} from malate at pH, C/N, and light intensity of 7.4, 27.5, and 126 W m^{-2} respectively. The main reasons for obtaining different results can be due to using various carbon sources, in addition to applying significantly different light intensities. Assawamongkholsiri and Reungsang [108] also reported that the carbon source is a significant factor affecting the metabolism of photofermentative hydrogen production. The second reason can be the variations on the light intensity. Uyar et al. [27] found that the rate of hydrogen production increased with increasing the light intensity.

Light conversion efficiency of 0.31 % is comparable with results found in the literature (Table 4.4). For example, Eroğlu et al. [17] and Eroğlu et al. [44] determined η as 0.27 % from olive mill wastewater by *Rhodobacter sphaeroides* O.U. 001, while Uyar et al. [27]and Nath and Das [234] reported η of 0.5% from malate by *Rhodobacter sphaeroides* O.U. 001.

4.4 Summary

The present study was designed to investigate the interactive effects of pH, C/N and light intensity on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM and optimize the experimental conditions of the batch process by applying response surface methodology (RSM) based on Box-Behnken design (BBD). The optimal conditions were 7.4, 27.5, and 126 Wm^{-2} of pH, C/N, and light intensity, respectively. Under these conditions the experimental responses of HPP, HPR, and η were 960 mL. L^{-1}, 41.7 mL. $L^{-1}.h^{-1}$, and 0.31 respectively**.** The study showed that both C/N ratio and *I* have crucial and significant effect on the HPP, HPR and η followed by

pH, the synergistic effect of pH–*I* and C/N–*I* on the light conversion efficiency (η) was significant while pH –C/N was insignificant. The clear peak of each 3D response surface plots led to conclude that the maximum response of bio hydrogen can be achieved with the applied ranges of design boundary. The current findings explain the interactive effects of the most important variables and contribute to optimize the experimental conditions with high goodness of fit. This means that the models can be applied to describe the impacts of pH, C/N, and light intensity on HPP, HPR, and η with high agreement between predicted and experimental responses. The results reveal that RSM with BBD was a useful tool to facilitate improvements in the biohydrogen production process.

Chapter 5

Enhancement of the photofermentative hydrogen production from brewery wastewater using banana peels waste as an effective pre-treatment stage

Chapter 5 Enhancement of the photofermentative hydrogen production from brewery wastewater using banana peels waste as an effective pre-treatment stage

5.1 Introduction

The climate change and energy security problems lead researchers to explore cleaner and more sustainable fuels than the conventional ones [235]. Hydrogen is a significant substance which widely used in several industries such as petroleum and petrochemical industries [236]. In addition, hydrogen is considered as a clean fuel with the high energy content of 122 kJ g^{-1} [217]. It can also be generated by applying both sustainable and non-sustainable resources [7, 237]. Currently, hydrogen is obtained mostly from the fossil fuel using thermo-chemical processes [238]. Biological processes of biohydrogen production especially photo fermentative H_2 technologies have received a considerable attention due to its ability for utilizing a wide range of substrates with a high conversion [11, 239]. Biohydrogen production from wastes is a renewable way to supply part of the global hydrogen demand [14, 240]. The use of organic wastes as substrate sources for biohydrogen production has gained a noticeable attention due to its dual action of waste treatment along with clean energy generation [2, 241].

The brewery industry produces high amounts of wastewater which requires further treatment before its disposal [242]. For example to produce 100,000 m^3 $year^{-1}$ of beer; 400,000 m^3 $year^{-1}$ of fresh water is required, which generates around 220,000 m^3 brewery effluent (BE) [243]. It has a chemical oxygen demand (COD) value between 2000 and 6000 mg L^{-1} and nitrogen compounds value between 25 to 80 mg L^{-1} [244, 245]. Discharge of the untreated BE with high amount of organic material into the surrounding streams could lead to the exhaustion of oxygen in water and degradation in the aquatic life [246]. The majority of previous studies applied a pure substrate as a carbon source to produce photo fermentative hydrogen [89, 96, 247, 248]. Various organic wastewater, including olive wastewater [17], pulp and paper mill effluent [49],

dairy wastewater [43], and sugar refinery wastewater [20] have been investigated to generate biohydrogen using photo fermentation processes. However, only few studies investigated the photo fermentative hydrogen production from brewery wastewater. Seifert et al. [19], investigated the production of biohydrogen from brewery wastewater using *Rhodobacter sphaeroides* O.U. 001. Hay et al. [246] applied brewery wastewater to produce hydrogen from *Rhodobacter sphaeroides* NCIMB 8253.

There have been several studies in the literature reporting the uses of feedstock pre-treatment processes to enhance biohydrogen yield [19, 45-51]. For example, Eroğlu et al. [44] investigated the impact of the adsorption pre-treatment stage of olive mill wastewater with clay on biohydrogen production from *Rhodobacter sphaeroides* O.U.001. They found that significant amounts of total phenol (81%) and colour (65%) were removed after pre-treatment stage and hydrogen production potential increased from 16 to 31.5 m^3 m^{-3}. Pintucci et al. [47] also observed that the pre-treatment process of olive mill wastewater with dry-Azolla and granular active carbon decreased both poly phenols and black-brownish colour of this wastewater which have an inhibitory impact on photo fermentative hydrogen production. They concluded that pre-treated mill wastewater was very suitable as a substrate for *Rhodopseudomonas palustris* 6A to produce hydrogen. According to Budiman and Wu [50], ultra-sonication pre-treatment stage of a combined effluent of palm oil and pulp and paper mill led to enhance the production of from 4.67 to 8.72 mL H_2 mL^{-1} medium by increasing soluble carbohydrates concentration in the wastewater. However, the investigations about the uses feed stock pre-treatment technologies prior to photo fermentation for improving hydrogen production are still limited.

Although banana peels wastes have been used effectively for heavy metal removals from aqueous solution [249-252], no study in the current literature attempted to apply banana peels waste as pre-treatment stage to remove the inhibitors and enhance the nutritional value of the feedstock, thereby improving bio hydrogen production. Hence, banana peels waste as pre-treatment an effective technique was introduced in current study to enhance photo fermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM using BE.

5.2 Materials and methods

5.2.1 Brewery effluent (BE)

Brewery effluent (BE) was collected from a local brewery production plant in Perth, Western Australia, Australia. The wastewater effluent samples were immediately filtered using 0.2 μm nylon micro filters and then autoclaved at 120 °C for 15 min to ensure sterile conditions. Sterilized BE was stored in the fridge (4 °C). The characteristics of the BE used in this study were described previously in Chapter 3.

5.2.2 Pre-treatment stage of BE with banana peels

Preparation and characterization of banana peels have been discussed in chapter 3. BE sample was treated with 1, 2, 3 or 4 g L^{-1} of banana peels particles. Processed banana peels were thoroughly mixed with BE at an agitation speed of 250 rpm for various time intervals (1, 2, 3 and 4 h). This process was carried out at a pH value of around 7.2. At the end, the mixture was rested for 2 h to settle down. After settlement, the above liquid was pre-filtered by Whatman^{TM} filter paper (0.45 μ) to separate liquid from the solids.

5.2.3 Photofermentative hydrogen medium

Rhodobacter sphaeroides DSM 158 was anaerobically activated and grown in modified Biebl and Pfennig medium [223] under sterile conditions and light irradiated by the procedure described previously in sections 3.2 and 3.3. Hydrogen production medium was established by adding 2.68 g L^{-1} of DL_malic acid and 0.52 g L^{-1} of L_ glutamic acid to the aforementioned medium. The batch experiments of hydrogen production were accomplished in 120 mL clear glass photo-bioreactor at 30 ∓ 2 ℃ . The produced gas mixture was collected by water displacement method and gas compositions were analysed by a gas chromatograph (Agilent 7890B GC). The preparation of hydrogen production medium, experimental setup and hydrogen production procedure were detailed in Chapter 3. In this study the standard medium (SM) was blended with untreated (raw) brewery wastewater (Bu) and treated one (Bt) in different volume ratios (Table 5.1).

Table 5. 1 Medium of different blending ratios investigated.

Medium used	Description
Raw brewery effluent	
100Bu:0SM	100% BE + 0% standard medium
75Bu:25SM	75% BE + 25% standard medium
50Bu:50SM	50% BE + 50% standard medium
25Bu:75SM	25% BE + 75% standard medium
Treated brewery effluent (by banana peels)	
100Bt:0SM	100% BE + 0% standard medium
75Bt:25SM	75% BE + 25% standard medium
50Bt:50SM	50% BE + 50% standard medium
25Bt:75SM	25% BE + 75% standard medium

Bu: untreated brewery effluent; Bt: treated brewery effluent; SM: standard medium

5.2.4 Analytic methods

Gas compositions, biomass, light intensity, chemical oxygen demand (COD), total nitrogen (TN), total organic carbon (TOC) and pH measurements have been described in chapter 3.The ammonium concentration was measured spectrophotometrically [253]. The functional groups of the banana peels particles were determined by Fourier-transformed infrared (FTIR) spectroscopy (Perkin Elmer spectrum 100). SEM analyses of banana peels samples were performed using Tescan Mira3 FESEM with Oxford Instruments X-Max SDD X-ray detector. The data of all measurements represented the average of three independent analyses with standard deviation less than 5 %.

5.3 Result and discussion

5.3.1 Characterization of banana peels particles

To understand the nature of the functional groups present in banana peels particles, FT-IR spectra was performed. Banana peels particles with a particle size of <250 µm (before and after the pre-treatment stage) were analyzed and results were shown in Figure 5.1. FTIR spectrum of banana peels particles (Figure 5.1) shows number of

peaks that indicate a complex nature of these particles. It is apparent from this figure that the following peaks 1735-1559 cm^{-1}, 1531.5- 1409 cm^{-1} and 1413.6 – 1024 cm^{-1} were shifted due to the pre-treatment stage. The peaks 1735-1559 cm^{-1}, 1531.5- 1409 cm^{-1} and 1413.6 – 1024 cm^{-1} were assigned as C=O stretch, C=C- stretch and C=N stretch, respectively [254, 255]. However, the peak of 2326-2048.9 cm^{-1} that was attributed to the OH stretch [254, 256] was not shifted.

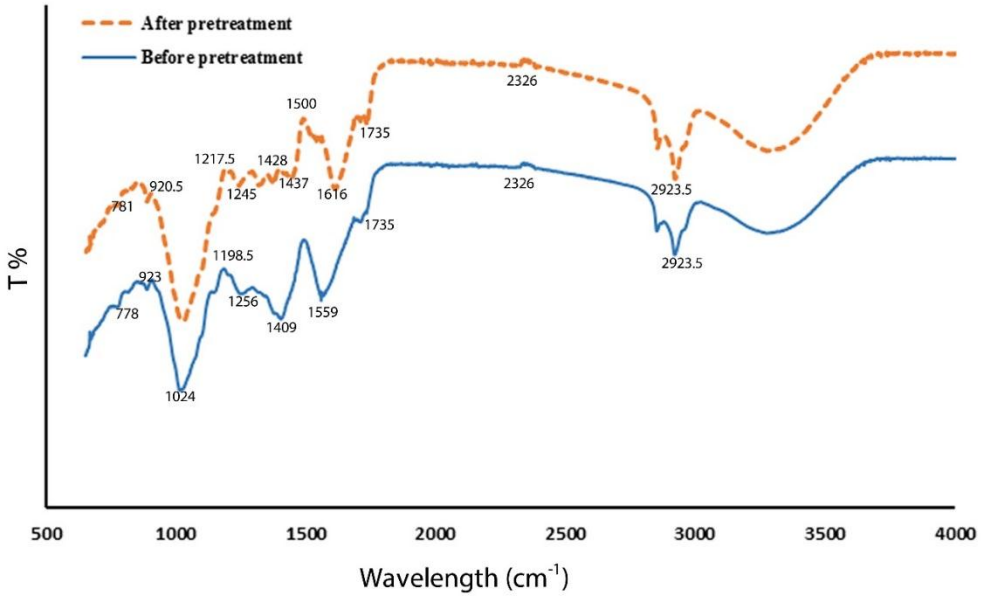

Figure 5. 1 FTIR spectra of bananas peels before and after pre-treatment stage.

SEM showing the surface morphology of banana peels particles before and after the pre-treatment stage are shown in Figure 5.2. Figure 5.2a shows that microporous structure and rough texture were observed for this sample, which can enhance the performance of banana peels particles as biosorbents. Similar observations were also previously reported when using banana peels as treatment agents [257, 258]. After pre-treatment stage (Figure 5.2b), it was observed that the porous structure of banana peels particles was increased and the binding sites were occupied. This can be attributed to the possible transfer of carbon compounds from banana peels particles into BE and the adsorption of ammonium.

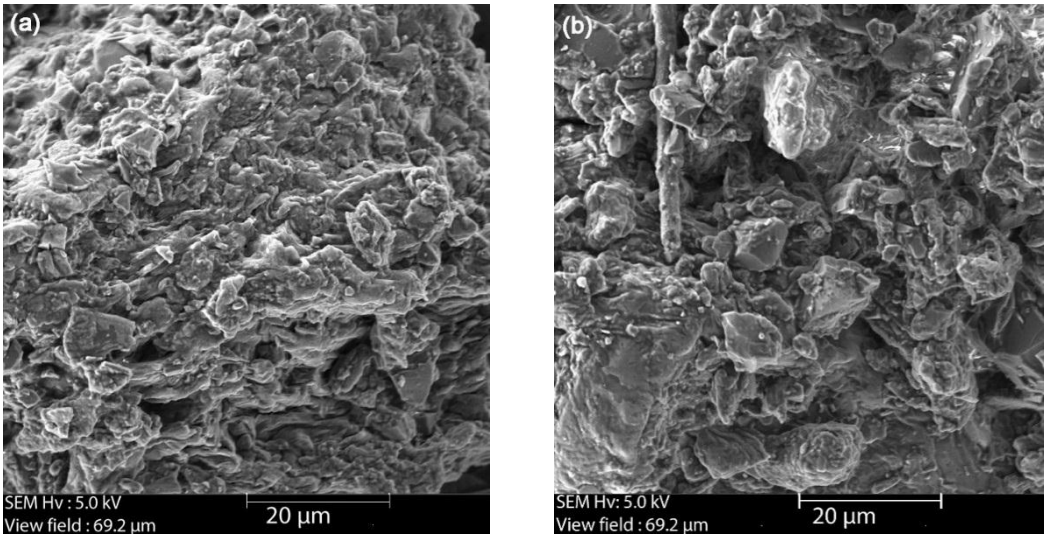

Figure 5. 2 SEM images of banana peels samples. (a) before pre-treatment, (b) after pre-treatment

5.3.2 Effect of the blending ratio on photofermentative hydrogen production

Influence of different blending ratios of treated and untreated brewery effluent with standard medium (Table 5.1) on the biohydrogen production using *Rhodobacter sphaeroides* DSM 158, were investigated. Figure 5.3 illustrates cumulative biohydrogen for different blending ratios. The results revealed that the pre-treatment stage for the raw brewery wastewater with banana peels significantly enhanced hydrogen production. The highest hydrogen production was obtained when blending equal ratios of treated brewery wastewater blended and standard medium (50Bt:50SM). The maximum hydrogen production potential (HPP) was 358.33 mL H_2 L^{-1}, which is 2.7-folds higher than those applying the same percentage of untreated (raw) brewery wastewater (50Bu:50SM). This significant enhancement in biohydrogen production can be attributed to the increase in C/N ratio and a decrease in the ammonium concentrations (Table 5.2). The increase in C/N ratio of treated brewery effluent is mainly due to the carbon content of banana peels. Achak et al. [249] reported that the carbon content of banana peels was about 40% (w/w). Furthermore, previous studies stated that banana peels can be applied as a promising adsorbent to remove ammonium from wastewater [252, 259].

It is well-known that C/N ratio plays a significant role in biohydrogen production process [87, 260]. A low C/N ratio was found to inhibit the nitrogenase enzyme and hence biohydrogen production while increase the biomass concentration [261]. The increase in biomass concentration leads to reduce the light penetration into photo-

bioreactor, thus reducing biohydrogen production [262]. However, on the other hand, very high C/N ratio was indicated to reduce biohydrogen production as well [260, 262]. Nitrogen limitation can force purple-non-sulphur (PNS) bacteria to dump their excess energy and reducing power during biohydrogen production process [87, 263]. Therefore, nitrogen limited conditions should be applied carefully to achieve a balance between the necessary amount of nitrogen for the growth while non-inhibiting the generation of biohydrogen [260]. The optimum value of C/N ratio in the literature for *Rhodobacter sphaeroides* has a wide range, from 13 to 35 [42, 92, 96, 229]. The presence of ammonium is known to cause the reduction of nitrogenase activity which is responsible for the biohydrogen production [264]. Waligórska et al. [265] reported that the wastewater sample with an ammonium concentration of higher than 1 mmol L^{-1} (17 mg L^{-1}) can reduce the amount of biohydrogen production. A similar observation was reported by Zheng et al. [266] as they demonstrated that the presence of ammonium with a concentration more than 17 mg L^{-1} inhibited fermentative hydrogen yield by negatively affecting the nitrogenase activity. This critical value of ammonium was reported as 34 mg L^{-1} by Akköse et al. [264], where no biohydrogen production at ammonium concentrations higher than 34 mg L^{-1}.

Table 5. 2 Effects of pre-treatment stage on various substrates

Substrate	Ammonium concentration mg L^{-1}		C/N		TOC mg L^{-1}		COD mg L^{-1}	
	Before	After	Before	After	Before	After	Before	After
100B:0SM	36.4	24.0	7.40	15.22	350.0	456	2130.0	2350.0
75B:25SM	27.3	18.0	12.42	18.29	387.5	527	2247.5	2512.5
50B:50SM	18.2	12.1	17.45	21.36	425.0	548	2365.0	2675.0
25B:75SM	9.1	6.0	22.47	24.43	462.5	584	2502.5	2837.5

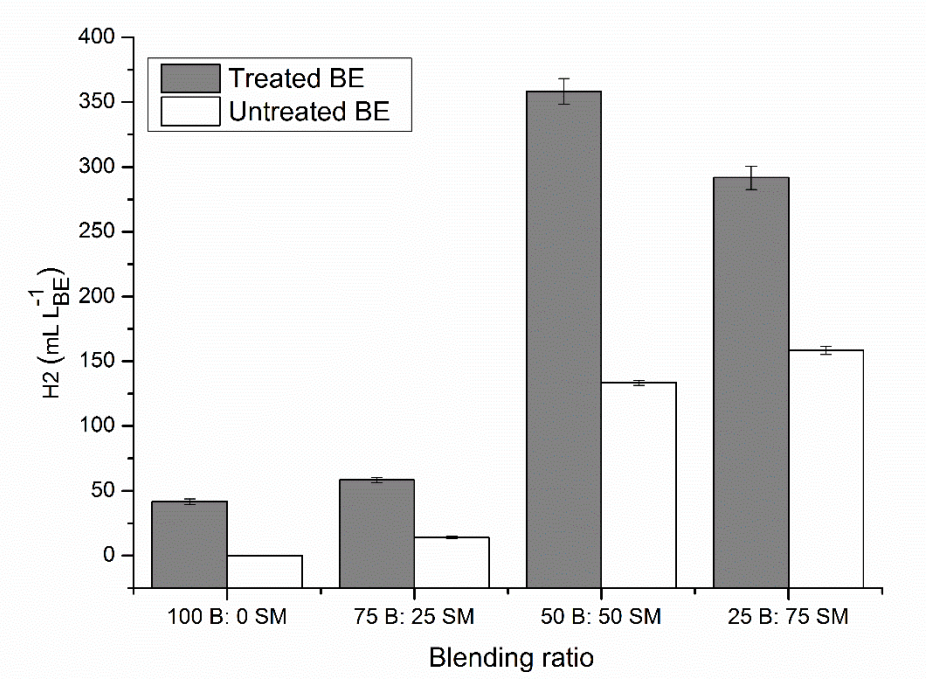

Figure 5. 3 Effect of banana peels pre-treatment of different concentration of BE on hydrogen production potential (T: 30 ^{o}C; agitation speed: 250 rpm; contact time: 60 min; dose: 1 g L^{-1}; particle size: <250 µm).

5.3.3 Effect of contact time

In order to enhance biohydrogen production, the effect of contact time for the raw BE pre-treatment by banana peels on biohydrogen production was investigated at 30 ^{o}C, agitation speed of 250 rpm, 1 g L^{-1} of banana peels dosage, particle size < 250 µm. The contact time range was 30, 60, 90,120, 180, and 240 min. Then the treated BE were blended with 50% SM (50Bt:50SM) and fed into photo-fermentation process in order to study the impact of the duration of pre-treatment on biohydrogen production yield. As shown in Figure 5.4, hydrogen production potential (HPP) was observed to be significantly enhanced (from 208.3 to 408.3 mL H_2 L^{-1} of BE) with increasing the contact time of pre-treatment from 30 to 120 min, representing the positive impact of contact time on biohydrogen production. Possibly, this result might be attributed to the presence of free binding sites on the banana peels particles [254]. However, there were no increases found after 120 min, where equilibrium stage achieved and the active binding sites of the banana peels fully occupied at used agitation speed of 250 rpm. This finding was also concluded for other biosorbents by various researchers [267, 268].

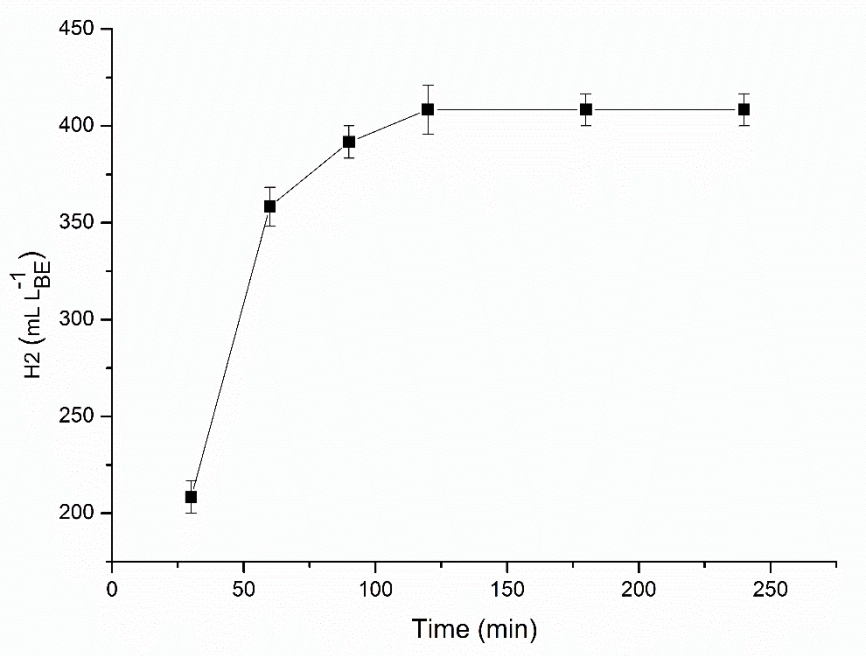

Figure 5. 4 The effect of contact time for banana peels pre-treatment on hydrogen production potential.

5.3.4 Effect of banana peels dosage on biohydrogen production process

The effects of various banana peels doses (Table 5.3) on biohydrogen production was investigated at 30 ^{o}C, agitation speed of 250 rpm, 120 min contact time, and particle sizes less than 250 µm.

Table 5. 3 Medium of equal blending ratio obtained at different banana peels dosage.

Medium used	Description
50Bu:50SM	50% BE + 50% standard medium
50Bt:50SM-1	50% BE with 1 g L^{-1} of banana peels + 50% standard medium
50Bt:50SM-2	50% BE with 2 g L^{-1} of banana peels + 50% standard medium
50Bt:50SM-3	50% BE with 3 g L^{-1} of banana peels + 50% standard medium
50Bt:50SM-4	50% BE with 4 g L^{-1} of banana peels + 50% standard medium

NOTE: Bu: untreated brewery effluent; Bt: treated brewery effluent; SM: standard medium

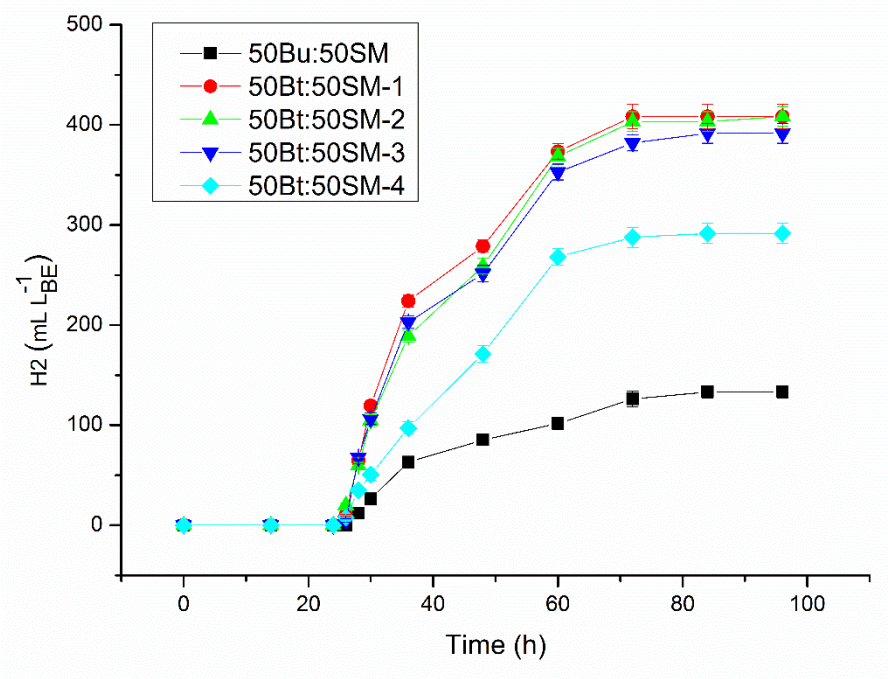

Figure 5. 5 Hydrogen production potential versus time for 50% BE pre-treated with different concentrations of banana peels.

Influences of banana peels dosage on biohydrogen production, under batch mode, are given in Figure 5.5. The amount of banana peels were observed to affect biohydrogen production. As illustrated in Figure 5.5, the hydrogen production using banana peel pre-treatment at any concentrations were higher than untreated control substrate (50Bu:50SM). The results indicated that the maximum hydrogen production (408.3 mL H_2 L^{-1} of BE) was achieved from the substrates (50Bt:50SM-1) and (50Bt:50SM-2). However, a gradual decrease in hydrogen production corresponding to further increase in banana peels concentration (above 2 g L^{-1}) implies that the pre-treatment of BE with a relatively high concentrations of banana peels led to a negative impact on biohydrogen production. The plausible reason for this decrease in hydrogen production yield can be attributed to having higher than optimum C/N values for the substrates (50Bt:50SM-3) and (50Bt:50SM-4) (Table 5.4) In our previous study, Al-Mohammedawi et al. [42]investigated the impact of C/N and other two variables on photofermentative hydrogen production by *R. sphaeroides* DSM 158. They noticed that HPP enhanced when C/N extended from 15 to 30 and then declined with further

increased in C/N. This could be due to the insufficient amount of nitrogen source, which preserves the metabolism of microorganisms [25].

Table 5. 4 The effects of pre-treatment with various banana peels concentrations on ammonium concentration, C/N, TOC and COD

Substrate	Ammonium concentration mg L^{-1}		C/N		TOC mg L^{-1}		COD mg L^{-1}	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
50Bu:50SM	18.2	-	17.45	-	425	-	2365	-
50Bt:50SM-1	18.2	12	17.45	21.3	425	548	2365	2675
50Bt:50SM-2	18.2	10.1	17.45	25.1	425	610	2365	2960
50Bt:50SM-3	18.2	8.7	17.45	30.6	425	695	2365	3150
50Bt:50SM-4	18.2	6.9	17.45	37.8	425	780	2365	3370

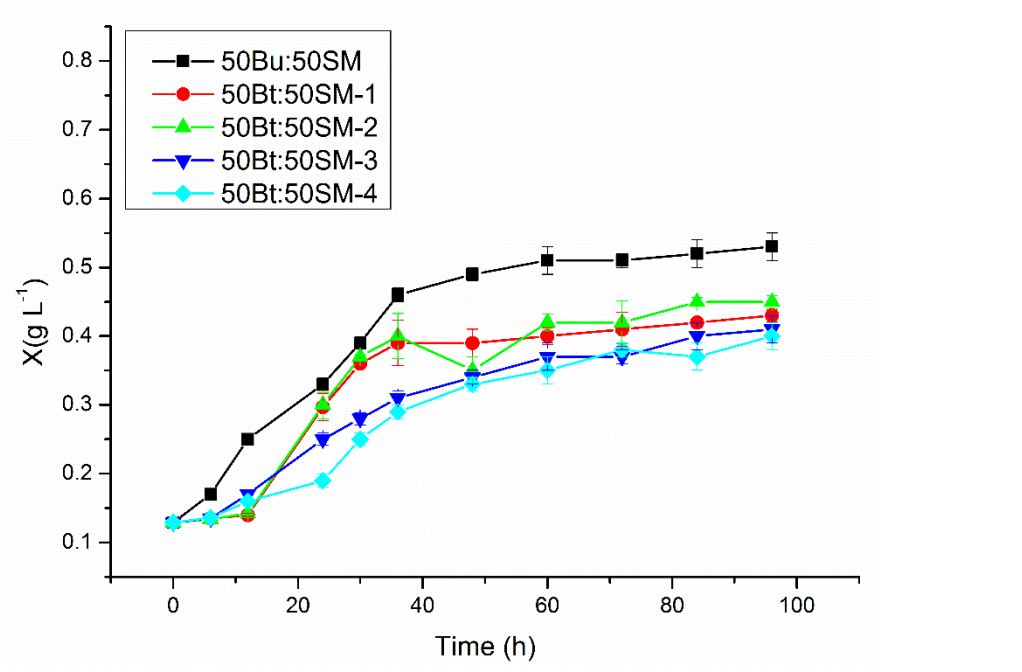

Figure 5. 6 Bacterial growth versus time for 50% BE pre-treated with different concentrations of banana peels.

Figure 5.6 illustrates the time course of the biomass concentration of *Rhodobacter sphaeroides* DSM 158 for 50Bu:50SM, 50Bt:50SM-1, 50Bt:50SM-2, 50Bt:50SM-3,

50Bt:50SM-4 substrates, during hydrogen production experiments. Lag phase was observed for all substrates, which is the adaption stage of the microorganism to the new conditions. Figure 5.6 also illustrates that the bacterial growth of 50Bu:50SM substrate was higher than other pre-treated substrates. This can be attributed to the concentration of ammonium in 50Bu:50SM was sufficient to support the growth of *Rhodobacter sphaeroides* DSM 158 (Table 5.4). Waligórska et al. [265] reported that ammonium acts as a nitrogen source to enhance the biomass growth and they found that the biomass was duplicated when the concentration of ammonium was increased from 1 to 10 mmol L^{-1}. Similarly, Akköse et al. [264] stated that the growth was 1.6 fold higher as a result of increasing ammonium from 1 to 10 mmol L^{-1}.

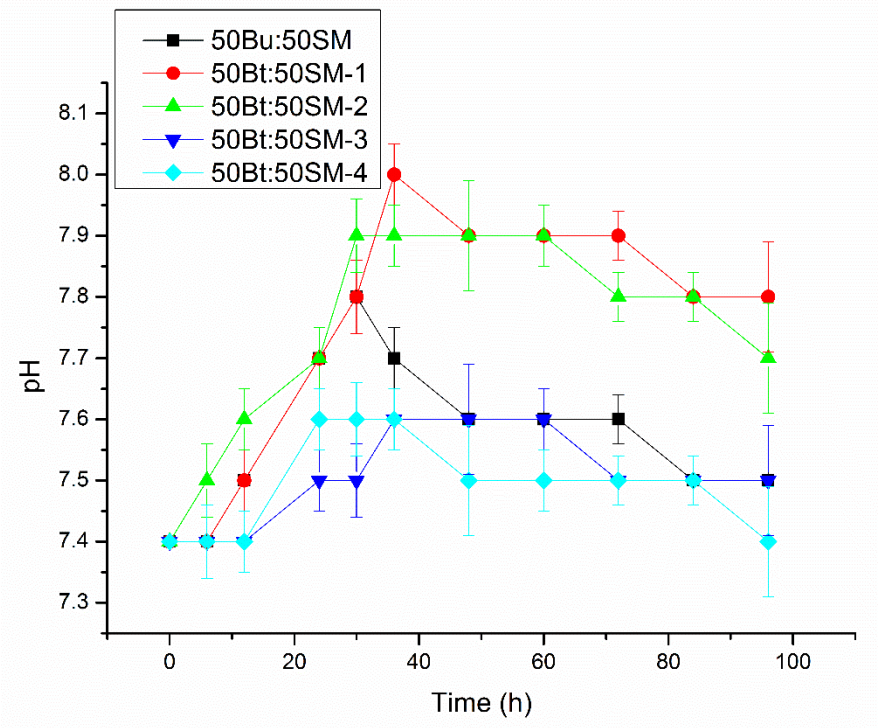

Figure 5. 7 Variation of pH with time for 50% BE pre-treated with different concentrations of banana peels.

The pH changes of 50Bu:50SM, 50Bt:50SM-1, 50Bt:50SM-2, 50Bt:50SM-3, 50Bt:50SM-4 substrates during hydrogen production by *Rhodobacter sphaeroides* DSM 158 are shown in Figure 5.7. Initial pH values of the substrates were adjusted to 7.4 and the pH values varied between 7.6 and 8.2 throughout the hydrogen production. Analysis of pH values indicated that the initial pH for all substrates increased during the period of 0 to 36 h, where the pH values reached 7.7, 8.0, 7.9, 7.6 and 7.6 of 50Bu:50SM, 50Bt:50SM-1, 50Bt:50SM-2, 50Bt:50SM-3, 50Bt:50SM-4 substrates, respectively. After this increase in pH, the most pH values slightly dropped. A similar

observation was indicated by Akroum-Amrouche et al. [226], as they observed that pH values increased during the first 70 h of culture age and dropped afterwards. This behavior could be attributed to the accumulation of reducing equivalents. The excess reducing equivalents lead to produce by-products which can increase pH [42]. The Figure 5.5 and Figure 5.7 indicate that, the biohydrogen production by *Rhodobacter sphaeroides* DSM 158 for different substrates of BE starts to generate at pH range 7.5-7.7. These finding is in agreement with the study of Akroum-Amrouche et al. [226] who indicated that the biohydrogen production by *Rhodobacter sphaeroides* CIP60.6 generated at pH range 7.5∓0.1.

The comparison of photofermentative hydrogen production performance of this study with other studies applying different wastewater samples after pre-treatment stages is summarized in Table 5.5. Table 5.5 clearly indicates that the percentage increases in photo fermentative hydrogen production yield after the pre-treatment stage used in the current study is at a relatively higher range with respect to those achieved by the previous studies (Table 5.5). However, Hay et al. [48] investigated the impact of ultra-sonication pre-treatment on biohydrogen production yield from pulp and paper mill effluent by *R. sphaeroides* NCIMB. They found that the ultra-sonication led to increase the biohydrogen yield from 1.1 mL H_2 mL^{-1} medium to 5.77 mL H_2 mL^{-1} medium. This means that compared to the control, the percentage increase in photofermentative hydrogen yield was about 424 %. The best HPP obtained in present study was comparable with those reported in previous studies [19, 47]. Pintucci et al. [47] reported that HPP of 0.316 mL H_2 L^{-1} of olive mill wastewater by *Rhodopseudomonas palustris* 6A using dry-Azolla and granular active carbon as a pre-treatment agent. In another study, Seifert et al. [19] applied *Rhodobacter sphaeroides* O.U. 001 and brewery wastewater to produce photo fermentative hydrogen under light intensity of 116 Wm^{-2}, and observed best result was 220 mL H_2 L^{-1} of BE at 10 % BE in standard medium. Comparing the HPP of 408.33 mL H_2 L^{-1} of BE at 50% BE in standard medium, the current study indicates the significant positive impact of banana peels pre-treatment on biohydrogen production process. However, it should also be mentioned that HPP from BE achieved in this study was lower than other studies using different wastewater such as olive wastewater and pulp and paper mill effluents (Table 5.5). The differences in HPP could be due to the COD value of used wastewater and the applied microorganism. For example, Eroğlu et al. [44] determined HPP as 13.5 mL H_2 L^{-1} of wastewater by *R. sphaeroides* O.U.001 from olive wastewater with high

COD of 52100 mg L^{-1}, while the COD of the wastewater applied in the present study was only 2130 mg L^{-1}.

Table 5. 5 Comparison among various studies of pre-treatment stage using wastewater as substrate to produce photo fermentative hydrogen.

Substrate	Microorganism	Pre-treatment	H_2 after the pre-treatment (mL H_2 mL^{-1} wastewater)	The increase percentage in hydrogen [a]	Reference
Olive mill wastewater	*R. sphaeroides* O.U.001	Clay	31.5	96.87 %	[44]
Olive mill wastewater	*R. sphaeroides* O.U.001	Zeolite-4A	17.6	10 %	[45]
Olive mill wastewater	*R. sphaeroides* O.U.001	Ozone	4.70	NE[b]	[45]
Olive mill wastewater	*R. sphaeroides* O.U.001	Fenton's reagent	2.10	NE[b]	[45]
Olive mill wastewater	*R. sphaeroides* O.U.001	UV radiation	3	NE[b]	[45]
Brewery wastewater	*R. sphaeroides* O.U.001	Thermal	0.22	-	[19]
Olive mill wastewater	*Rhodopseudomonas palustris* 42OL	dry-Azolla and granular active carbon	1.03	-	[46]
Olive mill wastewater	*Rhodopseudomonas palustris* 6A	dry-Azolla and granular active carbon	0.31	-	[47]
Pulp and paper mill effluent	*R. sphaeroides* NCIMB8253	Ultra-sonication (amplitude 60%, time 45 min)	5.77	424 %	[48]
Pulp and paper mill effluent	*R. sphaeroides* NCIMB8253	Ultra-sonication (amplitude 30%, time 10 min)	9.62	66.7 %	[49]

Combination of palm oil and pulp and paper mill effluents	*R. sphaeroides* NCIMB8253	Ultra-sonication on combined effluents (amplitude 70%, time 45 min)	8.72	86.7 %	[50]
Combination of palm oil and pulp and paper mill effluents	*R. sphaeroides* NCIMB8253	Ultra-sonication of both the bacterial cells and combined effluents	14.43	44.6 %	[51]
Brewery wastewater	*R. sphaeroides* DSM 158	Banana peels	0.40	182 %	This study

[a] The increase percentage in biohydrogen yield after pre-treatment was calculated from given data according the following equation: $\frac{Hydron\ Yield_{after\ pretreatment} - Hydrogen\ Yield_{before\ pretreatment}}{Hydrogen\ Yield_{before\ pretreatment}}$ x100; NE[b]: negative effect.

5.5 Summary

This study was designed to investigate the impacts of banana peels pre-treatment on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM using brewery effluent (BE) in a batch bioreactor. The standard medium (SM) was blended with untreated brewery wastewater (Bu) and treated one (Bt) in different volume ratios. The maximum hydrogen production yield (408.33 mL H_2 $L^{-1}_{wastewater}$) was achieved from the substrate, which was composed of 50 % BE (pre-treated with 1 g L^{-1} of banana peels for 2 h) and 50 % SM. This best result was 2.7-folds higher than those applying the same percentage of raw BE without any pre-treatment. The applied banana peels pre-treatment stage led to decrease ammonium concentration and enhance C/N ratio of BE which resulted in enhanced biohydrogen production. The results of this study indicated that banana peels pre-treatment can significantly enhance the cumulative hydrogen production. In current, study brewery wastewater hasn't been applied alone as a single substrate and it was supplemented with standard medium. This approach of supplementations needs to improve to overcome the economic limitations of use expensive synthetic medium. Therefore, further studies can be achieved to supplement the brewery wastewater with another real wastewater to complement each other.

Chapter 6

Impact of metal ions and EDTA on photo-fermentative hydrogen production by *Rhodobacter sphaeroides* using a mixture of pre-treated brewery and restaurant effluents

Chapter 6 Impact of metal ions and EDTA on photo-fermentative hydrogen production by *Rhodobacter sphaeroides* using a mixture of pre-treated brewery and restaurant effluents

6.1 Introduction

Hydrogen gas is recognized as a future energy carrier with a high energy content and non-polluting emissions [15, 217, 269]. It can be generated from fossil fuels by different methods such as steam reforming [270, 271]. Hydrogen can be also produced via several biological methods such as photofermentation and dark fermentation [23, 272]. In photofermentation, purple nonsulfur (PNS) bacteria can reduce H^+ to hydrogen gas by utilizing the derived energy from both the light and oxidation of organic substrates [11]. It is well known that the nitrogenase enzyme plays a crucial role in photo-fermentative hydrogen production pathways by catalysing the reduction of protons to hydrogen [10, 203]. In the absence of nitrogen (N_2), nitrogenase enzyme catalyses the following reaction (Equation 6.1) to generate biohydrogen [87].

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP \quad (6.1)$$

Molybdenum (Mo), iron (Fe) and vanadium (V) are present in the catalytic positions of nitrogenase enzyme, accordingly the enzyme can be classified as Mo– nitrogenase, V–nitrogenase, Fe-nitrogenase respectively. Mo-nitrogenase can be considered the most effective nitrogenase enzyme [11]. The active sites of Mo-nitrogenase are Fe protein and Mo-Fe protein [10]. The presence of iron and molybdenum in the structure of nitrogenase indicates that these metals have significant influences on enzyme activities that catalyse bioreactions [30]. The presence of Fe can be considered as a stimulator for photofermentative hydrogen production since Fe is available in the structure of nitrogenase [273]. Furthermore, it is available in different electron carriers in photosynthetic system such as ferredoxin [30].

It was reported that in the absence of iron and molybdenum the nitrogenase activity reduced [35, 204]. It has conclusively been confirmed that the addition of iron and

molybdenum metals leads to enhance photofermentative hydrogen production [30-32]. However, the optimum concentrations for these metals showed significant variations in the literature due to using various substrates and microorganisms [31-34]. Zhu et al. [37] reported that biohydrogen production of *Rhodobacter sphaeroides* was enhanced from 53 to 459 mL when Fe concentration was elevated from 0 to 2.4 mg L^{-1}. However, further increase in Fe concentration to resulted in decrease biohydrogen production. Özgür et al. [274] also observed that the higher Fe concentration (1 mM) resulted in inhibit photofermentative hydrogen production due to the toxic impact of higher Fe concentration. Kars et al. [35] also found that at the optimum Mo concentration (0.16 µM), biohydrogen production yield was 64 mmol H_2 L^{-1}. However, it was only 15 mmol H_2 L^{-1} at no Mo supplementation. Yokoi et al. [275] observed that the maximum cumulative photofermentative hydrogen of *Rhodobacter sphaeroides* M-19 using starch waste was 112 mL under 100 µM and 10 mg L^{-1} of Mo and EDTA concentrations, respectively, while cumulative photofermentative hydrogen was 52 mL under control condition. Furthermore, extensive literature reviews have indicated that the majority of the studies used defined mediums for enhancing the photofermentative hydrogen production by supplementing with iron and molybdenum metals [32-37]. It should be noted, however, that very few studies have applied actual wastewater as a substrate for biohydrogen production via photo-fermentation [30, 31, 275].

It was found that ethylenediaminetetraacetic acid (EDTA) could enhance the activity of nitrogenase enzyme and reduce hydrogenase activity. Ren et al. [36] reported that at 0.3 g EDTA L^{-1} culture medium, the maximum photofermentative hydrogen production achieved was 3325 mL H_2 L^{-1} culture, with a maximum nitrogenase activity for *Rhodopesdomonas faecalis* RLD-53 at 1331.9 µL-C_2H_4 h^{-1} mg dry $weight^{-1}$. However, high concentrations of EDTA in the culture medium (0.6-0.7g EDTA L^{-1}) inhibit both photofermentative hydrogen generation and the bacterial growth. Kern et al. [34] investigated the impact of EDTA on hydrogen generation and nitrogenase activity of *Rhodospirillum rubrum.* They found that the photofermentative hydrogen generation increased from 2.3 to 2.6 L H_2 L^{-1} culture when EDTA increased from 0 to 0.5 mM.

Restaurants generate high quantities of oily wastewater which could be produced by different restaurant activities such as cooking food. The restaurant wastewater is heavily loaded with organic compounds, but its composition is varied with time and

the type of prepared food [276]. A high amount of brewery wastewater with chemical oxygen demand (COD) value between 2000 - 6000 mg L^{-1} is also generated by the brewery industry. Previously, brewery effluent was applied as substrate to produce photofermentative hydrogen production by mixing/blending with standard medium [19 , 42]. Seifert et al. [19] reported that the maximum result of 220 mL H_2 L^{-1} of *Rhodobacter sphaeroides* O.U.001 was achieved at 10% brewery effluent blended with standard medium. Al-Mohammedawi et al. [42] applied *Rhodobacter sphaeroides* DSM 158 and brewery effluent to produce biohydrogen under the light intensity of 126 Wm^{-2} and observed the maximum hydrogen production of 408.3 mL H_2 L^{-1} using equal ratios of banana peels pre-treated brewery effluent blended with standard medium. However, blending the brewery effluents with standard medium could be considered infeasible for large scale application. Therefore, combining different wastewater/effluents qualities could be a cost-effective options.

In this study, the influence of sole-addition of iron, molybdenum, EDTA, and co-addition of (iron + molybdenum) on the photo fermentative hydrogen production using pre-treated blended brewery and restaurant effluents are investigated and analysed.

6.2 Materials and methods

6.2.1 Brewery and restaurant effluents

Effluents of brewery (BE) and restaurant (RE) were collected from a local brewery production plant and a Chinese restaurant in Perth, Western Australia, respectively.

Both wastewater samples were immediately filtered using 0.2 μm nylon microfilters and sterilized at 121 °C for 15 min and then stored in the fridge (4 °C). The characteristics of both brewery (BE) and restaurant (RE) effluents used in this study were described previously in Chapter 3.

6.2.2 Pre-treatment stage of effluents with banana peels

The waste banana peels particles were introduced in current study as an effective pre-treatment stage to reduce the inhibitors (such asNH_4^+) and enhance the nutritional value of the feedstock, thereby improving photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM. Preparation and characterization of banana peels have been discussed in chapter 3. A mixture of different ratios of brewery and restaurant effluents (Table 6.1) was treated with 1 g L^{-1} of waste banana peels particles. Processed banana peels were thoroughly mixed with the effluent mixture at an

agitation speed of 250 rpm for 2 h. This process was carried out at a pH value of around 7.2. At the end, the mixture was rested for 2 h to settle down. After settlement, the above liquid was pre-filtered by Whatman^{TM} filter paper (0.45 µ) to separate treated effluents from the solids. Prior to start of each photo fermentation experiment, the treated effluent was also sterilized by autoclaving at 120 °C for 15 min.

Table 6. 1 Applied medium at different blending percentages.

Medium	%(v/v) of Brewery effluent (BE)	%(v/v) of Restaurant effluent (RE)
M1	0	100
M2	10	90
M3	20	80
M4	30	70
M5	40	60
M6	100	0

6.2.3 Photofermentative hydrogen medium

Rhodobacter sphaeroides DSM 158 was anaerobically activated and grown in modified Biebl and Pfennig medium [223] under sterile conditions and light irradiated by the procedure described previously in sections 3.2 and 3.3. The treated effluent in Table 6.1 was used as a sole medium for biohydrogen production in this study. To investigate the influence of Iron, Molybdenum, and EDTA on the biohydrogen production, different concentrations (30 -110 µM of Iron, 8-20 µM of molybdenum, 0.1 -0.5 g EDTA L^{-1}) were added to the medium. $FeSO_4.7H_2O$ and $Na_2MoO_4.2H_2O$ were used as a source for Iron and Molybdenum, respectively.

The batch experiments of hydrogen production were accomplished in 180 mL clear glass photo-bioreactor. The produced gas mixture was collected by water displacement method. The experimental setup and hydrogen production procedure were detailed in Chapter 3. Gas compositions, biomass, light intensity, chemical oxygen demand (COD), total nitrogen (TN), total organic carbon (TOC), concentrations of Fe and Mo and pH measurements have been described in chapter 3. The data of all measurements represented the average of three independent analyses with standard deviation less than 5 %.

6.3 Result and discussion

6.3.1 Influence of wastewater concentration on biohydrogen production

Different treated blended effluents of brewery and restaurant effluents (M1- M6) (Table 6.2) were applied to determine the most effective blended effluent as an alternative medium for biohydrogen production. As shown in Figure 6.1, the cumulative hydrogen produced using only restaurant effluent (M1) enhanced 74% (from 49 to 83 ml) by increasing the brewery effluents from 10 to 30%. However, it was declined when the brewery effluents increased higher than 30%. This behaviour can be attributed mainly to the impact of C/N ratio and ammonium concentration. The best result of 83 mL was achieved at 30% BE and 70% RE (M4), where C/N ratio and NH^{+}_{4} are 27.32 and 11.68 mg L^{-1} respectively (Figure 6. 1).

Table 6. 2 Cumulative biohydrogen and characteristics of the different treated mixtures of brewery and restaurant effluents.

Medium	%(v/v) BE	%(v/v) RE	pH	C/N	COD mg L^{-1}	NH^{+}_{4} mg L^{-1}	Fe mg L^{-1}	H_2 mL
M1	0	100	7.2	32.50	3290	6.40	2.30	49
M2	10	90	7.2	30.77	3196	8.16	2.18	54
M3	20	80	7.2	29.04	3102	9.92	2.06	71
M4	30	70	7.2	27.32	3008	11.68	1.94	83
M5	40	60	7.2	25.59	2914	13.44	1.82	57
M6	100	0	7.2	15.22	2350	24.00	1.10	5

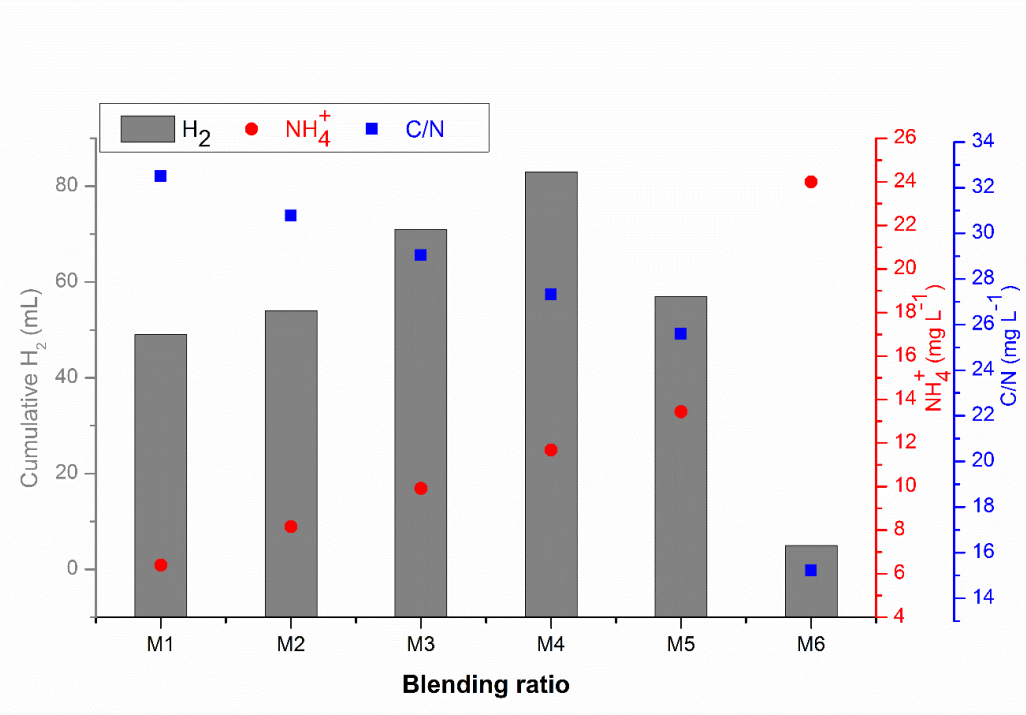

Figure 6. 1 Influence of the effluents blending ratio on the biohydrogen production, C/N ratio and ammonium concentration

It is obvious from literature that the value of C/N ratio is essential in biohydrogen production [87, 260]. A low C/N ratio was indicated to inhibit nitrogenase activity thus reducing biohydrogen production while increase the biomass concentration which reduces the light penetration into reactor [261, 262]. It was also reported that very high C/N led to decrease biohydrogen production [260, 262]. Previous studies have revealed the optimal value of C/N for *Rhodobacter sphaeroides* was from 13 to 35 [92, 96, 111, 229]. Nitrogenase activity is also impacted by the presence of ammonium. Zheng et al. [266] reported that with ammonium concentration higher than 17 mg L^{-1} could reduce the biohydrogen production. A similar finding was observed by Waligórska et al. [265] where they demonstrated that the presence of ammonium with concentration higher than 17 mg L^{-1} inhibited biohydrogen production.

6.3.2 Influence of iron on biohydrogen production and bacterial growth

In order to investigate the influence of Fe^{2+} on the biohydrogen production and bacterial growth, $FeSO_4.7H_2O$ was added to the treated medium M3 (blend of 30% brewery and 70% restaurant effluents). The iron supplementation was increased from 0 to 110 µM during batch experiments using *Rhodobacter sphaeroides* 158 DSM, while the experiment with no addition represents the control test. The concentrations

of iron supplementation were chosen based on previous literature [30, 33, 35]. The influence of Fe^{2+} on the photofermentative hydrogen production in batch process mode is shown in Figure 6.2.

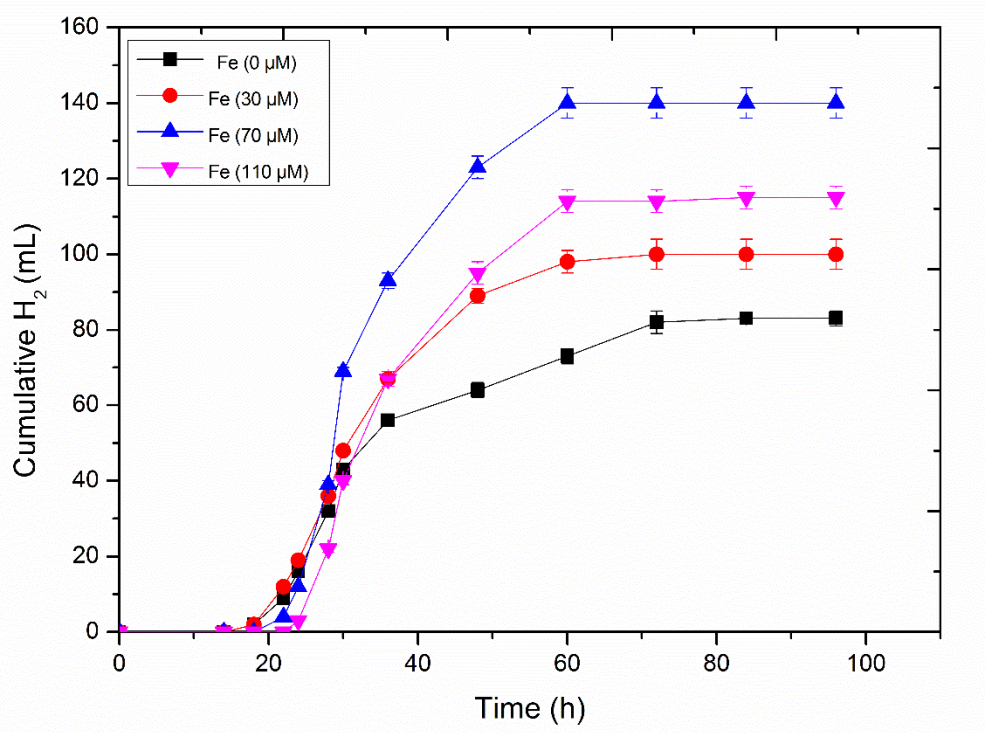

Figure 6. 2 Effects of iron supplementation on the photofermentative hydrogen production by *Rhodobacter sphaeroides* 158 DSM using a mixture of pre-treated brewery and restaurant elements.

Iron concentration in the culture medium was observed to impact the photo fermentative hydrogen generation. The cumulative hydrogen for all iron supplemented experiments was higher than the one obtained by the control test. The results indicated that the cumulative hydrogen production was enhanced from 83 to 140 mL by increasing the iron supplementation from 0 to 70 µM, showing the noticeable positive role of iron addition on photofermentative hydrogen production. However, a further increase of iron concentration (above 70 µM) reduced the cumulative hydrogen production, indicating a negative impact of high iron content on hydrogen production.

In purple non sulfur bacteria, photofermentative hydrogen production is impacted by the nitrogenase enzyme activity. The structure of this enzyme has 24 iron in each molecule. Also, several electron carriers of photosynthetic system such as ferredoxin have iron in their structure. Therefore, the iron concentration in the medium affects the photofermentative hydrogen production [30, 31, 33]. However, iron concentration above the optimum could cause the coagulation which changes the surface charge

distribution of the cells that would lead to their aggregation [37]. Compared to control, the maximum cumulative hydrogen of 140 mL was achieved at 70 µM representing a 69% enhancement. At control, cumulative hydrogen was generated after a lag time of 18 h. After the lag time, hydrogen production was increased at a rate of around 1.5 mL h^{-1}. On the other hand, the lag times were 18, 22, and 24 h for the iron supplemented conditions with Fe concentrations at 30, 70, and 110 µM, respectively. It was also observed that after the lag time, cumulative hydrogen production was increased at rates of 2.2, 3.5, and 3 mL h^{-1} for 30, 70, and 110 µM Fe, respectively. The apparent increase in the hydrogen production rates for iron supplemented conditions compared to control were also observed by Eroglu et al. [30], they found that after 24 h of a lag time, cumulative hydrogen production under iron supplemented conditions was increased at a rate of about 2 mL h^{-1}, while the lag time was 15 h and the rate of cumulative hydrogen production was 1 mL h^{-1} for the control.

Figure 6.3 shows the influence of iron on the bacterial growth under batch process conditions. The results indicated that the influence of iron on the bacterial growth was lower compared to its influence on biohydrogen production. Furthermore, the bacterial growth for all iron supplemented experiments was higher than control. It was also observed that the impact of iron supplemented medium was only observed after 18 h. The maximum growth was achieved around 48 h for 30 and 70 µM Fe, and 42 h for 110 µM iron supplemented cases, while the maximum growth of control was observed at 36 h. Initially, the bacterial growth of all iron supplemented experiments increased at a high rate during the first 30 h, after which, the bacterial growth increased with declined rate until reaching the stationary phase at 42-48 h.

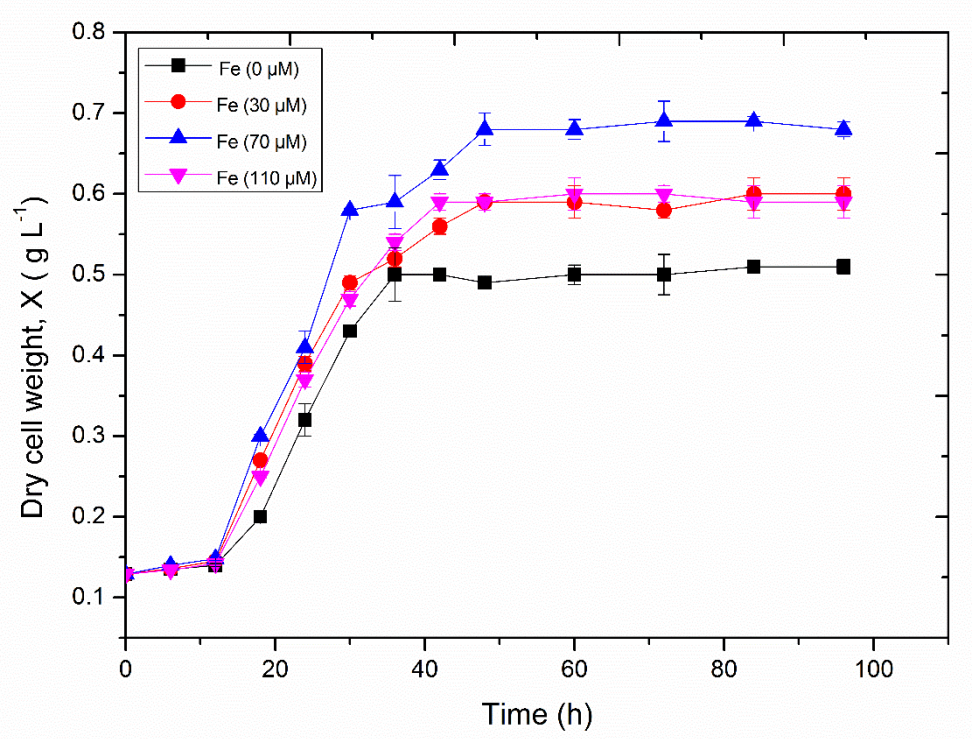

Figure 6. 3 Effects of iron supplementation on the bacterial growth.

6.3.3 Influence of molybdenum on biohydrogen production and bacterial growth

To understand the influence of molybdenum on the biohydrogen production and bacterial growth, different concentrations (8-20 µM) of Mo in the treated medium (M4: blend of 30% brewery and 70% restaurant effluents), were investigated. The concentrations of Mo supplementation were selected based on previous literature [30, 35]. Cumulative hydrogen is presented in Figure 6.4. As shown in Figure 6.4, cumulative hydrogen production was found to be enhanced with increasing Mo concentrations. At lower MO concentration (8 µM), the enhancement was only 10.6% compared to the control medium, while the maximum hydrogen production of 105 mL was achieved at Mo concentration of 14 µM representing an increase of 27% compared to control. However, further increase in the concentration of molybdenum (above 14 µM) reduced the cumulative hydrogen. The structure of nitrogenase enzyme has molybdenum, showing that the cumulative hydrogen production can be impacted by the concentration of molybdenum [30, 35]. As mentioned previously, under control condition, cumulative hydrogen was produced after a lag time of 18 h with a rate of about 1.5 mL h^{-1}. Similarly, the lag time for all Mo supplemented experiments was 18

h. Furthermore, the rates of hydrogen generation were about 2.1, 3.4- and 1.8 mL h^{-1}, for 8, 14, and 20 µM Mo, respectively.

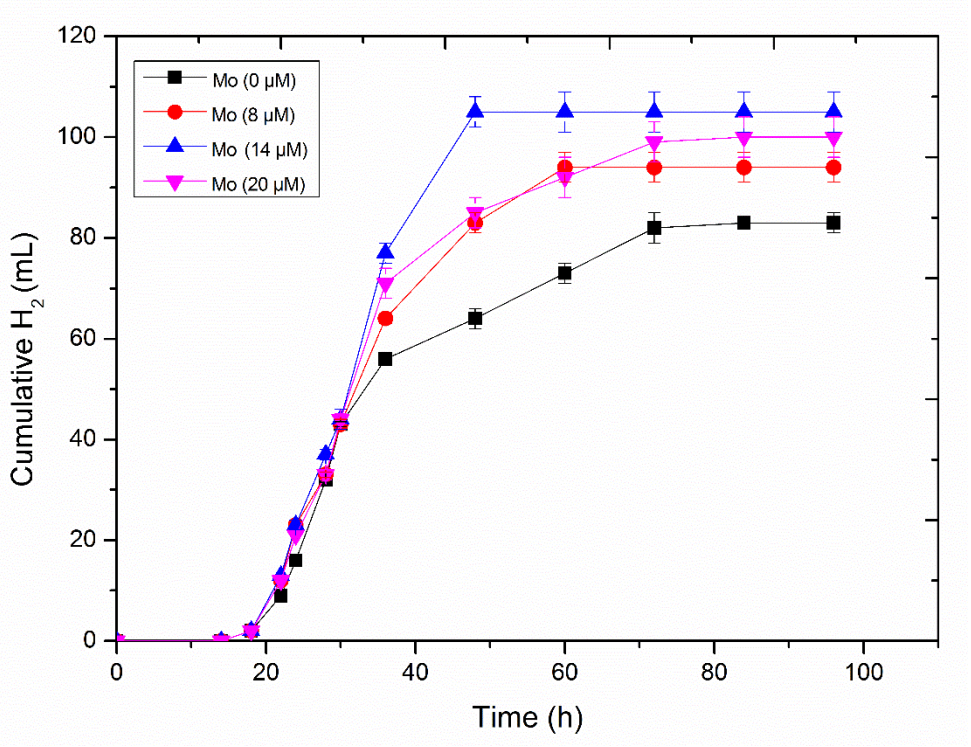

Figure 6. 4 Effects of molybdenum supplementation on the photofermentative hydrogen production by *Rhodobacter sphaeroides* 158 DSM using a mixture of pre-treated brewery and restaurant elements.

Figure 6.5 shows the influence of Mo on bacterial growth during batch experiments. From Figure 6.3 and Figure 6.5, it can be seen that the influence of Mo on bacterial growth was lower than the influence of iron on bacterial growth. As shown in Figure 6.5, the bacterial growth of media with 8 and 14 µM molybdenum was higher than the growth of control and 20 µM Mo. Figure 6.4 and Figure 6.5 indicated that the maximum hydrogen production was obtained at Mo concentration of 14 µM, while the maximum biomass was achieved at Mo concentration of 8µM. The behaviour of bacterial growth curves for control and all Mo supplemented were comparable. This observation is in agreement with the previous study by Kars et al. [35], which reported that the maximum biohydrogen production was achieved at Mo concentration of 16.5 µM, while the maximum bacterial growth was achieved at 0.0165 µM Mo and control medium (without Mo). They concluded that the lack in Mo concentration did not interfere with bacterial growth.

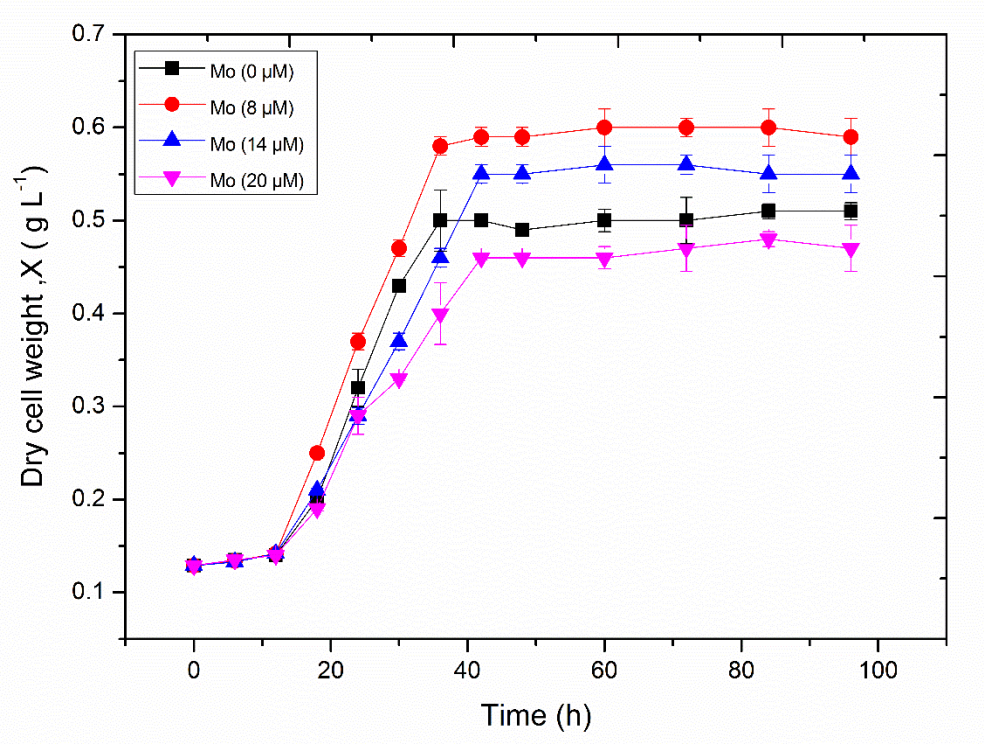

Figure 6. 5 Effects of molybdenum supplementation on the bacterial growth.

6.3.4 Influence of iron and molybdenum co-addition on biohydrogen production and bacterial growth

From the previous sections, it was clear that the positive impact of sole-addition of iron on biohydrogen production was higher than that achieved by sole-addition of molybdenum, where the maximum enhancement of cumulative biohydrogen production was 69% under iron-supplemented condition at 70 µM. Therefore, in order to investigate the influence of iron plus molybdenum on the biohydrogen production and bacterial growth, the concentration of iron was kept constant at 70 µM, while molybdenum supplementation was varied from 0 to 20 µM in the treated medium (M4: blend of 30% brewery and 70% restaurant effluents).

Figure 6.6 shows the influence of iron plus molybdenum co-addition on the photofermentative hydrogen production under batch processes. As shown in Figure 6. 6, the amount of hydrogen production obtained from all co-addition experiments was higher than the control experiment. The highest obtained hydrogen production was 160 mL, when the concentrations of both iron and molybdenum are 70 µM and 8 µM, respectively, indicating an enhancement of 93% with respect to control experiment. However, further increasing the concentration of molybdenum (>8 µM) will reduce the cumulative hydrogen production. The results also revealed that co-addition of iron

plus molybdenum favourable photofermentative and produced the highest hydrogen (160 ml), than sole-addition of iron (140 mL, 70 µM) and molybdenum (105 mL, 14 µM). Furthermore, the optimum concentration of molybdenum (8 µM) under co-addition conditions was lower than that found during the sole-addition experiments (14 µM). It is apparent from Figure 6.6 that lag times of hydrogen production for the control and other tests were around 18 h.

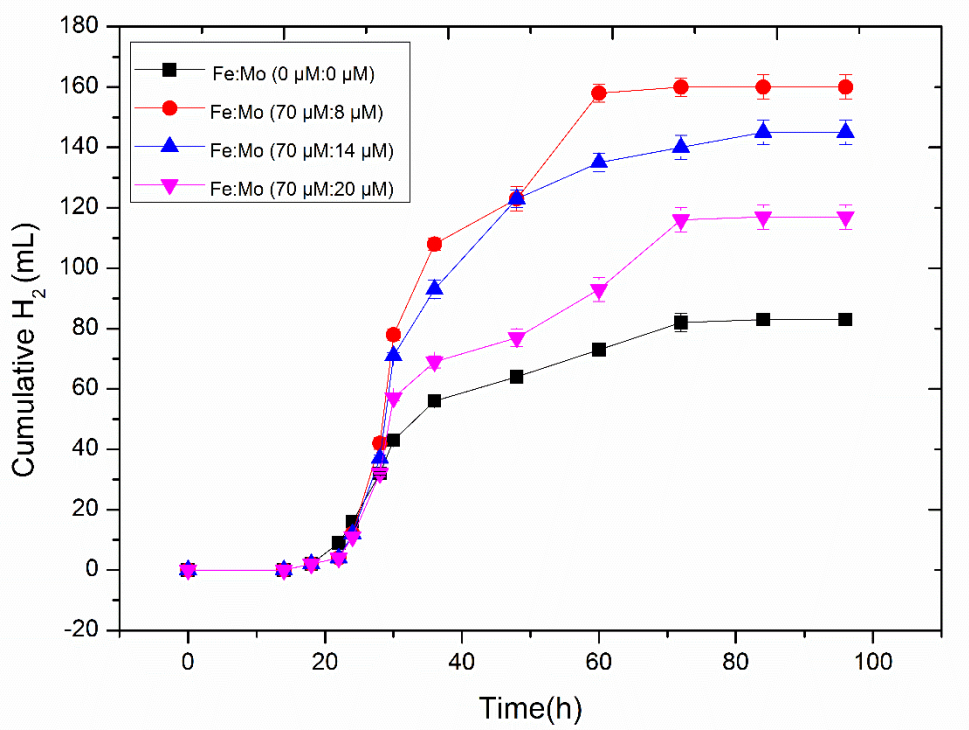

Figure 6. 6 Effects of iron and molybdenum co-addition on the photofermentative hydrogen production by *Rhodobacter sphaeroides* 158 DSM using a mixture of pre-treated brewery and restaurant elements.

Figure 6.7 illustrates the influence of iron plus molybdenum co-addition on the biomass growth under batch process. As can be seen from this figure the biomass produced (0.7 g L^{-1}) for Fe:Mo 70:8 and 70:14 supplementation tests were higher than the biomass obtained from the control experiment (0.5 g L^{-1}). However, the obtained biomass using high Mo concentrations (Fe:Mo = 70:20) was lower than the one obtained by the control. Figures 6.3, 6.5, and 6.7 indicate that the behaviour of bacterial growth for Fe:Mo = 70:8 and 70:14 were comparable with those achieved by sole-addition of iron-supplemented. On the other hand, the behaviour of bacterial growth curve for Fe:Mo = 70:20 was similar with those achieved by sole-addition of molybdenum-supplemented.

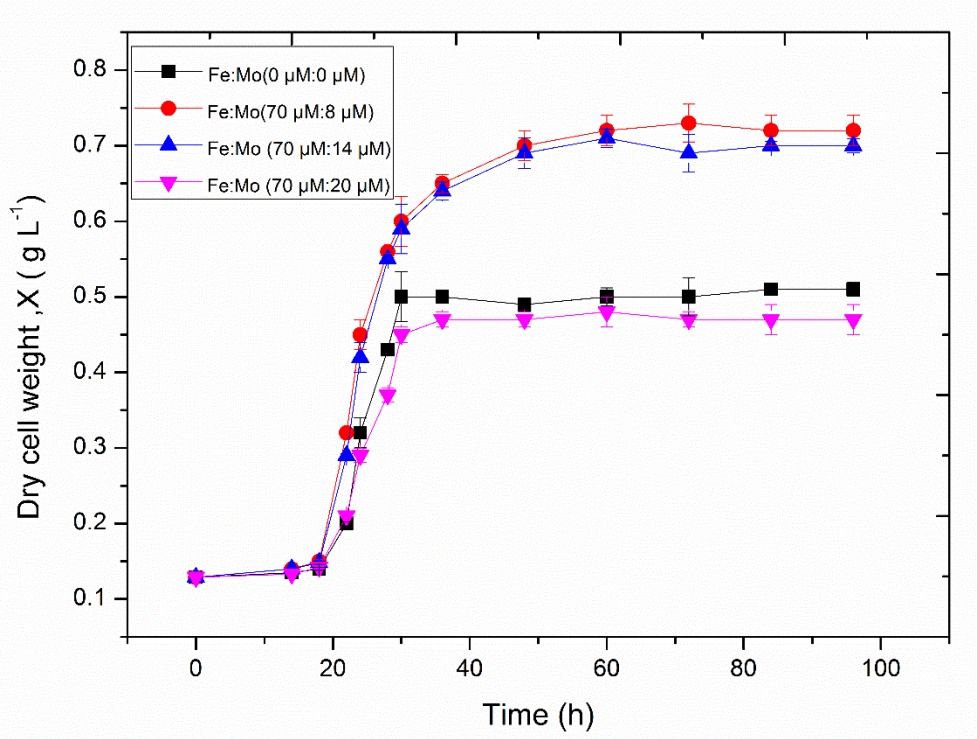

Figure 6. 7 Effects of iron and molybdenum co-addition on bacterial growth.

6.3.5 Influence of EDTA on biohydrogen production and bacterial growth

In order to investigate the influence of EDTA on the biohydrogen production and bacterial growth, the EDTA supplementation was varied from 0 to 0.5 g L^{-1}, while the concentrations of iron and molybdenum supplementation were kept constant at 70 µM and 8 µM of iron and molybdenum, respectively. The concentrations of EDTA supplementation were selected based on previous literature [34, 36]. The cumulative hydrogen production as a function of time is represented in Figure 6. 8. As shown in this figure, the lower concentration of EDTA supplementation (0.1 g L^{-1}) enhanced the hydrogen production, while the higher concentration of EDTA (0.5 g L^{-1}) declined the hydrogen production. It can be also seen that the EDTA addition of 0.2 g L^{-1} considerably increased the cumulative hydrogen production (192 mL), representing 131% enhancement compared to control. EDTA is able to increase the solubility of metal ions that lead to increase nitrogenase activity [34, 36]. However, high concentration of EDTA is also known to chelate the metal ion and decline the availability of metal ions in the culture medium [33, 36]. Figure 6.8 shows that the lag time of photofermentative hydrogen production was nearly 18 h when EDTA

supplementation was increased from 0 to 0.3 g $L^{-1,}$ while it was about 28 h at 0.5 g L^{-1}.

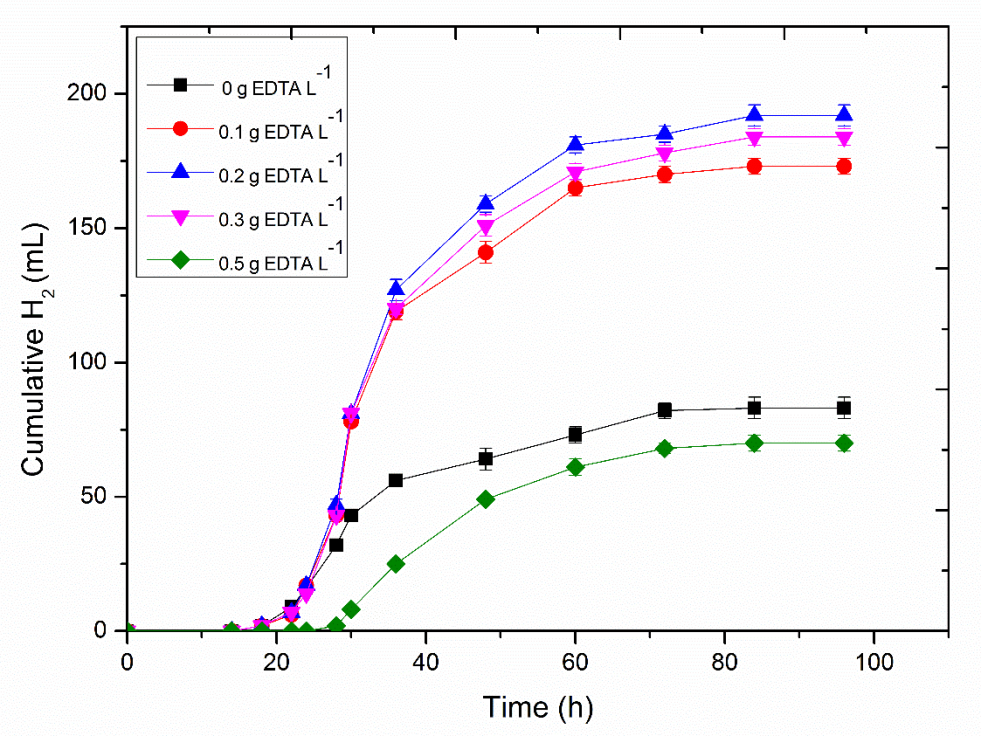

Figure 6. 8 Effects of EDTA on the photofermentative hydrogen production by *Rhodobacter sphaeroides* 158 DSM using a mixture of pre-treated brewery and restaurant wastewater, at Fe:Mo concentrations of 70 µM:8 µM.

Figure 6.9 illustrates the influence of EDTA on the biomass production. As shown in this figure, the biomass at EDTA supplementations of 0, 0.1, 0.2, 0.3 and 0.5 g L^{-1} increased rapidly as a function of time with high growth rates during the first 30 h, however, beyond this time the growth rate decreased and then it reached to a stationary phase at around 60 h after inoculation. It is apparent from Figure 6.9 that biomass produced were comparable for all EDTA supplemented conditions. This finding is consistent with findings of a previous study by Ren et al. [36], which found that the bacterial growth patterns were comparable for different EDTA concentrations (0 - 0.5 g L^{-1}). Concluding that the biomass was not a crucial factor impacting biohydrogen production under the above-mentioned EDTA concentration.

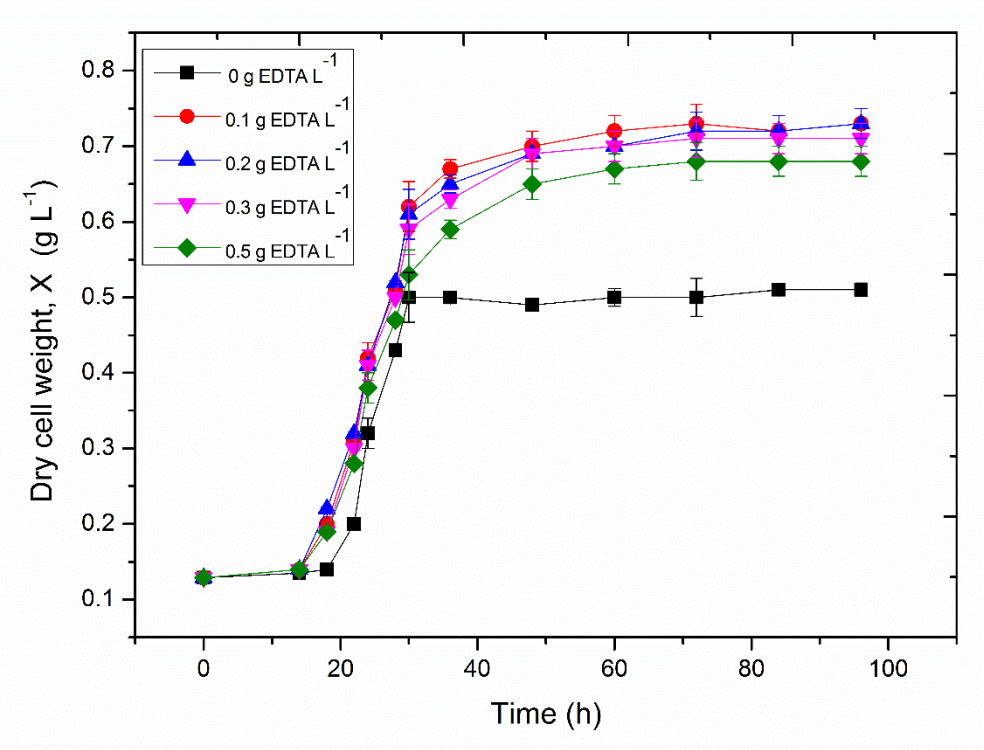

Figure 6. 9 Effects of EDTA addition on the bacterial growth, at Fe:Mo concentrations of 70 µM:8 µM.

There have been several studies in the literature reporting the impact of metal ions or EDTA on photofermentative hydrogen production. Liu et al. [33] reported that the photofermentative hydrogen production from acetate was enhanced from 2.33 to 2.78 mol H_2 mol^{-1} acetate when increasing the iron concentration from 0 to 80 µM. They also reported that higher iron concentration (above 80 µM) inhibited the photo fermentative hydrogen production.

Eroglu et al. [30] investigated the effects of iron and molybdenum on photofermentative hydrogen from olive mill wastewater by *Rhodobacter sphaeroides* O.U.001. They observed that compared to control (40 mL), the maximum cumulative hydrogen (125 mL) was achieved under iron supplementation (100 µM), while it was only 62 mL under molybdenum supplementation (16.5 µM). Zhu et al. [37] showed that the photofermentative hydrogen production from sodium lactate was increased from 53 to 459 mL when increasing the iron concentration from 0 to 2.4 mg L^{-1}. They also observed that further extend of iron concentration to 3.2 mg L^{-1} led to decline the photofermentative hydrogen production.

The addition of Mo into the medium was also reported to impact biohydrogen production and bacterial growth of *R. sphaeroides* O.U. 001 [35]. It was observed that

biohydrogen yield was 0.84 L L^{-1} medium at Mo optimum concentration of 16.5 µM. However, in the absence of Mo, the experimental results indicated that there was no biohydrogen production. The enhancement of photofermentative hydrogen production rate with Mo supplementation also reported by Salih and Maleek [277], who noticed that at 0.3 mg L^{-1} of Mo, the rate of biohydrogen production was 625 mL L^{-1} day^{-1} while it was 680 mL L^{-1} day^{-1} at 1.6 mg L^{-1} of Mo. In different study Özgür et al. [274] studied the influence of Mo addition on biohydrogen production by *R.capsulatus* DSM1710 using potato steam peels hydrolysate as feedstock. They observed that Mo supplementation with concentration of 0.16 µM led to produce about 64 mmol H_2 L^{-1}, while the biohydrogen production yield was only 15 mmol H_2 L^{-1} at no Mo supplementation.

Yokoi et al. [275] investigated the impact of molybdenum and EDTA on photofermentative hydrogen from starch waste by *Rhodobacter sphaeroides* M-19. They observed that compared to control (52 mL), the maximum cumulative hydrogen of (112 mL) was achieved under molybdenum supplementation (100 µM) and EDTA concentration of 10 mg L^{-1}. Kern et al. [34] found that the photofermentative hydrogen production by *Rhodospirillum rubrum* increased from 2.3 to 2.6 L H_2 L^{-1} culture when EDTA increased from 0 to 0.5 mM. It was noted that the co-addition of 0.5 mM of EDTA and 42 µM of Fe^{2+} resulted in the highest nitrogenase and photofermentative hydrogen yield of 6.8 L L^{-1} medium. The influence of different concentrations EDTA (0 – 0.7 g L^{-1}) addition on photofermentative hydrogen production by *Rhodopesdomonas faecalis* RLD-53 was also studied by Ren et al. [36], they revealed that at 0.3 g EDTA L^{-1} supplemented culture medium, the maximum amount of photofermentative hydrogen production by *Rhodopesdomonas faecalis* RLD-53 was achieved (3325 mL H_2 L^{-1} culture). However, they observed that high concentrations of EDTA (above 0.6 g EDTA L^{-1} culture medium) led to inhibit photofermentative hydrogen generation and bacterial growth.

6.4 Summary

The major objectives of this study were investigation the influence of metal ions and EDTA on photofermentative hydrogen production using pre-treated (by banana waste) mixture of brewery and restaurant effluents as a feedstock to complement each other. The influence of different concentrations of iron, Fe (30 -110 µM), molybdenum, Mo (8-20 µM), and EDTA (0.1-0.5 g L^{-1}) on the photofermentative

hydrogen production and bacterial growth by *Rhodobacter sphaeroides* 158 DSM was investigated and discussed. The medium employed in this study is a blend/mixture of pre-treated brewery and restaurant effluents. Comparing with the control experiment, the results show that sole-addition of Fe at 70 µM, sole-addition of Mo at 14 µM, and co-addition of Fe and Mo at Fe:Mo of 70 µM:8 µM to the pre-treated mixture of 30% brewery and 70% restaurant effluents, could enhance the cumulative biohydrogen production to 140 mL (69% increased), 105 mL (27% increased), and 160 mL (93% increased), respectively. The results also revealed that the addition of EDTA should be optimized to avoid the chelation of the added metal ions (Fe, Mo). At constant Fe:Mo concentrations of 70 µM:8 µM, the highest biohydrogen production of 192 mL was obtained when 0.2 g L^{-1} of EDTA was added to the blended effluents. Hence, co-addition of (iron+ molybdenum+ EDTA) was observed to have a higher impact on photo fermentative hydrogen production by *Rohodobacter sphaeroides* 158 DSM more than those achieved by the other addition.

Chapter 7

Integrated banana peels waste and ultra-sonication technology as feedstock pre-treatment to enhanced photo fermentative hydrogen production.

Chapter 7 Integrated banana peels waste and ultra-sonication technology as feedstock pre-treatment to enhanced photo fermentative hydrogen production.

7.1 Introduction

Hydrogen can be considered as one of the clean and future source of renewable energy. Different techniques have been implemented for hydrogen production, such as, physicochemical processes and biological processes [23, 70]. The biological method for hydrogen production (biohydrogen) includes heterotrophic (photo and dark fermentation) and autotrophic (bio photolysis) [278]. Among various biohydrogen production, photo fermentation by purple non-sulfur bacteria has been reported widely, due to its ability to (i) utilize a wide wavelength of light (520-680 nm), (ii) utilize wastewater as a carbon source [185], (iii) produce considerable amounts of biohydrogen under different environmental conditions [48].

Different wastewater qualities (pre-treated or raw; sole or mixed with standard medium) have been utilized for photofermentation hydrogen production, such as brewery wastewater [19, 42, 246], olive mill wastewater [17, 47], palm oil mill effluent [51], dairy wastewater [43], and tofu [266]. Thus, photofermentation process can address both the production of a clean fuel (biohydrogen) and the waste reduction.

Restaurants wastewater can be considered as serious pollution source for environmental [279]. The effluent has been commonly generated from the leftovers of food due to washing activities such as cleaning the raw food and washing dishes [280]. Restaurants produce a considerable amount of organic rich wastewater [276] and organic matters can be very biodegradable [281] . Also, huge amounts of brewery wastewater are produced by brewery industry which has a chemical oxygen demand (COD) up to 6000 mg L^{-1} and nitrogen compounds up to 80 mg L^{-1} [244, 245]. Brewery wastewater has been also applied to produce biohydrogen using photo fermentation [19, 42, 282].

However, the previous studies applying brewery wastewater have not used it alone as a single substrate [19, 42]. Thus, brewery wastewater was supplemented with standard

medium. Al-Mohammedawi et al. [42] applied *R. sphaeroides* DSM 158 and brewery wastewater to generate biohydrogen and observed the maximum hydrogen production potential was 408.33 mL H_2 L^{-1} at 50% brewery wastewater in 50% standard medium. In different study, Seifert et al. [19] achieved 220 mL H_2 L^{-1} using *R. sphaeroides* O.U.001 10% brewery wastewater in 90% standard medium. This approach of supplementations reduced the importance of use brewery wastewater as feedstock to produce photofermentative hydrogen. Therefore, one objective of current study was supplementation of brewery wastewater with restaurant wastewater to complement each other to enhance photofermentative hydrogen production. Different strategies could be applied to enhance the photofermentative hydrogen production utilizing the wastewater effluents, of these; blending two different wastewaters effluents with and without pre-treatment stage [50, 185, 246].

In the literature, serval adsorption pre-treatment steps techniques have been applied before photofermentative hydrogen production process to decrease the toxic characteristics of wastewater that have inhibitory impact on photosynthetic bacteria [42, 44, 46, 47]. In addition, previous studies indicated that use ultra-sonication technique led to enhance photo fermentative hydrogen. For example, Budiman and Wu [50] examined the impact of ultra-sonication pre-treatment stage on biohydrogen production using a combined effluent of palm oil and pulp and paper mills. They found that at amplitude of 70% and ultra-sonication time of 30 min, photofermentative hydrogen production enhanced from 467 (no pre-treatment) to 872.5 mL H_2 due to increase SCOD/TCOD ratio of a combined effluent. In another study, Hay et al. [48] reported that use amplitude of 60% and ultra-sonication time of 45 min gave the maximum biohydrogen yield of 5.77 mL H_2 from pulp and paper mills and *R. sphaeroides* NCIMB. However, no study has been conducted to investigate integrated adsorption (by banana peels waste) and ultrasonic technology as feedstock pre-treatment to enhanced photo fermentative hydrogen production. Thus, integrated banana peels waste and ultra-sonication was predicated to increase the bioavailability of organic content and reduce the toxic properties of wastewater. Therefore, integrated two pre-treatment technologies was the novelty and main objective of the current study.

7.2 Materials and methods

7.2.1 Brewery and restaurant effluents

Effluents of brewery (BE) and restaurant (RE) were collected from a local brewery production plant and a Chinese restaurant in Perth, Western Australia, respectively. Both wastewater samples were immediately filtered using 0.2 μm nylon microfilters and sterilized at 121 °C for 15 min and then stored in the fridge (4 °C). The characteristics of both brewery (BE) and restaurant (RE) effluents used in this study were described previously in Chapter 3.

7.2.2 Pre-treatment stage of effluents with banana peels

Preparation and characterization of banana peels have been discussed in chapter 3. A Different blended ratios of brewery and restaurant effluents (0BE:100RE " sole restaurant effluent", 10BE:90RE, 20BE:80RE, 30BE:70RE, 40BE:60RE, 50BE:50RE, and 100BE:0RE "sole brewery effluent") were first pre-treated with 2 g L^{-1} of banana peels particles at an agitation speed of 250 rpm at 30 ^{o}C, and contact time of 90 min

7.2.3 Pre-treatment stage of effluents with ultra-sonication

In order to understand the influence of the ultra- sonication on the photofermentative hydrogen production the pre-treated effluents (30%BE:70%RE) from the first stage (banana peels) was further subjected to a second treatment stage (ultra-sonication) at different amplitudes (A) of 30, 60, and 90%, and ultra-sonication duration (T) of 15, 25, 35 and 45 min. Ultra-sonication was conducted using Q sonica Q700 Sonicator (QSonica LLC., Connecticut, USA) with transducer probe of 12.5 mm dimeter.

7.2.4 Photofermentative hydrogen medium

Rhodobacter sphaeroides DSM 158 was anaerobically activated and grown in modified Biebl and Pfennig medium [223] under sterile conditions and light irradiated by the procedure described previously in sections 3.2 and 3.3.

The batch experiments of hydrogen production were accomplished in 120 mL clear glass photo-bioreactor. The produced gas mixture was collected by water displacement method. The experimental setup and hydrogen production procedure were detailed in Chapter 3. Gas compositions, biomass, light intensity, chemical oxygen demand (COD), total nitrogen (TN), total organic carbon (TOC), concentrations of Fe and Mo and pH measurements have been described in chapter 3. The data of all measurements

represented the average of three independent analyses with standard deviation less than 5 %.

7.3 Result and discussion

7.3.1 Influence of blending ratio on biohydrogen production

Influence of different blending ratios of brewery (BE) and restaurant (RE) effluents (0BE:100RE" sole restaurant effluent", 10BE:90RE, 20BE:80RE, 30BE:70RE, 40BE:60RE, 50BE:50RE, and 100BE:0RE "sole brewery effluent") on photofermentative hydrogen production by *R. sphaeroides* DSM 158, have been investigated before and after pre-treatment with banana peels. As shown in Figure 7.1, all the treated blended ratios have significantly enhanced the accumulated hydrogen in comparison with the untreated same blending ratios, except 100BE:0RE (sole brewery effluent) that shows low accumulated hydrogen even after the pre-treatment, that could be attributed to the high ammonium concentration (24 mg L^{-1}) and low C/N ratio (15.22) (Table 7.1). The maximum cumulative hydrogen of 74 mL was achieved for treated effluents at blending ratio of 30BE:70RE, comparing with 47 mL obtained from the same blending ratio effluent but untreated. The plausible reason for this enhancement in biohydrogen is that both C/N ratio and ammonium concentration improved to the level that favours the biohydrogen production after the pre-treatment with banana peels (Table 7.1).

Table 7. 1 Effects of banana peels pre-treatment stage on different blended ratios of brewery and restaurant effluents.

Substrate	C/N		SCOD (mg L^{-1})		Ammonium (mg L^{-1})	
	Before	After	Before	After	Before	After
0BE:100RE	24.5	32.50	3100	3290	10.20	6.40
10BE:90RE	22.79	30.77	3003	3196	12.82	8.16
20BE:80RE	21.08	29.04	2906	3102	15.44	9.92
30BE:70RE	19.37	27.31	2809	3008	18.06	11.68
40BE:60RE	17.66	25.58	2712	2914	20.68	13.44
50BE:50RE	15.95	23.86	2615	2820	23.30	15.20
100BE:0RE	7.40	15.22	2130	2350	36.40	24.00

In this study, the carbon content of banana peels led to increase C/N ratio of the pre-treated effluents. Achak et al. [249] indicated that 40% (w/w) of the banana peels are carbon compounds. It is well-known that biohydrogen production depends on the

optimum value of C/N ratio [87, 260]. Savasturk et al. [261] reported that a lower C/N ratio inhibited nitrogenase enzyme and hence biohydrogen. On the other hand, higher C/N ratio was also reported to decrease biohydrogen production [260, 262] . Literature reviews have indicated that there are a wide range (13 – 35) for the optimum C/N ratio for *R. sphaeroides* [92, 96, 111, 229]. In addition, previous research reported that banana peels can be applied to remove ammonium from waste effluents [252, 259]. The higher ammonium concentration is known to inhibit the activity of nitrogenase enzyme and hence photo fermentative hydrogen production [264]. Previous studies have shown that the presence of ammonium in wastewater with concentration higher than 17 mg L^{-1} led to reduce biohydrogen production [265, 266]. However, it was reported that biohydrogen was totally inhibited when ammonium concentration was higher than 34 mg L^{-1} [264].

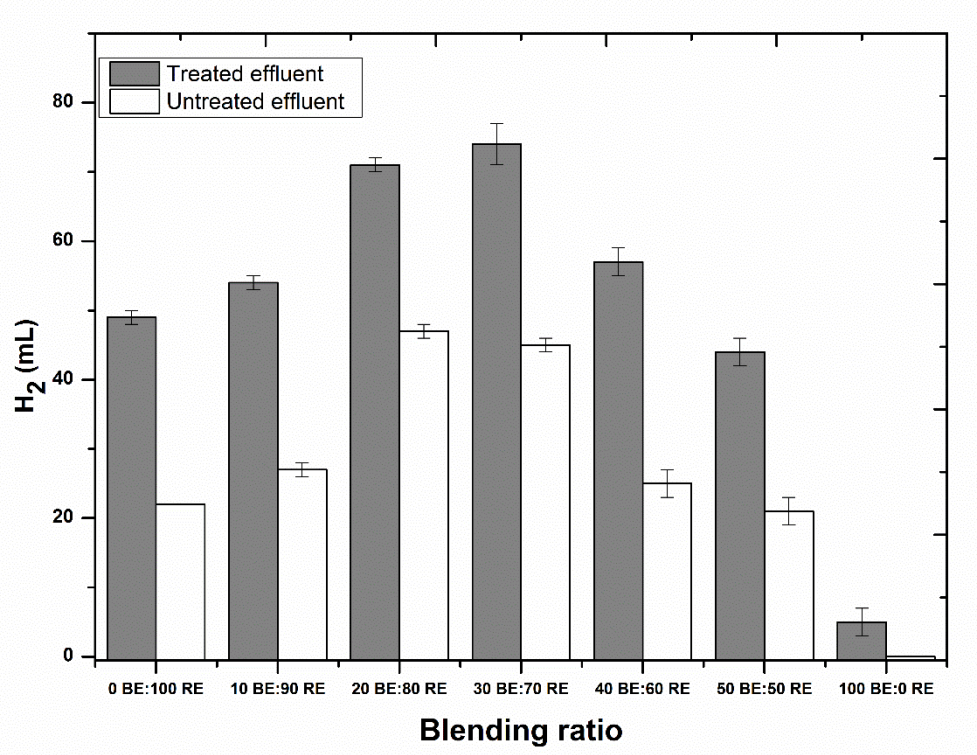

Figure 7. 1 Effect of different blending ratios of treated (by banana peels) and untreated mixed brewery and restaurant effluents on photofermentative hydrogen production (T: 30 ºC; agitation speed: 250 rpm; contact time: 60 min; dose: 2 g L^{-1}; particle size: <250 µm).

7.3.2 Influence of ultra-sonication pre-treatment on biohydrogen production

The influence of the untreated (UE) and pre-treated (TE) mixed effluents (30BE:70RE) on the photofermentative hydrogen production was investigated under different ultra-sonication conditions (Table 7.2).

Table 7. 2 A combined of 30% BE and 70% RE treated by banana peels and ultra-sonication at different amplitudes and ultra-sonication duration

Mixed effluent (30BE:70RE)	Banana peels treatment (stage 1)	Ultra-sonication treatment (stage 2)	
		Amplitude (A)	*Duration (min)*
Untreated (UE – Control 1)	No	No	
Pre-treated (TE– Control 2)	Yes	No	
Pre-treated ($TE_{(A30:T15)}$)	Yes	30	15
Pre-treated ($TE_{(A30:T25)}$)	Yes	30	25
Pre-treated ($TE_{(A30:T35)}$)	Yes	30	35
Pre-treated ($TE_{(A30:T45)}$)	Yes	30	45
Pre-treated ($TE_{(A60:T15)}$)	Yes	60	15
Pre-treated ($TE_{(A60:T25)}$)	Yes	60	25
Pre-treated ($TE_{(A60:T35)}$)	Yes	60	35
Pre-treated ($TE_{(A60:T45)}$)	Yes	60	45
Pre-treated ($TE_{(A90:T15)}$)	Yes	90	15
Pre-treated ($TE_{(A90:T25)}$)	Yes	90	25
Pre-treated ($TE_{(A90:T35)}$)	Yes	90	35
Pre-treated ($TE_{(A90:T45)}$)	Yes	90	45

Figure 7.2 illustrates that the cumulative photofermentative hydrogen production using all the pre-treated effluents (by both stages 1 and 2) was higher than that produced using the untreated effluent (UT-control 1) and the pre-treated effluent using only stage 1 (TE - control 2). The highest cumulative hydrogen of about 110 mL was achieved when using the effluents $TE_{(A60:T35),}$ $TE_{(A60:T45)}$ and $TE_{(A90:T25)}$, which represent 144 % and 32 % enhancement when compared with that produced using UT- control 1 and TE- control 2, respectively.

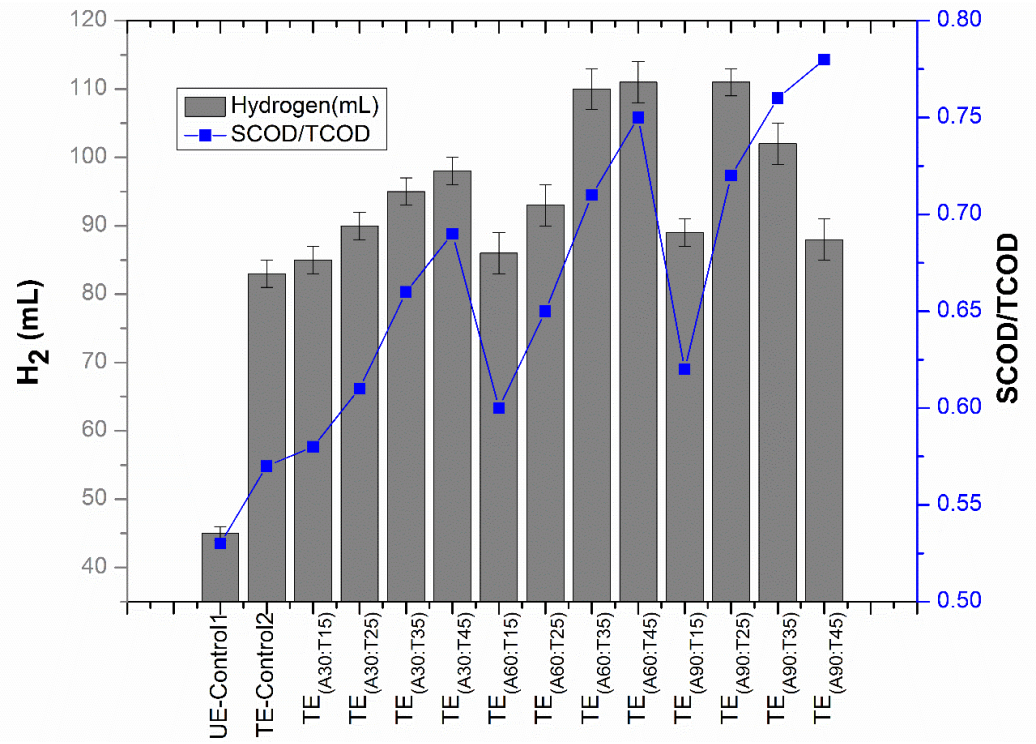

Figure 7. 2 Cumulative biohydrogen with different amplitudes and ultra-sonication time

The solubilisation of the mixed effluent increased significantly by applying the ultra-sonication. The results clearly show that at constant sonication amplitude (30, 6, and 90), the solubilisation degree of organic compounds, represented by SCOD/TCOD ratio, increased by increasing the sonication time from 15 to 45 min. The SCOD/TCOD ratio of $TE_{(A90:T45)}$ enhanced by 50% comparing with the untreated effluent (control 1). This enhancement can be attributed to the dissolution of organic compounds from solid and complex state into liquid phase [48, 50], which in turn enhance the bioavailability of the soluble organic compounds which can be used by microorganisms[283]. According to Tiehm et al. [284], mechanical forces created by ultra-sonication lead to decomposition of the solid components and transfer the organic matter from solid phase to liquid phase.

With the enhancement of the solubilisation a corresponding enhancement for the cumulative photofermentative hydrogen was evident at sonication amplitude of 30 and 60. Similar outcomes were reported by Wang et al. [285] and Leaño and Babel [286], they found that pre-treated cornstalk and cassava effluents by ultra-sonication led to enhance biohydrogen compared to raw effluents. Hay et al. [48] reported that the produced biohydrogen of any ultra-sonicated substrate was higher than raw substrate (control) and the highest hydrogen production of 437.8 mL was achieved at A60:T45.

Similar observation was reported by Budiman and Wu [50], where biohydrogen production of ultra-sonicated feedstock of combined effluent of palm and pulp and paper mills at A70:T45 was increased 86% to 872.4 mL compared to control sample.

It appears from Figure 7.2 that for amplitude values of 30% and 60%, the cumulative biohydrogen increased with increase ultra-sonication times from 15 to 45 min. However, at 90 % amplitude, the cumulative biohydrogen declined from 110 mL (25 min sonication time) to 103 mL and 88 mL when the sonication time increased to 35 min and 45 min, respectively. Higher sonication amplitude and time might help the re-polymerization of degradable compounds and convert to complex polymers. Therefore, the hydrolysis might be inhibited and this led to decrease biohydrogen production [285]. Similar finding was observed by Budiman and Wu [50] , Hay et al. [48] and Wang et al. [285], in which excessive ultra-sonication pre-treatment for 45 and 60 min (at all sonication amplitude applied) led to decrease biohydrogen production and did not have a significant impact on SCOD/TCOD ratio at higher sonication amplitude. Therefore, higher sonication amplitude (in this study > 90) should be avoided.

Figures (7.3, 7.4 and 7.5) demonstrate the cumulative biohydrogen, bacterial growth, and pH profiles, respectively, using amplitude value of 60% at different ultra-sonication times (15, 25, 35 and 45 min). Figure 7.3 shows that the cumulative biohydrogen for all the applied ultra-sonication time was higher than that obtained using control 1 and control 2 effluents. The highest cumulative photofermentative hydrogen (110 mL) was achieved for the treated effluents $TE_{(A60:T35)}$ and $TE_{(A60:T45)}$. The results show that the biohydrogen production rate for the untreated effluent (control 1) enhanced from 1.3 mL hr^{-1} to 1.8 mL hr^{-1}, while the lag time changed from 28 hr to and 14 hr after the pre-treatment with banana peels and ultra-sonication ($TE_{(A60:T35)}$).

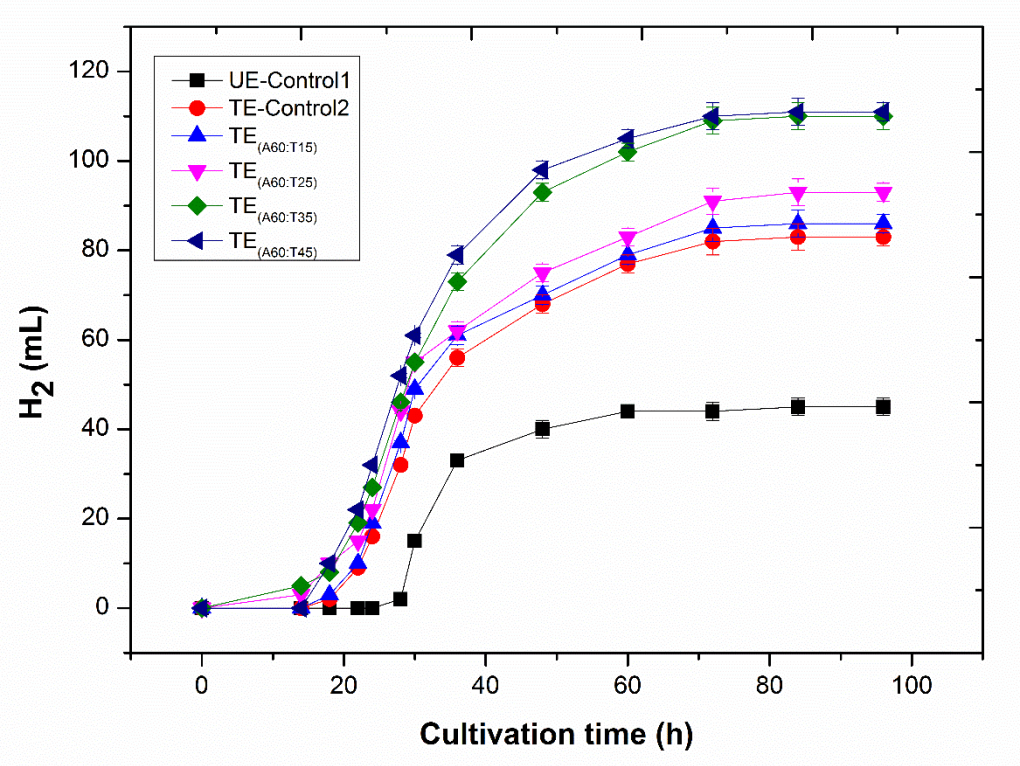

Figure 7. 3 Cumulative biohydrogen production versus time for amplitude of 60%with different ultra-sonication times (15, 25, 35 and 45min).

Figure 7.4 illustrates the growth trend of *R. sphaeroides* DSM 158 during the photofermentative hydrogen production using the treated and untreated effluents. Similar lag phase was observed for all the effluents. Maximum biomass of 0.58 g L^{-1} was achieved for the untreated effluents (control 1), while the minimum biomass of 0.51 g L^{-1} was achieved for the effluent treated with only banana peels (control 2). This observation can be attributed to the concentration of ammonium in control1 (18.06 mg L^{-1}) was enough to promote and enhance the growth of microorganism during the photofermentative hydrogen production (Table 7.1). The biomass produced using all the effluents treated by both banana peels and ultra-sonication was higher than that obtained from control 2 and lower than that obtained from control 1. It was noticed also that increasing the sonication period from 15 min to 45 min enhanced the biomass from 0.53 g L^{-1} to 0.57 g L^{-1}. The plausible reason for this behaviour can be attributed to applied ultra-sonication which can increase the bioavailability of the soluble organic substances as indicated by an increase in SCOD/TCOD in present study, which can be effortlessly used by microorganisms to support the growth.

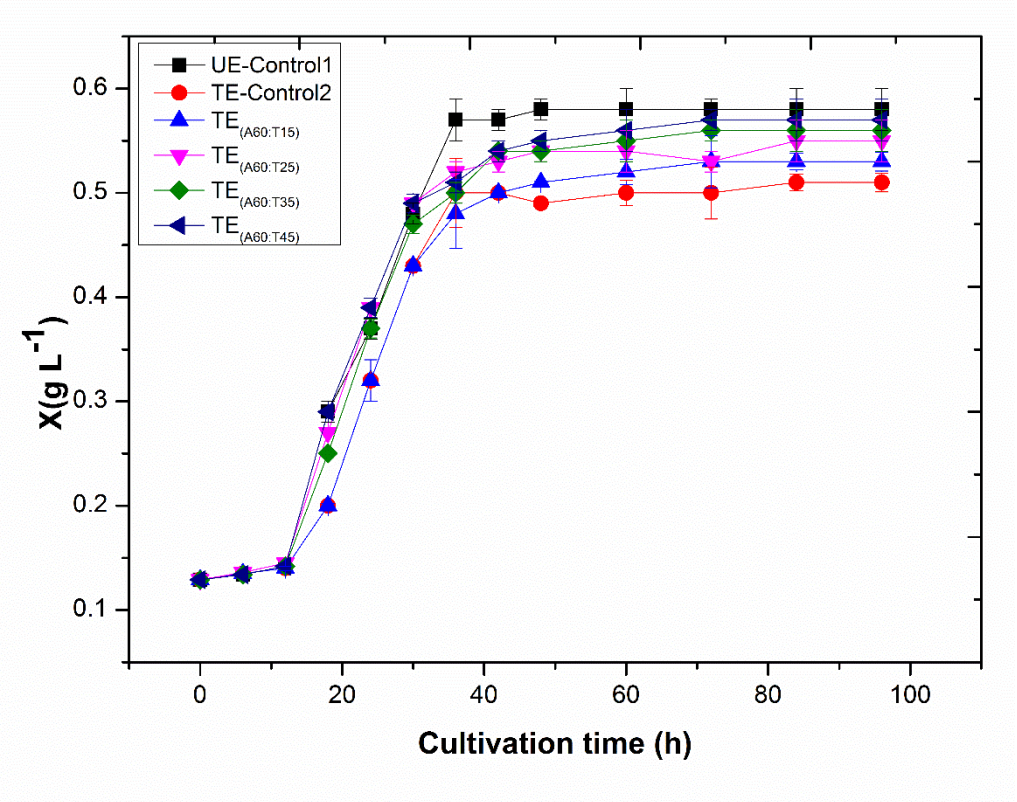

Figure 7. 4 Bacterial growth versus time for amplitude of 60%with different ultra-sonication times (15, 25, 35 and 45min)

Figure 7.5 shows variation of pH during the photofermentative hydrogen production by *R. sphaeroides* DSM 158 for all the treated and untreated effluents. As shown in Figure 7.5, initial pH of all the effluents rapidly increased during the first 48 h from 7.4 to the maximum value of 8.3 for control 2 effluent, then the pH values slightly decreased. According to Akroum-Amrouche et al. [226], initial pH values of biohydrogen production medium increased during the period of 0-70 h and decrease after that. This observation could be attributed to the accumulation of reducing equivalents in cells. The surplus of reducing equivalents employed to produce by-products such as poly β-hydroxybutyrate (PHB) which could increase pH value of the medium. When biohydrogen starts to produce, the co-evolved CO_2 might help to moderate the pH increases [35, 37, 111, 233].

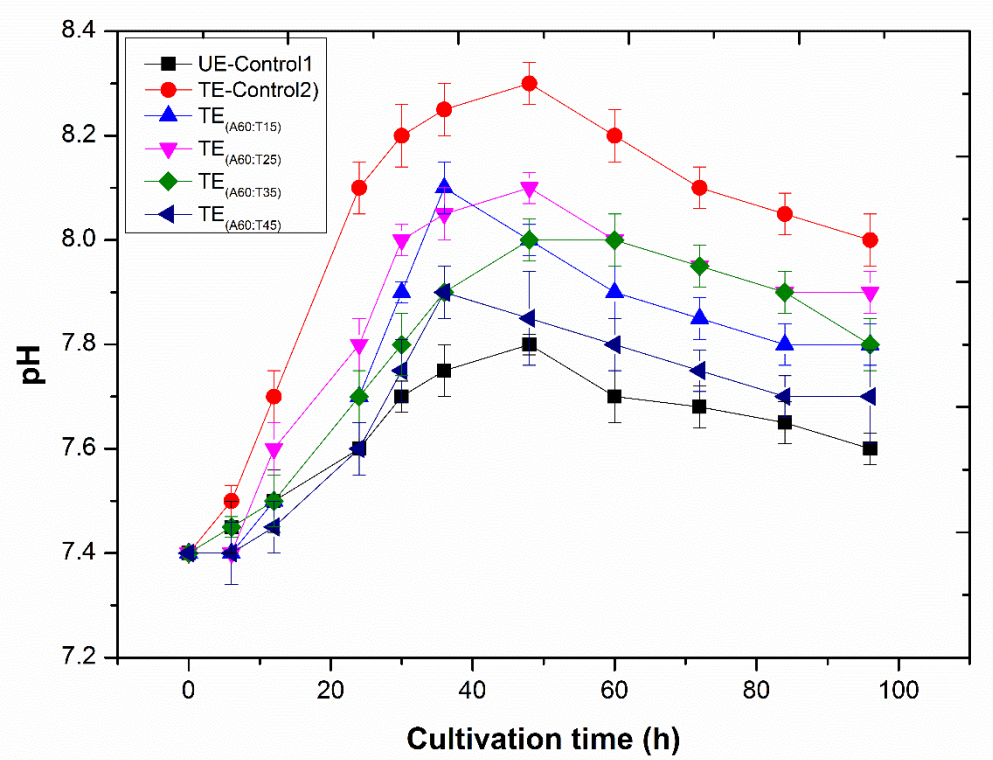

Figure 7. 5 pH versus time for amplitude of 60%with different ultra-sonication times (15, 25, 35 and 45min)

Table 7.3 comparing the present finding with other studies that used different effluents and pre-treatment methods. The maximum hydrogen production potential (HPP) achieved in the present study was 0.95 mL H_2 mL^{-1} which is comparable with those reported in prior studies [19, 47]. Pintucci et al. [47] reported HPP of 0.31 mL H_2 mL^{-1} from *Rhodopseudomonas palustris* 6A using pre-treated olive mill wastewater by applying dry-Azolla and granular active carbon. In different study, Seifert et al. [19] examined *Rhodobacter sphaeroides* O.U. 001 and 10 % brewery wastewater mixed with the standard medium, they reported a maximum HPP of 0.22 mL H_2 mL^{-1}. In our previous research [42] a maximum HPP of 0.4 mL H_2 mL^{-1} was achieved from *R. sphaeroides* DSM 158 using 50 % brewery wastewater mixed with standard medium. The variation of HPP among the previous studies (Table 7.3) attributed differences in COD of the applied wastewaters and used microorganisms. For example, Eroğlu et al. [44] indicated that maximum HPP was 31.5 mL H_2 mL^{-1} of olive wastewater with high COD of 52100 mg L^{-1} by *R. sphaeroides* O.U.001 while the COD of the 70RE:30BE effluents used in this study was only about 3008 mg L^{-1}.

Table 7. 3 Comparison among different studies.

Pre-treatment process	Biohydrogen production medium	Microorganism	COD mg L^{-1}	Maximum HPP (mL H_2 mL^{-1})	Reference
Ultra-sonication	Pulp and paper mill effluent	*R. sphaeroides* NCIMB8253	318	5.77	[48]
Banana peels	Brewery wastewater	*R. sphaeroides* DSM 158	2675	0.40	[42]
Clay	Olive mill wastewater	*R. sphaeroides* O.U.001	52100	31.5	[44]
Ultra-sonication	Combination of palm oil and pulp and paper mill effluents	*R. sphaeroides* NCIMB8253	22900	8.72	[50]
Ultra-sonication of both the bacterial cells and combined effluents	Pulp and paper mill effluent	*R. sphaeroides* NCIMB8253	318	9.62	[49]
Ultra-sonication of both the bacterial cells and combined effluents	Combination of palm oil and pulp and paper mill effluents	*R. sphaeroides* NCIMB8253	25200	14.43	[51]
Zeolite-4A	Olive mill wastewater	*R. sphaeroides* O.U.001	52100	17.6	[45]
Dry-Azolla and granular active carbon	Olive mill wastewater	*Rhodopseudomonas palustris* 6A	2830	0.31	[47]
Thermal	Brewery wastewater	*R. sphaeroides* O.U.001	202	0.22	[19]
Banana peels	Combination of and restaurant brewery wastewater	*R. sphaeroides* DSM 158	3008	0.72	This study
Banana peels+ Ultra-sonication	Combination of and restaurant brewery wastewater	*R. sphaeroides* DSM 158	3944	0.95	This study

7.3.3 Organic compound reduction during photofermentative hydrogen production

To determine the environmental advantages of photofermentative hydrogen production, COD removal efficiency (%) was measured in the present study. Figure 7.6 shows initial SCOD and SCOD removal efficiency (%) after photofermentative hydrogen production by *R. sphaeroides* DSM 158 for all the applied effluents. The highest SCOD removal efficiencies of 46% and 45.5% were achieved when using the effluents $TE_{(A60:T45)}$ and $TE_{(A60:T35)}$ respectively. It is noteworthy to see that the trend

of the SCOD removal efficiency following the same trend of the accumulated hydrogen production (see Figure 7.2), this could confirm the fact that the reduction in the SCOD attributed to the consumption of organic compounds for producing biohydrogen and supporting the bacterial growth [185].

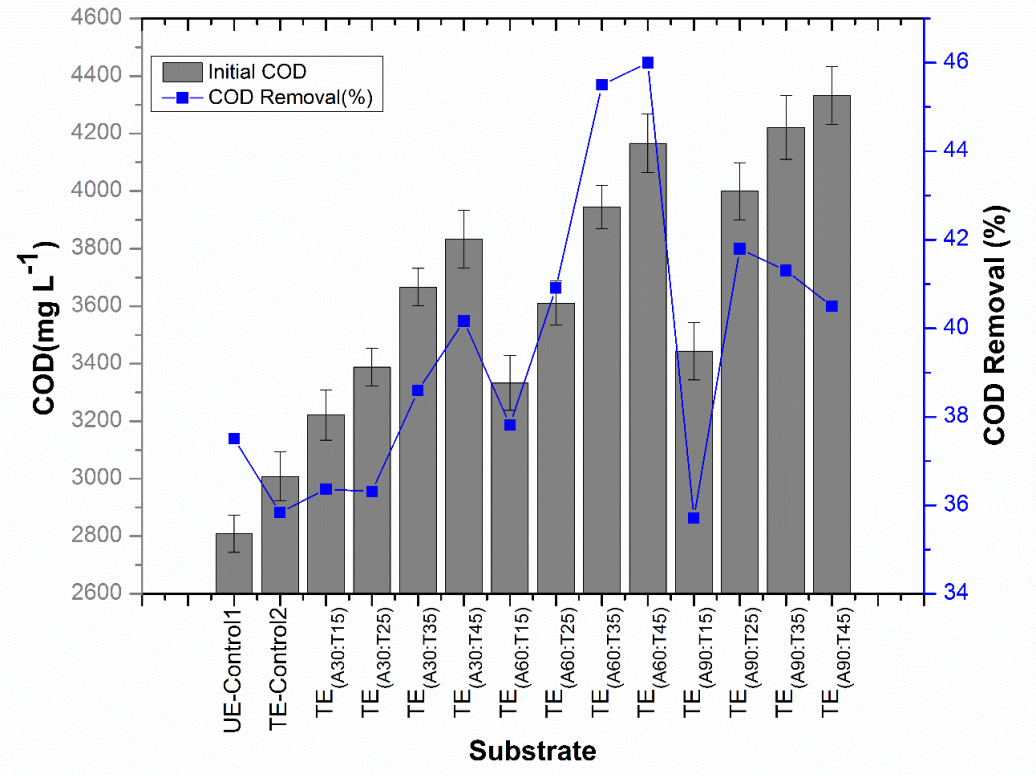

Figure 7. 6 Initial SCOD and SCOD removal efficiency (%) for all the treated and untreated effluents after photofermentative hydrogen production.

The current study indicated that the highest cumulative hydrogen was achieved at $TE_{(A60:T35)}$ effluent. Therefore, further investigation of COD consumption during photofermentative hydrogen production by *R. sphaeroides* DSM 158 was performed. Figure 7.7 illustrates SCOD profile for UE-control 1, TE- control 2, and $TE_{(A60:T35)}$. The significant SCOD removal was observed during the period (12-36 h), this could be due to the high rates of photofermentative hydrogen production and the bacterial growth at exponential phase [185]. After that, the rate of SCOD removal was slower during stationary phase of bacterial growth. According to Xie et al. [287], the bacterial growth utilize 37% of medium during photofermentative hydrogen production, while only 16% organic acid was used for biohydrogen production. Pintucci et al. [47] indicated that COD declined progressively from 2830 to 1270 mg L^{-1}, approximately 55% of COD removal during biohydrogen production by *Rhodopseudomonas*

palustris 6A using pretreated olive mill effluents. Also, it was reported by the same group that COD removal was 47.1% using olive waste by *Rhodopseudomonas palustris* 42OL under light intensity of 74 Wm^{-2} [46]. Hay et al. [246] determined COD removal of 36.7% for combined of brewery wastewater, palm oil, and pulp and paper mill effluents using *R. sphaeroides* NCIMB8253. Eroğlu et al. [17] reported that SCOD removal was 35% from olive mill wastewater using *R. sphaeroides* O.U.001. In another study, COD removal of 21% was achieved from dairy wastewater using *R. sphaeroides* O.U.001[43]. Budiman et al. [185] reported that SCOD removal was 20.5% from combination of palm oil and pulp and paper mill effluents using *R. sphaeroides* NCIMB8253.

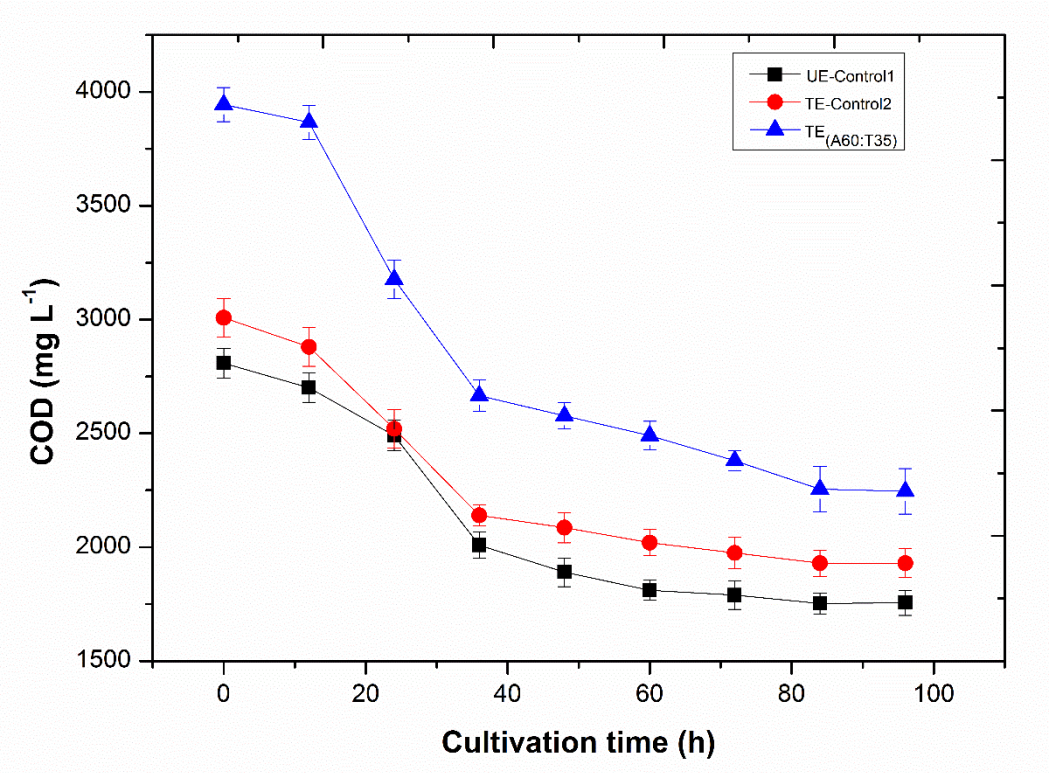

Figure 7. 7 COD versus time for amplitude of 60% and ultra-sonication duration of 35 min

7.4 Summary

The integrated banana peels waste and ultra-sonication as an integrated pre-treatment stage was found to enhance the bioavailability of soluble organic compounds and reduce the toxic properties of wastewater which resulted in enhanced biohydrogen production. Compared to the control (no pre-treatment), the experimental results demonstrated that integrated technique of banana peels waste and ultra-sonication using the combined effluents of 70% RE and 30% BE pre-treated with 2 g L^{-1} of

banana peels waste and A60:T35 (amplitude=60% and ultra-sonication time=35) led to increase the cumulative biohydrogen production to 110 mL (144% enhanced), while applying single pre-treatment stage (by banana peels only) enhanced the cumulative biohydrogen production to 83 mL (84% enhanced). This enhancement can be attributed to the increase in SCOD/TCOD ratio due to the dissolution of organic compounds in the medium. The result indicated that about 46% of soluble chemical oxygen demand (SCOD) removal was achieved during photo-fermentative hydrogen production process.

Chapter 8

Conclusions & Recommendations for future research

Chapter 8 Conclusions & Recommendations for future research

8.1 Conclusions

The following main conclusions could be drawn from the current study;

1. The optimization analysis conducted in this thesis for photofermentative hydrogen production, reveals the following;
   i) The predicted optimal conditions were pH, C/N, and *I* of 7.4, 27.5, and 126 W m^{-2}, respectively. Under these conditions the experimental responses of HPP, HPR, and η were 960 mL L^{-1}, 41.7 mL $L^{-1}h^{-1}$, and 0.31 respectively. This was confirmed through experimental validation.
   ii) The HPP, HPR, and η appear to have been well described by quadratic models developed using BBD according to multiple linear regression analysis of the outputs, with ANOVA analysis confirming the relative importance of the different parameters. Regression models can be applicable with high agreement between predicted and experimental responses in certain experimental domain used to develop these models. However, these models have limited ability to extrapolate beyond experimental domain.
   iii) The analysis reveals all the three factors (*I*, C/N, pH) were found to have significant impacts on HPP, HPR, and η. However, for HPP and HPR, the most important factors were C/N and *I,* followed by pH. While for η, light intensity had a greater impact than other factors, followed by pH and C/N. The synergistic effect of pH–*I* and C/N–*I* on the light conversion efficiency (η) was significant while pH –C/N was less significant.
   iv) Optimizing and balancing the C/N ratio and *I* appear to be critical in achieving higher HPP, HPR, and η.

2. The study of the impacts of banana peels pre-treatment stage on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM using brewery effluent (BE) revealed the following;
   i) Banana peels pre-treatment can significantly enhance the cumulative hydrogen production and can be considered as a promising bio-sorbent to

pre-treat wastewater samples, which can be further used as the substrate source for photofermentative hydrogen production.

ii) The maximum hydrogen production (408.3 mL H_2 $L^{-1}_{wastewater}$) was achieved with equal ratios of treated brewery wastewater blended and standard medium (50Bt:50SM), which was treated with 1 g L^{-1} of banana peels for 2 h. This best result was 2.7-folds higher than those applying the same percentage of raw BE without any pre-treatment.

iii) As a result of banana peels pre-treatment stage, ammonium concentration of BE was decreased, while C/N ration increased which resulted in enhanced biohydrogen production.

3. The study of the influence of different concentrations of Fe , Mo , and EDTA on the photofermentative hydrogen production and bacterial growth by *Rhodobacter sphaeroides* 158 DSM revealed the following;

   i) In the presence of metal ions (Fe and Mo), only the optimum concentration of EDTA could enhance the biohydrogen production.

   ii) At constant Fe:Mo concentrations of 70 µM:8 µM, the highest biohydrogen production of 192 mL was obtained when 0.2 g L^{-1} of EDTA added to the blended effluents.

   iii) A blend of pre-treated brewery (30%) and restaurant (70%) effluents was used successfully as sole medium for the photofermentative hydrogen production, producing a cumulative biohydrogen.

4. Combining ultra-sonication and adsorption with banana peels as an integrated pre-treatment stage was concluded to be a promising technique for photo fermentative hydrogen production processes. The experimental results demonstrated that integrated ultrasonic technology and adsorption of banana peels waste for mixture of 30% brewery and 70% restaurant effluents can increase the cumulative biohydrogen production to 110 mL (144% increased), while pre-treating the same mixture with only banana peels pre-treatment can enhance the cumulative biohydrogen production to 83 mL (84% increased) with compared to the control (no pre-treatment).

8.2 Recommendations for future research

Based on the findings of current study, several recommendations can be suggested for future expanding work;

1. This study indicated that RSM was effective tool to model the effects of multiple parameters and it can reduce the time and cost of hydrogen production. Therefore, it will be very useful to apply this technique with other parameters such temperature and light source.
2. One of the promising by products of hydrogen production from photo fermentation is polyhydroxybutyrate (PHB). This polymer has biodegradability and thermoplastic properties so it can alternative material for petrochemical plastics. Therefore, utilization of pre-treated wastewater for biohydrogen and biodegradable polyhydroxybutyrate formation can be investigated to open a new avenue for the utilization of sustainable energy resources.
3. The current study demonstrated that the brewery wastewater cannot be used as a sole substrate to produce biohydrogen, while mixture of brewery wastewater with restaurants wastewater led to enhance biohydrogen production. Therefore, there is need to mix the brewery wastewater with other wastewater qualities such as dairy wastewater or olive wastewater.

4. The current study has been carried out with the batch bioreactor. A batch mode is more appropriate for initial optimization and laboratory experiments. However, a continuous mode is preferred by industries. Therefore, continuous reactors such as continuous stirred tank reactors (CSTR) can be investigated for continuous production. CSTR has many advantages such as effective homogeneous mixing and easy construction with simple operation.

5. The current study demonstrated that co-addition of iron and molybdenum led to enhance biohydrogen production. The sole addition and co-addition of iron and molybdenum nanoparticles can be investigated because nanoparticles have high specific surface area and high catalytic activity.

6. The results of this study indicated that banana peels pre-treatment can be considered as a promising biosorbent to pre-treat wastewater samples, which

can be further used as the substrate source for photo fermentative hydrogen production. Therefore, further studies can be achieved to apply a new biosorbent such as chicken bones.

7. To make the photo or dark fermentation processes more economical, and feasible, future studies should be focused on genetic and metabolic engineering to enhance biohydrogen production. Different metabolic pathway engineering strategies can be applied such as increase substrate utilization, increase oxygen-resistant hydrogen-evolving enzymes, and elimination polyhydroxyalkanoate (PHA) synthesis and knocking out the uptake hydrogenase because accumulation of PHA compounds competes for electrons used in biohydrogen production. One of the most normally applied technique for achieving metabolic engineering is the knockout of specific chromosomal genes by creating mutations either by ultraviolet radiation or by chemical techniques after that screening them.

8. The main disadvantage related to photo fermentation systems could be the high-power consumption of the artificial light sources such as halogen lamp or tungsten lamp. To overcome this problem, outdoor bioreactor system utilized solar light energy can be used. Several benefits have been found for using solar light energy, as it is free and the most abundant natural light source on earth source with full spectrum of light energy. Hence, effective utilization of sunlight can simultaneously address the hindrances of high operation cost related with artificial light source.
9. In this study, lab-scale experiments were conducted, and the small amount of residual banana peels disposed by applying the standard protocol. In large-scale, it will be very useful to use residual banana peels as fertilizer.
10. In current study, residual wastewater disposed by applying the standard protocol while in large-scale residual wastewater can be reused in agriculture.

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Appendix A Articles copyright

Copyright of Synergistic effects and optimization of photo-fermentative hydrogen production of *Rhodobacter sphaeroides* DSM 158.

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Synergistic effects and optimization of photo-fermentative hydrogen production of *Rhodobacter sphaeroides* DSM 158

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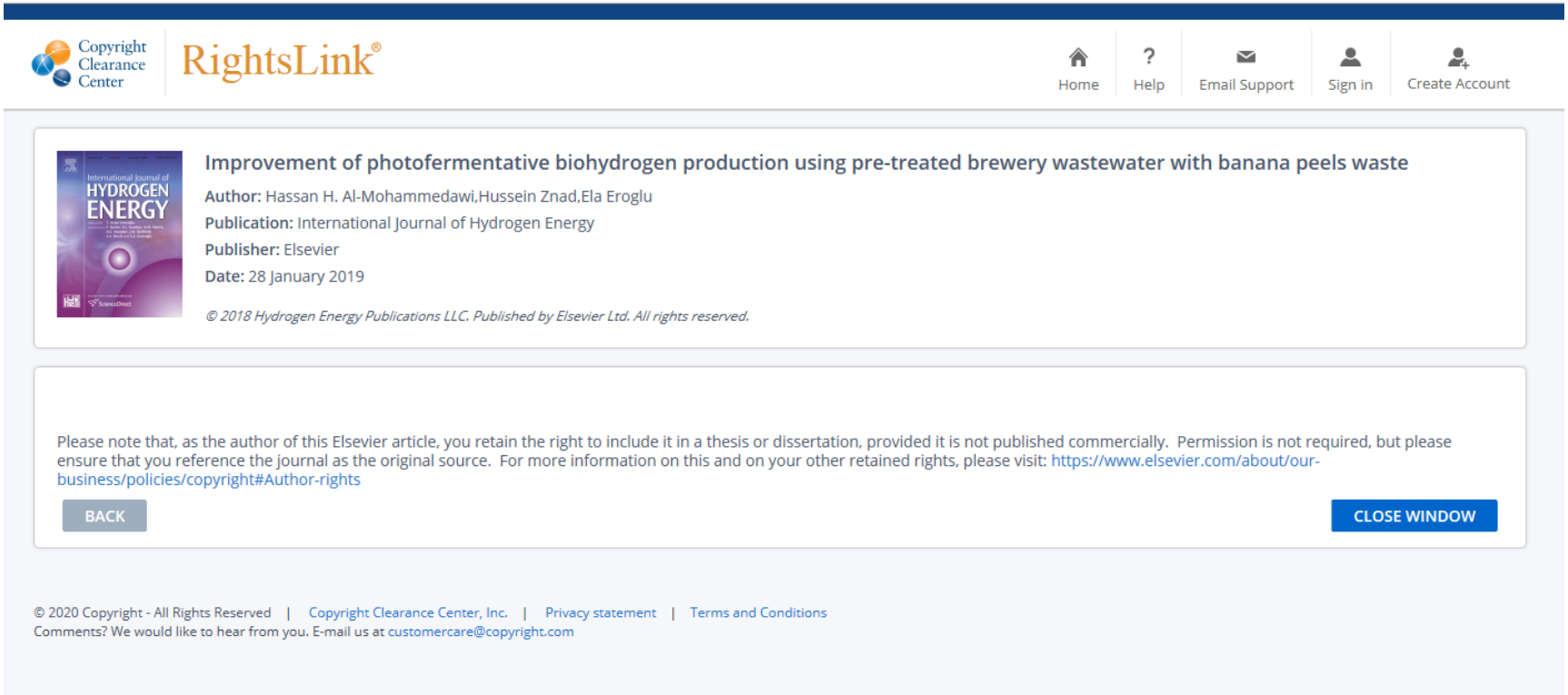
ABSTRACT

The synergistic effects and optimization of pH, carbon-to-nitrogen ratio (C/N), and light intensity (I) on the photo-fermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM and light conversion efficiency have been investigated under different conditions of pH (6.5–8); C/N (15–35); and light intensity (35–185 W m^{-2}). Response surface methodology (RSM) and Box-Behnken experimental design (BBD) were used to identify the optimum values of the three key parameters of pH, C/N, and I, based on the impact on hydrogen production potential (HPP), hydrogen production rate (HPR), and light conversion efficiency η. With desirability value of 0.91, the optimum values of 7.4, 27.5, and 126 W m^{-2} were identified for pH, C/N, and I respectively, with HPP, HPR and η reaching 960 mL L^{-1}, 41.74 mL L^{-1} h^{-1}, and 0.31 respectively. Regression analysis indicated a good fit between experimental and model data. The study showed that both C/N ratio and I have crucial and significant effect on the HPP, HPR and η, followed by pH, the synergistic effect of pH–I and C/N–I on the light conversion efficiency (η) was significant while pH – C/N was insignificant. The results and analysis obtained could be very useful for better optimizing the photo-fermentative hydrogen production.

Synergistic effects and optimization of photo-fermentative hydrogen production of *Rhodobacter sphaeroides* DSM 158. International Journal of Hydrogen Energy. 2018;43:15823-34.

Authors	Conception and design	Acquisition of data and method	Data conditioning and manipulation	Analysis & statistical method	Interpretation & discussion	Final Approval
Dr. Hussein Znad			**X**		**X**	**X**
I acknowledge that these represent my contribution to the above research output Singed.						
Dr. Ela Eroglu					**X**	
I acknowledge that these represent my contribution to the above research output Singed. **Ela Eroglu**						

Copyright of Improvement of photofermentative biohydrogen production using pre-treated brewery wastewater with banana peels waste.



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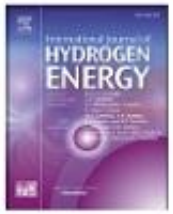

Improvement of photofermentative biohydrogen production using pre-treated brewery wastewater with banana peels waste

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ABSTRACT

In this study, the impacts of banana peels pre-treatment stage on photofermentative hydrogen production of *Rhodobacter sphaeroides* 158 DSM using brewery wastewater (BWW) were investigated in a batch bioreactor. The experimental results indicate that banana peels pre-treatments can significantly enhance the cumulative hydrogen production. The maximum hydrogen production yield (408.33 mL H_2 $L^{-1}_{wastewater}$) was achieved from the substrate, which was composed of 50% BWW pretreated with 1 g L^{-1} of banana peels for 2 h and 50% standard medium. This highest amount of hydrogen production was 2.7-folds higher than those that applied the same percentage of raw BWW as the substrate source.

Improvement of photofermentative biohydrogen production using pre-treated brewery wastewater with banana peels waste. International Journal of Hydrogen Energy. 2019;44:2560-8.

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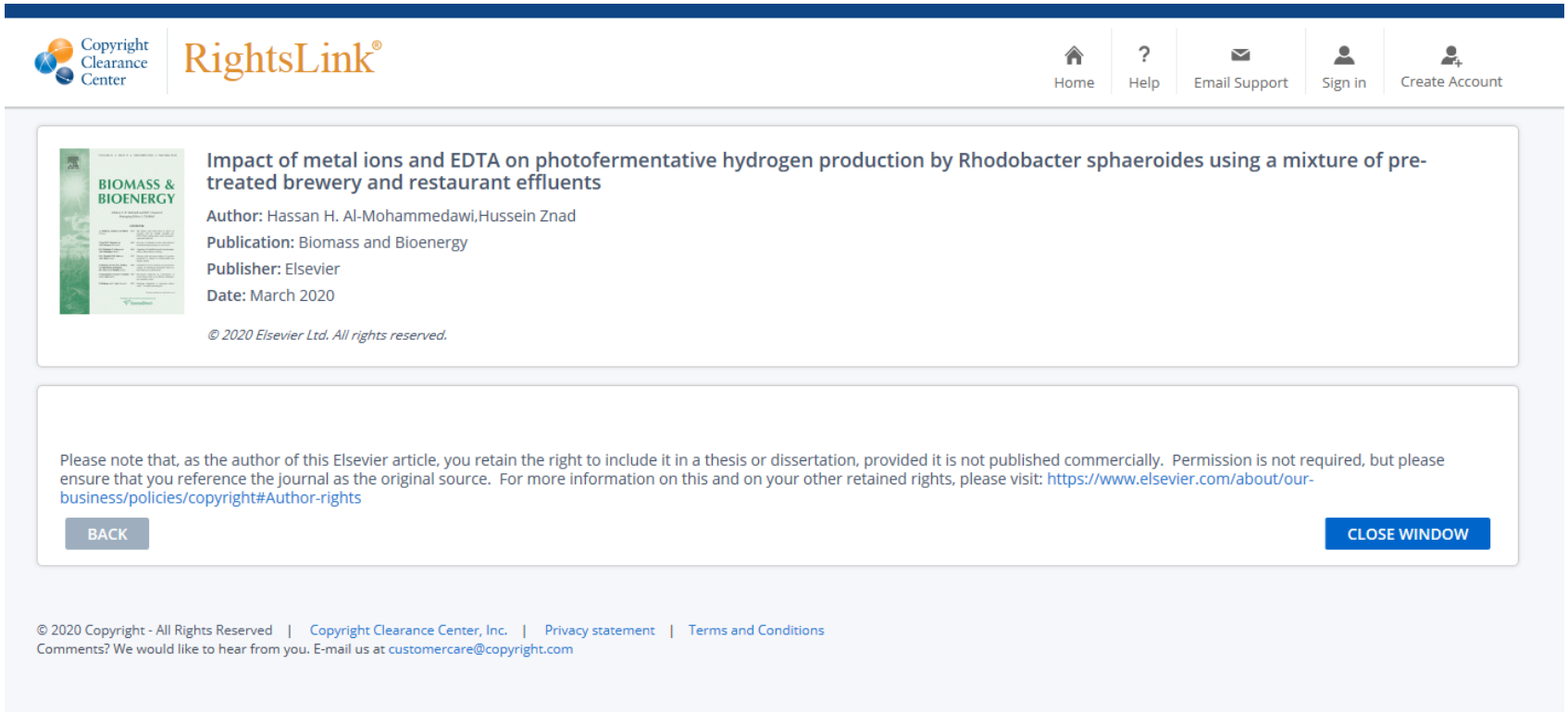
- **Copyright of** Impact of metal ions and EDTA on photofermentative hydrogen production by *Rhodobacter sphaeroides* using a mixture of pre-treated brewery and restaurant effluents.



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Research paper

Impact of metal ions and EDTA on photofermentative hydrogen production by *Rhodobacter sphaeroides* using a mixture of pre-treated brewery and restaurant effluents

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ABSTRACT

The influence of different concentrations of iron, Fe (30–110 μM), molybdenum, Mo (8–20 μM), and ethylenediaminetetraacetic acid, EDTA (0.1–0.5 $g\ L^{-1}$) on the photofermentative hydrogen production and bacterial growth by *Rhodobacter sphaeroides* 158 DSM was investigated and discussed. A blend of pre-treated brewery (30%) and restaurant (70%) effluents was used successfully as sole medium (without using the standard medium) for the photofermentative hydrogen production, producing a cumulative biohydrogen of 83 mL. The results show that sole-additions of Fe at 70 μM, Mo at 14 μM, and co-addition of Fe:Mo at 70 μM:8 μM to the mixture of pre-treated brewery and restaurant effluents, could enhance the cumulative biohydrogen production to 140 mL (69% increased), 105 mL (27% increased), and 160 mL (93% increased), respectively. The results also revealed that the addition of EDTA should be optimized to avoid the chelation of the added metal ions (Fe, Mo). The biohydrogen production was further enhanced to 192 mL, which represent 131% increase compared to control, when the optimized EDTA of 0.2 $g\ L^{-1}$ was added the blended effluents at Fe:Mo concentrations of 70 μM:8 μM. Furthermore, the study shows that the addition of Fe, Mo and EDTA to the blended effluent enhances the biomass growth as well. Utilizing the wastewater for biohydrogen production as a sole medium could open new era for renewable energy production.

- **Impact of metal ions and EDTA on photofermentative hydrogen production by *Rhodobacter sphaeroides* using a mixture of pre-treated brewery and restaurant effluents.** Biomass and Bioenergy. 2020;134:105482.

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