

**Study on the Effect of Pretreatment Methods of Aquatic Biomass for  
Improving Bioenergy Generation**

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**Study on the Effect of Pretreatment Methods of Aquatic Biomass for  
Improving Bioenergy Generation**

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## Abstract

Bioenergy has great prospects in overcoming the barriers, considering the worldwide yield of biomass residue is 220 billion tons every year, which can be converted to bioenergy in the form of biogas, biofuels, bioethanol and so on. In this research, emphasis is made on biohydrogen as an alternative source of energy due to its carbon neutral eco-friendly nature and high energy density. Although, a variety of biomass has been reported to produce biohydrogen efficiently, in this research, aquatic biomass such as *Ipomoea aquatica* and *Chlorella vulgaris* were employed as substrates to study the effect of pretreatment methods for improving biohydrogen production.

Digested sludge (DS) was used as inoculum and it was subjected to thermal and acid pretreatment to identify the optimum pretreatment method. From, the results, thermal pretreatment was better than acid pretreatment as it enhanced the H<sub>2</sub> production. Response surface methodology (RSM), a statistical method useful for evaluating the optimal conditions for desirable responses was employed to further identify the optimum thermal pretreatment condition. Conclusively, thermal pretreatment of DS at 90°C for 60 min was identified as the optimum pretreatment condition. It was used as inoculum in all further hydrogen fermentation experiments.

*Ipomoea aquatica* a semi aquatic, tropical plant with high percentage of carbohydrate (54%) was used as one of the substrate. Pretreatment of *I. aquatica* was optimized under conditions like freezing, boiling, and alkali pretreatment to attain high hydrogen yield (HY). Frozen dried *I. aquatica* demonstrated the highest HY of 217.2 mL/g-VS, which was manifold higher than control and other treatment conditions.

Although *I. aquatica* showed remarkable results in bioenergy generation, it can be harvested only once a year in colder countries, posing a disadvantage for practical application in these countries. To overcome this problem *Chlorella vulgaris* was chosen as a substrate as

its widely distributed. However, to improve the hydrolysis of microalgal biomass and enhance biohydrogen production, pretreatment methods like acid and thermal pretreatment were employed. As a result, thermal pretreatment at 100°C for 60 min was identified as the optimum condition for the pretreatment of *C. vulgaris*. Furthermore, experiments were also carried out to identify the optimum substrate to inoculum ratio (S/I) for the process. S/I of 8 generated the highest hydrogen yield of 190.90 mL H<sub>2</sub>/g-VS. Moreover, comparing the energy production from the two-aquatic biomass fermentation process, *C. vulgaris* was 1.3 times higher than *I. aquatica*.

Although the energy obtained from thermal pretreatment of *C. vulgaris* was high, but there is also some amount of energy consumed for pretreatment which cannot be neglected. Moreover, microalgal biomass has drawn worldwide attention because of its lipid content which has a great value.

Conclusively, study on the effect of pretreatment methods of aquatic biomass for improving bioenergy generation could be very important for solve the future energy problems.

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## Chapter 1 Introduction

### 1.1 Background

Currently, on a global scale we consume the equivalent of over 11 billion tons of oil in fossil fuels. Crude oil reserves are vanishing at the rate of 4 billion tons a year (CIA world fact book), at this rate without any increase for our growing population, known oil deposits will be gone by 2042 [1]. In addition, due to the utilization of fossil fuels, a vast amount of greenhouse gases (GHGs) is produced upon combustion, causing the worldwide climate change [2]. To overcome these problems, explorations of alternative renewable energy as well as increased efforts in energy efficiency has drawn worldwide attention [3, 4]. Bioenergy, as one of the alternative energy is an unavoidable option and bioenergy of renewable and sustainable nature, such as biogas or bio-diesel can be the ultimate solution to this global crisis. Bio-hydrogen due to its high-energy density (141.9 kJ/g) which is 2.75 times higher than fossil fuel [5], and non-polluting nature as the only by-product on combustion is water vapor has taken scientific interest.

So far 40% of hydrogen is produced from natural gas or steam reforming of hydrocarbons, 30% from oil (mostly consumed within factories), 18% from coal, and the remaining 4% via water electrolysis around the world [6]. However, these are energy-exhaustive, expensive and not environmental friendly. Owing to these issues, one option would be to use biological means such as potential substrates, wastes and biomass to produce biohydrogen through fermentation. Biological dark and photo fermentation of biomass to liberate biohydrogen has found light in recent times due its efficiency and sustainability. But the availability of substrates that are sustainable, easy to be degraded and those which do not interfere with food security are few. Aquatic plants such as water hyacinth, *Ipomoea aquatica*, and *Chlorella vulgaris* have the capacity to treat polluted wastewater and then later be used as biomass for biohydrogen

production. Thereby, *I. aquatica*, and *C. vulgaris* were chosen as substrate for enhanced biohydrogen production.

Another setback is the substrate pretreatment which is necessary for biomass conversion. Commonly used pretreatment methods like freezing, boiling, drying, acid and thermal pretreatment were used in this research for improved biohydrogen production from *I. aquatica* and *C. vulgaris*. To further enhance efficient biomass conversion by utilizing the lipids from microalgal biomass, photocatalysis which can operate under ambient conditions and has shown great potential in mineralization of hazardous chemicals was used as a pretreatment method for *C. vulgaris*. Consecutively, along with other conventional pretreatment methods, photocatalysis was also investigated, as a novel pretreatment method in this research.

One of the other bottleneck in the fermentation process is the inoculum and its optimization for higher hydrogen yield. So far, a lot of research has been done in finding the best optimum condition for inoculum pretreatment and it has resulted in a wide range of temperature at different time intervals reported by different authors. Another stand point in this research is finding the best optimum condition among the various conditions reported in literature.

Therefore, this research aims at achieving a sustainable process of biohydrogen production using aquatic biomass as substrate and digested sludge as inoculum. Along with the investigation of photocatalysis as a novel pretreatment method for bioenergy generation.

## **1.2 Digested sludge as a source of inoculum**

Digested sludge (DS) obtained from waste water treatment plant has been an efficient source of bacteria for biohydrogen production from various biomass like agricultural waste, food residues, plant residues, waste water and so on [7, 8]. DS is a sustainable source for bacteria as sludge will exist as long as the existence of human race. DS contain mixed culture with a wide range of microbial organisms. However, processes using mixed cultures are more

practical than those using pure cultures, because the former are simpler to operate and easier to control, and may have a broader source of feedstock; thus, mixed cultures are preferred [9]. However, in a mixed culture system, under anaerobic condition the hydrogen produced from hydrogen producing bacteria (HPB), such as *Clostridium* and *Enterobacter*, are often readily consumed by hydrogen consuming bacteria (HCB), such as methanogens, homoacetogens and *Archaea* [10-13]. Therefore, to harness hydrogen using a mixed culture system, the seed sludge needs pretreatment to suppress as much hydrogen-consuming bacterial activity as possible while still preserving the activity of the hydrogen-producing bacteria.

### **1.2.1 Different pretreatment methods employed for optimization of DS**

Pretreatment of the seed sludge is used to stimulate the HPB and suppress the HCB to improve the hydrogen yield. The main pretreatment methods reported in literature are temperature shock, pH shock (acid or alkali), chemical pretreatment (Chloroform, 2-bromoethanesulfonic acid (BESA) and iodopropane), pressure shock, ultrasonic and combination of these methods as shown in Table 1.1. However, the first two methods are generally the most widely used for selecting HPB populations, but the best pretreatment is different for various inoculum sources [13]. Since, acid and thermal pretreatment have proved to be efficient in many researches; the same was used in this research.

Acid pretreatment was performed at pH 3 using HCl (2M) as reported in literatures [14-19]. Thermal pretreatment was another pretreatment method that was employed in this research. Wide ranges of temperature and residence time have been reported as optimum conditions in different literatures as the best pretreatment differs according to the inoculum sources [13]. The optimum thermal pretreatment conditions reported in literature are 100°C for 45 min [20], 100°C for 30 min [21, 22], 100°C for 60 min [14], 70°C for 60 min [23] and so on. These different ranges of temperature and residence times makes it a challenge to decide the best

thermal pretreatment condition that can be used in this research, as a wide range of conditions should be tested making it time and labor consuming.

### **1.2.2 Using RSM for optimizing thermal pretreatment**

Response surface methodology (RSM) is a statistical method useful for evaluating the relative significance of several independent variables, understanding the interactions of the various parameters affecting the process, and hence determining optimal conditions for desirable responses. It is particularly useful for developing empirical models and investigating uncertain phenomena. RSM is a statistical method introduced by Box and Wilson in 1951. Since then, it had been employed in many fields for designing experiments, improving the efficiency, evaluating the effects of diverse factors, and finding an optimal condition as desired. RSM has the advantage of reducing the number of experiments required making it easy for overall data analysis [24]. RSM has demonstrated its effectiveness for the optimization of many complex processes in chemical engineering [25], food sciences [26], wastewater treatment [27], biological fermentation [28], etc. In this experiment, the effects of the parameters (temperature and time) on hydrogen concentration, hydrogen production, hydrogen accumulated and methane concentration were investigated. The experiments could be designed with a few techniques in RSM procedure. The Central Composite Design (CCD) is a fractional factorial design useful for describing the effects of parameters and their interactions on a response with a second-order polynomial equation. It could be an effective alternative to a full factorial design which requires a lot of resources to obtain the data [29].

CCD was used to identify the best thermal condition from a range of 90°C to 110°C at residence time of 15 min to 60 min, as this range was found to be appropriate for higher hydrogen yield (HY) from the inoculum source used in this research. Using CCD in this



research makes the process easy as it designs an experiment with just 13 runs to identify the optimum condition which would otherwise take more than 50 runs.

The software generates the result or optimum condition after calculating the mean standard deviation and standard error based on the experimental results, which makes the outcome very accurate and acceptable. Based on the results generated by the software, the corresponding optimum condition was used in all further experiment for the pretreatment of inoculum for enhanced biohydrogen from water purification biomass.

### **1.3 *Ipomoea aquatica* as potential substrate**

*I. aquatica*, commonly known as ‘water spinach’, is a free floating semi - aquatic plant native to the tropics and consumed as leafy vegetable in tropical countries [30]. Water spinach is an herbaceous perennial plant belonging to the family *Convolvulaceae*. It has a long, hollow and viny stem, grow prostrate or floating and the roots are produced from the nodes and penetrate wet soil or mud. The leaf shape ranges from saggitate to lanceolate (USDA (2005), Wikipedia). The composition and other characteristics of *I. aquatica* are shown in Table 1.2 [31].

#### **1.3.1 Water treating capacity of *I. aquatica***

It plays an important role in nutrient removal from nutrient rich effluents of polluted lakes and ponds thus overcoming eutrophication. Nutrient removal and water quality were investigated by planting *I. aquatica* on artificial beds in 36 m concrete fishponds. After treatment of 120 days, 30.6% of total nitrogen (TN) and 18.2% of total phosphorus (TP) were removed from the total input nutrients by 6 m aquatic vegetable *I. aquatica*. The concentrations of TN, TP and chemical oxygen demand (COD), in planted ponds were significantly lower

than those in non-planted ponds ( $P < 0.05$ ). Transparency of water in planted ponds was much higher than that of control ponds [31].

### **1.3.2 Why choose *I. aquatica* as potential substrate?**

These plants after nutrient rich effluent removal become unfit for human consumption. However, in some polluted aquatic environment, the fast growth of *I. aquatica* makes it a secondary pollutant as it hinders irrigation, sailing, etc. *I. aquatica* can grow at a rate of 4 inches per day, producing 84 tons of fresh weight biomass per acre in 9 months (Houston Advanced Research Centre, 2006), indicating the huge amount of biomass readily available. *I. aquatica* also contains mineral elements like K, Na, Ca, Mg, Fe and Mn, along with a high percentage of carbohydrate (54%), making it a viable biomass to produce biohydrogen [30]. Until date *I. aquatica* has not been reported in literature as a potential substrate to produce hydrogen, which is of key importance in this research.

### **1.3.3 Different pretreatment methods employed for degradation of *I. aquatica***

Many literatures have shown that pretreatment of substrate is necessary for higher biohydrogen yield [7, 32]. Also, many pretreatment methods have been used for degrading the substrate such as microwave heating, acid pretreatment, alkali pretreatment, enzyme hydrolysis and so on. In this research, also pretreatment of *I. aquatica* was carried out to optimize the fermentation process. Since, *I. aquatica* is a leafy vegetable, common household methods to preserve vegetables were used as pretreatment methods. Pretreatment methods with easy operation such as freezing, boiling and alkali pretreatment were examined. Also, to rectify the errors and avoid compositional differences in the plant, the substrate was reduced to constant weight by drying after subjecting it to pretreatment and consecutively drying was investigated as pretreatment method as well.

## **1.4 Microalgal biomass as potential substrate**

Recently, microalgal biomass has drawn a lot of attention due to its characteristics such as high photosynthetic efficiencies, rapid aquatic growth and wide distribution [33, 34] which can be considered as a large potential application for bioenergy generation. Utilization of microalgae for biohydrogen or biodiesel production has important significance to relieve the energy crisis and environmental pollution.

### **1.4.1 Potential availability of microalgal biomass as third-generation feedstock**

Currently, microalgal biomass shows great promise to become the largest viable biomass source for bioenergy generation in the form of biohydrogen and biodiesel production. Some microalgae mainly contain carbohydrates, usually accounts for 12-55 % of biomass dry weight, which is also an important organic matter for hydrogen fermentation [34]. While some of them are rich in lipids 5-58 %, favorable for biodiesel generation [36].

Microalgal biomass considered as a potential feedstock because of some reasons, which include: 1. The high growth rate. Doubling of microalgal biomass occurs within 3.5 h; 2. The high bioenergy productivity; Biofuel yields from microalgae are 10-100 times higher than those from land-based crops in the same cultivation area; 3. Continuous supply. Microalgae can be cultivated continuously almost all the year around with a very short harvesting cycle (1-10 days), therefore providing a continuous and enough biomass supply; 4. Wide distribution. Microalgae can grow on salt or seawater even wastewater, thereby reducing freshwater consumption; 5. No requirement of herbicides or pesticides; 6. The ability to remove NO<sub>x</sub>. Microalgae can be cultured in industrial, municipal and other wastewater, resulting in bioremediation of wastewater by the removal of inorganic nitrogen and phosphorus as well as heavy metals; 7. Reduce GHGs emissions. Microalgae can make good use of CO<sub>2</sub> in photosynthesis procedure, mitigating greenhouse gas emissions efficiently; 8. The specific

compositions of microalgae. Microalgae biomass is mainly composed of carbohydrates, proteins and lipids; thus, it is suitable for biohydrogen and biodiesel production [2, 33, 37].

Among various microalgal biomass, *C. vulgaris* is a typical type of microalgal biomass which has 12-55 % carbohydrates, 42-58 % proteins and 5-58 % lipids, indicating great potential to be used as biomass feedstock Table 1.3 [36]. *C. vulgaris* and *Spirulina platensis* are consumed as food supplements as well and their products are used for different purposes such as dyes, pharmaceuticals, animal feed, aquaculture and cosmetics. They both are suitable choices as biomass feedstock for bioenergy generation. However, the composition of *Spirulina* is 15-25 % carbohydrates, 11% lipids and 50-70% proteins. The compositional difference between *Chlorella* and *Spirulina*, make the former a better candidate for biogas and biodiesel production. Therefore, in this research *C. vulgaris* was used as a viable biomass to generate bioenergy in the form of biogas and lipid.

#### **1.4.2 Different pretreatment methods employed for degradation of *Chlorella vulgaris***

Some previous literatures indicated that the intact and strong cell membranes and cell walls of *C. vulgaris* would result in a low biogas yield, limiting the efficient degradation in fermentation process. As a result, it is necessary to break the thick cell wall for releasing the intracellular organics and increasing the efficiency of the fermentation process. To help disrupt the cell walls, pretreatment or disintegration on the microalgal biomass is needed [38]. Until now, some different pretreatment methods on microalgal biomass have been investigated. For instance, the ultrasonic pretreatment uses high shear forces resulting in extracting the intracellular organic and thereby increasing the biodegradability [39]. Although ultrasonic pretreatment is a good choice for microalgal biomass, it is energy-intensive and cannot be used for practical application. Baccay and Hashimoto (1984) investigated that acid pretreatment can bring about swelling of organic structure at low pH, thus making the substrate easier to be

hydrolyzed [40]. Furthermore, it has the characteristics such as low cost and simple operation. Thermal hydrolysis has also been accepted as the optimum pretreatment method especially for agricultural wastes as it is effective in increasing biogas production by thoroughly destroying the cell membrane [41]. Nevertheless, the optimum pretreatment method for microalgal biomass is still subject to much debate. Therefore, the acid and thermal pretreatment methods were further investigated in this study. Furthermore, a RSM with a CCD was used to find the optimum thermal pretreatment conditions and analyze the data statistically.

## **1.6 Objective and outline of thesis**

Rising global warming, depletion of fossil fuels, rising pollutions are some of the major social and economic crisis we are facing worldwide. To combat these problems, a lot of researchers are working on different fields, trying to find alternative solutions to these world problems. From our knowledge and understanding, out of the immense research that has been carried out, the utilization of biomass to generate energy is a very good option. Statistics have shown that the annual global yield of biomass residue exceeds 220 billion tons which equals the energy of 60-80 billion tons of crude oil. Along with biomass residue, there are additional biomass such as the third generation microalgal biomass that are high sources of bioenergy. Although energy from biomass has great advantages, it still has some barriers due to the inefficiency of biomass conversion. Therefore, in this research we would like to try our best to overcome some of these barriers and achieve a sustainable way to utilize biomass for energy generation. By identifying the efficiency of aquatic biomass to produce bioenergy and attaining the utmost benefits from these biomass through various pretreatment methods is the objective of this research. Some of the specific objectives of this research is enlisted below:

- 1) Optimization of digested sludge used as inoculum to enhance biohydrogen production from aquatic biomass.

- 2) Identify and utilize the capacity of biohydrogen production from aquatic biomass, namely *I. aquatica* and *C. vulgaris* through different pretreatment methods.
- 3) Investigate the efficiency of pretreatment methods based on their capability to improve bioenergy.
- 4) Conduct a study on photocatalysis as a novel pretreatment method, using simulated solar light as an energy source, for bioenergy generation from *C. vulgaris*.

The objectives stated above are achieved in the following chapters of this dissertation.

overall structure of the thesis is as outlined below:

- 1) Introduction – Give the reader a clear background and prospects in this field of research. Also, giving a basic insight about the different concepts that are dealt with in this thesis.
- 2) Identification of *I. aquatica*, an aquatic biomass, as a substrate for biohydrogen production. Along with optimization of the process of biohydrogen production from *I. aquatica*, by investigating different pretreatment methods for substrate and inoculum (digested sludge). Widely accepted pretreatment methods like acid and thermal pretreatment were employed for optimizing the inoculum. Also, RSM software was used in extensive analysis of thermal pretreatment conditions. For the substrate, commonly used pretreatment methods for vegetables like freezing, boiling and drying was employed. The pretreatment methods were evaluated using their capacity to produce hydrogen and their energy generation.
- 3) Utilization of *C. vulgaris*, a microalgal biomass, as a substrate for enhanced biohydrogen production. The substrate was optimized for maximum biohydrogen generation through pretreatment methods such as acid and thermal pretreatment. Again, RSM software was used in extensive analysis of thermal pretreatment conditions. The concept of substrate to inoculum ratio (S/I) was also investigated to obtain the optimum

S/I of microalgal biomass to digested sludge. The pretreatment methods were again evaluated using their capacity to produce hydrogen and bioenergy generation.

- 4) Study was conducted on utilizing photocatalysis as a pretreatment method for disintegrating the cell wall of *C. vulgaris*. P/Ag<sub>2</sub>O/Ag/Ag<sub>3</sub>PO<sub>4</sub>/TiO<sub>2</sub> was used as photocatalyst under simulated solar light, which has been identified to possess excellent photocatalytic activity from previous researches in our lab. Along with disintegration of algal cell wall for enhanced biogas production, the photocatalyst was used as means of releasing lipid from *C. vulgaris* as a value-added product.
- 5) Conclusions – Demonstrates the results of the experiment and conclusions that can be drawn, giving prospective ideas for future research.

**Table 1.1** Previous studies about different pretreatments of various inoculum sources.

<b>Substrate</b>	<b>Inoculum source</b>	<b>Pretreatment methods</b>	<b>Best pretreatment</b>	<b>Ref.</b>
Sucrose	DS- palm oil biogas plant	Alkaline, acid, heat, load-shock, control	Load-shock, heat-shock	[55]
Starch	Thermophilic acidogenic sludge, mesophilic digested sludge, kitchen waste	Heat	Heat	[56]
Glucose	DS- anaerobic digester of sewage treatment plant	Heat, acid, alkaline, aeration, chloroform, control	Heat, alkaline	[57]
Glucose	Sludge from secondary settling tank of wastewater treatment plant	Heat, acid, alkaline, repeated-aeration, control	Repeated-aeration, heat	[58]
Glucose	Anaerobic sewage sludge from wastewater treatment plant, granules from UASB treating starch wastewater	Acid, heat, chloroform, control	Chloroform, heat	[59]
Sucrose	Anaerobic mixed microflora from an UASB treating soybean-processing wastewater	Heat, acid, alkaline	Heat, acid	[60]
Glucose	Aerobic activated sludge, anaerobic digested sludge, soil from watermelon field, soil from kiwi grove	Heat, acid, control	Heat, control	[61]

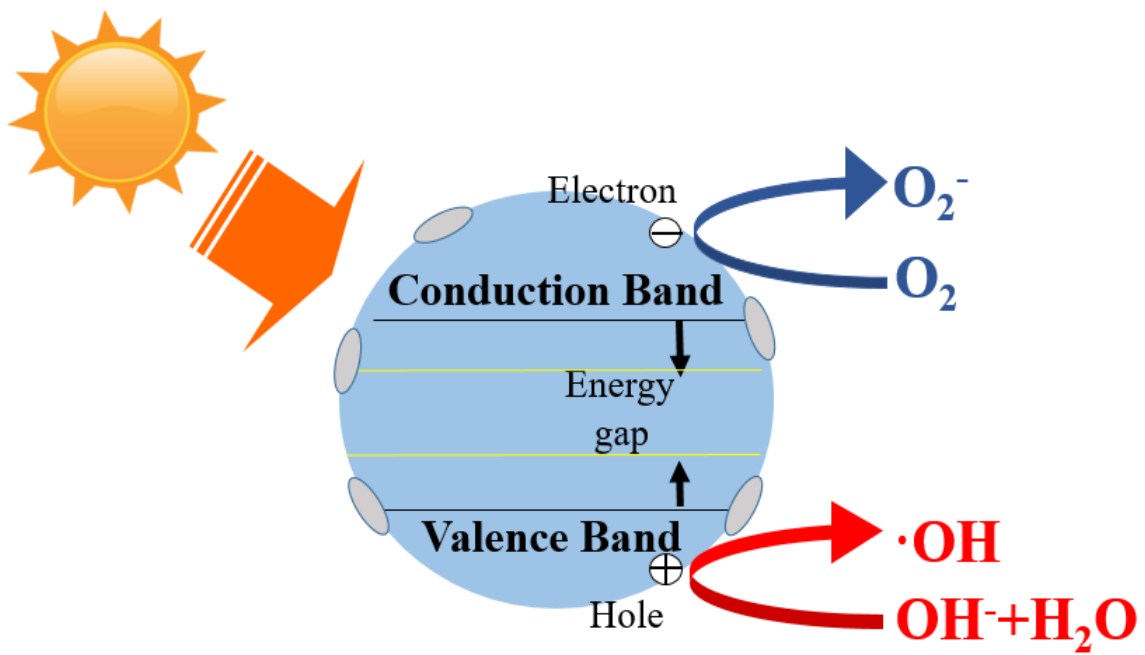


**Table 1.2** Characteristics of *I. aquatica* <sup>[31]</sup>.

Components	% Composition
Moisture	72.8±0.3
Ash	10.8±0.8
Crude lipid	11.0±0.5
Crude fiber	17.7±0.3
Carbohydrate	54.2±0.7
Crude protein	6.3±0.3

**Table 1.3** Characteristics of *C. vulgaris* <sup>[36]</sup>

Components	% Composition
Ash	5.8
Lipid	5 - 40
Carbohydrate	12 - 55
Protein	42 - 58
Pigments	1 - 2



**Fig. 1.1** Mechanism of photocatalysis <sup>[48]</sup>

## Chapter 2 Optimization of biohydrogen production from digested sludge and *I. aquatica*

### 2.1 Introduction

*I. aquatica*, commonly known as ‘water spinach’ has a high carbohydrate content of 54 % and contains mineral elements like K, Na, Ca, Mg, Fe and Mn, making it a viable source for biohydrogen production [30]. Although there are many reports on hydrogen production from plants, there are no reports showing *I. aquatica* as a suitable substrate for biohydrogen production. In our research, for the first time *I. aquatica* was used to produce biohydrogen. Furthermore, for the plant substrate to be readily hydrolysed by bacteria, pretreatment is necessary [7, 32]. In this chapter, commonly used pretreatment methods for substrates with easy operation such as freezing, boiling and alkali pretreatment were examined. Also, to rectify the errors and avoid compositional differences in the plant, the substrate was reduced to constant weight by drying after subjecting it to pretreatment.

On the other hand, DS is present in abundance all over the world and it is also an economical source of microorganisms. DS obtained from waste water treatment plant has been an efficient source of hydrogen producing bacteria (HPB), such as *Clostridium* and *Enterobacter* for biohydrogen production from various biomass. However, in a mixed culture system, under anaerobic condition some hydrogen consuming bacteria (HCB) existing in the DS, such as methanogens, homoacetogens and Archaea [10-13], often readily consume the hydrogen produced by HPB. Therefore, in order to harness hydrogen from a mixed culture system the HCB have to be inhibited by pretreatment [62]. Acid pretreatment using HCl [14-19] and thermal pretreatment [14, 21-23] are the most widely accepted pretreatment methods for inoculum and the same were employed in this chapter to identify the optimum method.

In case of thermal pretreatment, a wide range of temperature and residence time have been reported as optimum conditions for thermal pretreatment in different literature, as the best

pretreatment differs according to the inoculum source [13]. RSM, is a statistical method useful for evaluating the significance of several explanatory variables, understanding the interactions of the various parameters affecting the process, and hence determining optimal conditions for desirable responses [24]. In this chapter, the parameters (temperature and time) of thermal pretreatment of DS was optimized using RSM to enhance hydrogen production.

The objective of this chapter was to identify the capability of biohydrogen production from *I. aquatica* and optimize the process in order to obtain a practical fermentation system for higher biohydrogen production from aquatic biomass. Additionally, a comparison of the hydrogen yield with other researches was carried out.

## **2.2 Materials and Methods**

### **2.2.1 Inoculum preparation from digested sludge**

The DS was obtained from a wastewater treatment plant in Ibaraki prefecture, Japan. After sub packaging in plastic bottles, the digested sludge was stored in the refrigerator at 4°C before using. The pH, total solid (TS), volatile solid (VS) and DOC (dissolved organic carbon) of the DS were 6.8, 11.4 g/L, 7.8 g/L, and 808 mg/L respectively. DS was acclimatized by incubating them at 35°C in 500 mL serum bottles containing trace mineral solution (200 mL/L) for 15 days. The composition of trace mineral solution is as follows: FeSO<sub>4</sub>·7H<sub>2</sub>O (1.1 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (24.6 g/L), CaCl<sub>2</sub>·2H<sub>2</sub>O (2.9 g/L), NaCl (23.4 g/L), MnSO<sub>4</sub>·4H<sub>2</sub>O (111 mg/L), ZnSO<sub>4</sub>·7H<sub>2</sub>O (28.8 mg/L), Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O (29.2 mg/L), CuSO<sub>4</sub>·5H<sub>2</sub>O (25.2 mg/L), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (24.2 mg/L) and H<sub>3</sub>BO<sub>3</sub> (31 mg/L) [20]. In addition, 0.5 g glucose was added every alternate day to enable the acclimatization of DS. For acid pretreatment the DS was adjusted to pH 3 using 2 M HCl. In case of thermal pretreatment, the DS was subjected to heating using a hot air oven (SHIMADZU, Japan) at the corresponding temperature and residence time. Initially to identify the best pretreatment method among acid and thermal pretreatment, 100°C

for 30 min was used as thermal pretreatment condition. Later the DS was pretreated thermally according to the temperature and corresponding residence time as designed by RSM software.

RSM, including two factors and a CCD was used in this chapter to study the effect of independent variables on dependent variables. The maximum H<sub>2</sub> concentration, accumulated H<sub>2</sub> and CH<sub>4</sub> concentration were chosen as response or dependent variables, while temperature (Factor A: 90°C - 100°C) and pretreatment time (Factor B: 15 - 60 min) were chosen as independent variables. Design expert version 6.0.6 was used as the software. Based on the response variables the best thermal pretreatment condition was identified and reported in the form of 2-D contour plots and 3-D response surface models. The thermal pretreatment conditions at 114.14°C for 37.5 min and 85.85°C for 37.5 min were used to calculate standard error and deviation by the program.

### **2.2.2 Substrate preparation from *I. aquatica***

*I. aquatica* was obtained from a supermarket in Ibaraki prefecture, Japan. It was divided into two categories – one category of the plant was pulverized and packed in air-tight bags and frozen (Frozen substrate). The other category of the plant was boiled for 1 min, pulverized and frozen in air-tight bags (Boiled substrate). This was done to prevent decomposition and to study the effects of freezing and boiling as a pretreatment method. In the experiments to identify the optimum inoculum pretreatment condition only frozen *I. aquatica* was used as substrate.

In the further batches of fermentation to identify the optimum substrate pretreatment condition; the frozen and boiled substrates were dried using a hot air oven (SHIMADZU, Japan) at 105°C for 24 h and used as substrate.

For alkali pretreatment method of substrate, the dried substrate was treated using 1% NaOH for 24 h followed by microwave heating for 1 min [7]. Raw *I. aquatica* was used as control.

### **2.2.3 Hydrogen fermentation experiment**

To identify the best pretreatment method for inoculum, 50 mL serum bottles were used as bioreactors. Each bioreactor contained 2 g of frozen *I. aquatica* and 10 mL of thermal or acid pretreated inoculum and the control bioreactor contained untreated inoculum for the fermentation experiments. The pH in the bioreactors was adjusted to 5 using HCl (2 M) and NaOH (2 M) and the incubation temperature was set to 35°C. The bioreactors were then sparged with nitrogen gas (SHIMADZU, Japan) to create anaerobic conditions and the reactors were tightly sealed with rubber caps. The experiment was carried out in triplicate.

Further to identify the optimum thermal pretreatment temperature and time, RSM was used to design the experiments. For the RSM experiments, D-glucose (0.5 g) was used as substrate to identify the best thermal pretreatment condition, in order to maintain uniformity among the different runs of RSM, 25 mL of thermal pretreated inoculum was used and the other conditions were similar to the above experiments. The experiments were carried out in triplicate.

Using the optimum inoculum condition, the fermentation experiments to identify the best pretreatment method for *I. aquatica* was carried out. 2.5 g of frozen dried, boiled dried, alkali pretreated and unfrozen *I. aquatica* as control and 25 mL of optimized inoculum were used for fermentation respectively. The other experimental conditions were similar to the above experiments.

### **2.2.4 Analytical methods**

The biogas yield and composition was measured every day. Biogas was collected using 20 mL plastic syringes which were connected to the bioreactor using plastic tubes as connectors. The volume of the biogas was read directly using the scale on the syringe. The gas composition

was detected via gas chromatography (GC-8A, SHIMADZU, Japan) using a machine equipped with a thermal conductivity detector (80°C) and a Porapak Q column (60°C). Nitrogen was used as the carrier gas. Dissolved Organic Carbon (DOC), Volatile solids (VS) and hydrogen yield (HY) were determined in accordance with standard methods, and pH was detected using a pH meter. Also, the activity of the microorganisms which is indicated by the adenosine triphosphate (ATP) concentration [63] was evaluated on Day 2 using a Bac Titer-Glo™ Microbial Cell Viability Assay (Promega, USA).

### **2.2.5 Energy calculation**

In order to demonstrate the efficiency of biohydrogen production from *I. aquatica*, in terms of energy obtained at the end of the process, the energy was evaluated using equation 1 [64].

$$EH_2 = F \cdot PH_2 (T_w) \cdot HH_2 \quad (1)$$

Where  $EH_2$  is the energy produced from hydrogen,  $PH_2 (T_w)$  is the production of hydrogen per unit volume at the working temperature  $T_w$  and  $HH_2$  is the lower heating value of hydrogen which is around 119.9 mJ/kg.

## **2.3 Results and Discussion**

### **2.3.1 Effect of thermal and acid pretreatment methods of inoculum on H<sub>2</sub> production**

#### **from *I. aquatica***

Hydrogen fermentation experiment was conducted using different inoculum pretreatment methods and the results are as shown in Fig. 2.1. Thermal pretreatment of inoculum at 100°C for 30 min showed the highest concentration of hydrogen at 62.6%. Acid pretreatment of inoculum showed a hydrogen concentration of 53.7%, whereas the control showed the lowest



hydrogen concentration of 28.9%. This can be attributed to the fact that thermal pretreatment more effectively inhibited methanogens in the inoculum which resulted in higher H<sub>2</sub> concentration. In addition to higher H<sub>2</sub> concentration, thermal pretreatment also demonstrated the highest H<sub>2</sub> production of 132.5 mL/L followed by acid pretreatment (125.3 mL/L) and control (78.1 mL/L) as shown in Fig. 2.2.

In the control experiment, the untreated inoculum had different groups of bacteria. These bacteria followed an array of different types of fermentation as they are characterized by great metabolic versatility, both among species and within the same species or strain [21]. Therefore, the concentration of H<sub>2</sub> is drastically reduced in the control due to non-selection of HPB. In acid pretreatment, the acid concentration was favourable in hydrolyzation of inoculum but the high Cl<sup>-</sup> anion concentration inhibited the growth of HPB and resulted in reduced hydrogen producing ability [65]. These results were in accordance with results reported in various literatures where thermal pretreatment was used as an appropriate pretreatment method for inoculum [21, 23, 66 - 68].

Fig. 2.3 shows higher ATP value under thermal treatment condition than acid treatment conditions. The higher ATP value of the thermally pretreated inoculum also corresponds to the highest H<sub>2</sub> concentration as thermal pretreatment not only destroys the HCB but also increases the spore formation in HPB. The poor hydrogen concentration in case of acid pretreatment is due to the increased formation of acidic metabolites, which destroys the cells ability to maintain internal pH [69]. Acid pretreatment resulted in lowering the intracellular level of ATP, thereby, inhibiting glucose uptake which is shown by the low ATP value of acid pretreated inoculum [70]. Thus, ATP value could be an effective indicator of the activity of microorganism during H<sub>2</sub> fermentation.

To further validate the above results, analyses of various measurements such as VS, DOC and HY before and after fermentation were evaluated as illustrated in Table 2.1. A steep

decrease in final VS and DOC values as compared to the initial values showed higher degradation efficiency of the substrate thereby resulting in higher HY. Decrease in the DOC or soluble carbohydrate at the end of hydrogen fermentation indicates that the carbohydrate was readily consumed for hydrogen production [71]. Thermal pretreatment showed the highest  $\delta$ DOC (DOC difference between initial and final conditions) value of 595 mg/L and correspondingly the highest HY of 75.1 mL/g –VS (Table 2.1). Acid pretreatment also showed a comparable  $\delta$ DOC value with a HY of 52.1 mL/g –VS. On the other hand, control showed the lowest  $\delta$ DOC value and correspondingly the lowest HY. Also, the highest initial DOC of  $4653 \pm 12.0$  mg/L in case of thermal pretreatment of inoculum indicated that the pretreatment also helped in the solubilisation of the inoculum; which was otherwise inefficient in the case of acid pretreatment.

Therefore, the H<sub>2</sub> concentration, H<sub>2</sub> production, ATP values, VS, DOC and HY clearly demonstrated that pretreatment of DS enhanced the hydrogen production and that thermal pretreatment is more efficient than acid pretreatment. Consequently, the thermal pretreated DS can be used as an efficient source of HPB for biohydrogen production.

### **2.3.2 Identification of optimum thermal pretreatment condition of inoculum for higher HY, by using RSM analysis**

The previous results indicated that thermal pretreatment was the optimum pretreatment method of inoculum. However, as thermal pretreatment in various researches have varying conditions of temperature and residence time, RSM was employed to identify the best thermal condition. Table 2.2 shows the different runs of experiment carried out at various temperature (Factor A: 90°C - 100°C) and time (Factor B: 15 - 60 min). Fig. 2.4a uses 3 - dimensional (3-D) response surfaces and 2 - dimensional (2-D) contour lines to estimate the H<sub>2</sub> concentration over independent factors such as time and temperature. The H<sub>2</sub> concentration increased

significantly with the increasing residence time of heating, but did not show significant increase with increasing temperature. The value of H<sub>2</sub> concentration was around 60% at a temperature of 90°C for 60 min. This decreased remarkably with decreasing residence time and increasing the temperature. Thermal pretreatment at 90°C for 15 min showed the least significant results with H<sub>2</sub> concentration of 36.4 %, as denoted by the lowest point in the 3-D response surface model (Fig. 2.4a).

The effect of different thermal pretreatment condition on accumulated H<sub>2</sub> is shown in Fig. 2.4b. As seen from the 3-D model the accumulated H<sub>2</sub> showed a peak at 90°C for 60 min as indicated by the highest point on the 3-D model. At this point the methanogens were more effectively inhibited resulting in very high accumulated H<sub>2</sub>. The lowest point on the 3-D model depicts the lowest accumulated H<sub>2</sub> and at this point the methanogens were poorly inhibited leading to very low hydrogen production. The 2-D contour plots do not show symmetrical correlation between the temperature and time of thermal pretreatment as represented by the unsymmetrical contour plots. They show maximum surface response at high residence time as indicated by the contours surrounding the area around 60 min which shows that thermal pretreatment was more time dependent.

Fig. 2.4c demonstrates the 3-D model showing the relative effects of temperature and residence time of thermal pretreatment on CH<sub>4</sub> concentration. The methane concentration showed significant increase on increasing temperature and decreasing residence time, contrary to H<sub>2</sub> concentration (Fig. 2.4a). This is due to the poor inhibition of methanogens at low residence times which favoured methane production. The lowest CH<sub>4</sub> concentration was achieved at 90°C for 60 min which is the most suitable for the hydrogen production.

The above results were confirmed by further analyses of VS, DOC and HY as shown in Table 2.2. Pretreatment at 90°C for 60 min showed the highest HY of 1.0 mol H<sub>2</sub>/mol-glucose and correspondingly a drastic decrease in final DOC value indicating both a high degree of

degradation of substrate and that it was readily consumed to produce H<sub>2</sub> [71]. Accordingly, the HY reduces to 0.6 mol H<sub>2</sub>/mol-glucose for pretreatment at 110°C for 15 min and 110°C for 60 min, which is due to the fact that the former showed higher degradation efficiency but produced CH<sub>4</sub> (Fig. 2.3c) leading to reduction in HY. The latter showed higher inhibition of necessary HPB due to severe pretreatment conditions that lead to denaturation of hydrogenase resulting in low microbial activity and decrease in HY [72]. On further reduction of temperature and residence times (100°C for 37.5 min and 5 min), the HY drastically reduced to 0.5 mol H<sub>2</sub>/mol-glucose and 0.1 mol H<sub>2</sub>/mol-glucose respectively. This was due to low degree of degradation as demonstrated by the low <sup>o</sup>DOC value and was also inefficient in inhibiting the HCB as indicated in Fig. 2.3c.

In conclusion, thermal pretreatment at 90°C for 60 min was determined to be the optimum pretreatment condition and was used to optimize the inoculum in all further experiments.

### **2.3.3 Biohydrogen production from different pre-treated dry substrates of *I. aquatica* under optimum inoculum pretreatment condition**

After obtaining the optimum condition for thermal pretreatment of inoculum using RSM analysis, the optimized inoculum was used in the hydrogen fermentation of *I. aquatica*. Huibo et al. reported that, in order to overcome the negative effects of compositional difference in various parts of the plant, the plant has to be reduced to dry weight [21]. Therefore, in this experiment the boiled and frozen *I. aquatica* was reduced to dry weight before using it as substrate. The frozen dry substrate demonstrated H<sub>2</sub> concentration of over 60% (Fig. 2.5) in the first three days of fermentation, whereas the boiled dry substrate showed a concentration of less than 46%. The NaOH pretreated substrate showed a high concentration of H<sub>2</sub> (53.3%) only on the first day after which the concentration radically declined to less than 30.0%. There were no traces of methane throughout the fermentation experiment.

The accumulated H<sub>2</sub> for various substrate conditions of *I. aquatica* are shown in Fig. 2.6. Frozen dry substrate showed the highest accumulated H<sub>2</sub> of 40.9 mL which was 20 times higher than unfrozen control, 10 times higher than NaOH pretreated substrate and 2.8 times higher than boiled dry substrate. Higher accumulated H<sub>2</sub> demonstrated by frozen dry substrate can be attributed to the fact that freezing and reducing the substrate to dry weight favoured the conversion of more carbon source to simple sugars generating higher hydrogen yield [21]. Drying also led to an increase in concentration of sugars as the moisture was removed. On the contrary the NaOH pretreated substrate showed the lowest accumulated H<sub>2</sub>, the reason being the undesired secondary reactions of hydrolysis process and the decomposition of some useful components in *I. aquatica*. For example, glucose may have been degraded into hydroxyl methyl furfural (HMF). These by-products of sugar negatively affect fermentation efficiency because they are toxic to fermentative microorganisms and inhibit their metabolism [73]. Another reason could be the fibre content which also contributes as a carbon source for fermentation, may have been solubilized by NaOH (alkali soluble fibres) on pretreatment and thereby decrease the carbon content. This can be supported by the decreased initial DOC of NaOH pretreated substrate as indicated in Table 2.3.

To substantiate the above results analyses of VS, DOC and HY are defined in Table 2.3. Here again the frozen dry substrate of *I. aquatica* illustrated the highest HY of 217.2 mL/g-VS and initial DOC value of 6196±15.0 mg/L. Correspondingly a very high decrease in final DOC value, indicated a high degree of degradation of the substrate and that it was readily consumed to produce H<sub>2</sub> [70]. On the other hand, the unfrozen control showed a low initial DOC of 1400±20.0 mg/L and HY of 39.1 mL/g-VS. The HY and initial DOC value were 110.1 mL/g-VS and 5479±30.0 mg/L respectively for boiled dry substrate; which further decreased to 15.1 mL/g-VS and 2171±35.0 mg/L respectively for NaOH pretreated substrate. This explains the high organic carbon content in frozen dry substrate and boiled dry substrate that lead to

increased HY. Although the process of boiling is presumed to degrade and separate the lignocellulosic material, the crystalline structure of the cellulose embedded in the lignocellulosic material is difficult to degrade, which reduces the release of sugars [74]. This accounts for the comparatively low HY in case of boiled dry substrate.

Jun *et al.*, reported that alkali pretreatment was feasible in pretreating rice straw and demonstrated a maximum HY of 155.0 mL/g-VS in dark-fermentation [75]. These results were not in correlation with the results obtained in this research as alkali pretreatment demonstrated the lowest HY. This indicates that different plants could require different pretreatment method due to their differing structure and composition. It is presumed, *I. aquatica* having a very delicate structure as compared to rice straw demonstrated negative effects for alkali pretreatment. The negative effect of alkali pretreatment can further be attributed by the fact that even though unfrozen control had a lower DOC, it demonstrated 2.5 times higher HY than alkali pretreated substrate. While alkali pretreatment had a negative effect on *I. aquatica*, freezing and drying showed a positive effect on the HY (217.2 mL/g-VS). The low DOC content of unfrozen control as compared to frozen dry substrate indicate that freezing is effective as a pretreatment method. Further, the presence of mineral elements like K, Na, Ca, Fe, etc. in *I. aquatica* could have also contributed to the good performance of the bioreactor as these minerals support the growth of microorganisms [30, 76]. Therefore, *I. aquatica* shows interesting characteristics that can enhance both hydrogen production as well as the activity of microorganisms especially under optimum pretreatment conditions.

#### **2.3.4 Comparison of hydrogen yield with other research works**

Table 2.4 shows a comparison of HY from *I. aquatica* with other substrates obtained from various studies. The highest HY (217.2 mL/g-VS) was obtained in this study from frozen dry *I. aquatica* as substrate, which is manifold higher than wheat straw (62.8 mL/g-VS), corn stalk

(176.0 mL/g-VS), corncob (107.9 mL/g-VS), corn straw (9.0 mL/g-VS) and maize leaves (42.0 mL/g-VS) [77, 78 - 83]. This shows that *I. aquatica* is a potential substrate for hydrogen production using digested sludge as inoculum. Although many of the researches used pure culture, the hydrogen yield was still low [78 - 80]; moreover, pure cultures have the disadvantage of not being applicable on large scale owing to their high costs. In this research, digested sludge was used as the inoculum which is more economical and the microorganisms showed very high activity (Fig. 2.3) and therefore corresponded to high hydrogen yield as well.

Some researchers used thermophilic conditions to enhance the fermentation process [83], but the HY was still very low compared to this study, where mesophilic conditions were employed. Fermentation at mesophilic condition not only demonstrated a higher hydrogen yield but also proved to be more economical as the energy consumed was lower. Therefore, the overall process of biohydrogen production from *I. aquatica* and digested sludge was efficient and cost effective.

### **2.3.5 Proposal of a practical process for biohydrogen production from *I. aquatica***

The energy produced per unit volume of the reactor was calculated as 4797 kJ (Eq. 1), where the heating value of  $H_2$  was 119.9 mJ/Kg. From the lab scale experiment results, the energy production is high. However, some energy is consumed for pretreatment and it's necessary to propose a more attractive process for large scale practical applications.

Based on the results obtained we can promote a novel fermentation system (shown in Fig. 2.7). *I. aquatica* can be grown in ponds and lakes to treat eutrophication and then harvested and dried. After that, it can be subjected to freezing in the natural conditions during winter in countries where the temperature falls below 0°C. Subsequently, the *I. aquatica* frozen and dried under natural conditions can be used for hydrogen fermentation. This process would further reduce the freezing costs. Moreover, *I. aquatica* can grow at a rate of 4 inches per day,

producing 84 tonnes of fresh weight biomass per acre in 9 months (Houston Advanced Research Centre, 2006), indicating the huge amount of biomass readily available for biohydrogen production. Further, studies will be carried out to propose a practical system for countries which do not have severe winter conditions.

As for inoculum, the total sludge generated from treatment plants, for example in India alone is estimated to be around 12 billion L/day (Energy Alternatives India (EAI), 2014). This makes it a sustainable source of inoculum after subjecting it to thermal pretreatment at 90°C for 60 min. It follows that, a cost effective, renewable, eco-friendly and sustainable fermentation process for biohydrogen production from water purification plants like *I. aquatica* can be achieved.

## 2.4 Summary

*I. aquatica* for the first time, was used as a suitable substrate for biohydrogen production. Along with, the optimization of DS, used as inoculum was evaluated. Thermal pretreatment of DS showed better results than acid pretreatment and control. Furthermore, RSM results indicated 90°C for 60 min as the optimum pretreatment condition of inoculum with a HY of 1.0 mol H<sub>2</sub>/mol-glucose. Also, frozen dry *I. aquatica* demonstrated the highest HY (217.2 mL/g-VS) among all the other substrate pretreatment conditions. A comparison was made with HY from other substrates which clearly showed that *I. aquatica* demonstrated a HY manifold higher than wheat straw, corn stalk, corncob and maize leaves. The energy obtained from the process was around 4797 kJ. Conclusively, a sustainable process that favours water purification and clean bioenergy production was developed.



**Table 2.1** Variation of VS, DOC and HY according to different pretreatment methods of inoculum.

Pretreatment method	Initial		Final		Hydrogen yield (mL/g-VS)
	VS (%)	DOC (mg/L)	VS (%)	DOC (mg/L)	
Control	2.0±0.6	3857±15.0	1.4±0.2	3812±15.0	26.0
Acid Pretreatment <sup>a</sup>	2.0±0.4	4095±20.0	1.3±0.2	3576±10.0	52.1
Thermal Pretreatment <sup>b</sup>	2.0±0.2	4653±12.0	1.2±0.2	4058±20.0	75.1

<sup>a</sup> Acid pretreatment: 2 M HCl, pH - 3

<sup>b</sup> Thermal pretreatment: 100 °C, 30 min

(Note: The ± values indicate standard deviation in the triplicate experiments. The H<sub>2</sub> yield is calculated from the average values without showing the standard deviation.)

**Table 2.2** Variation of VS, DOC and HY according to different thermal pretreatment conditions of inoculum.

Run	Factor 1 A: Temp- erature (°C)	Factor 2 B: Time (min)	Initial		Final		Hydrogen yield (mol H <sub>2</sub> /mol- Glucose)
			VS (%)	DOC (mg/L)	VS (%)	DOC (mg/L)	
1	85.85	37.5	2.4±0.2	4684±46.0	1.8±0.3	4232±30.0	0.6
2	90	15	2.5±0.1	4935±20.0	2.0±0.1	3703±10.0	0.2
3	90	60	2.4±0.2	4626±35.0	1.7±0.2	2940±10.0	1.0
4	100	5	2.5±0.1	4813±32.0	2.0±0.2	3975±20.0	0.1
5	100	37.5	2.6±0.1	4752±30.0	2.0±0.1	4082±15.0	0.5
6	100	69.31	2.6±0.1	4950±20.0	2.0±0.1	3942±30.0	0.5
7	110	15	2.2±0.3	5008±35.0	1.6±0.2	3925±10.0	0.6
8	110	60	2.4±0.2	5055±50.0	1.7±0.2	4308±15.0	0.6
9	114.14	37.5	2.5±0.1	5108±40.0	1.9±0.0	4228±20.0	0.4

(Note: The ± values indicate standard deviation in the triplicate experiments. The H<sub>2</sub> yield is calculated from the average values without showing the standard deviation.)

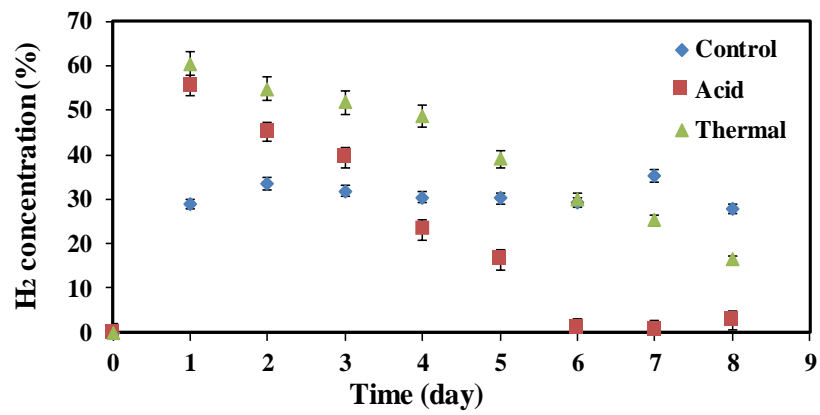
**Table 2.3** Variation of VS, DOC and HY according to the different substrate conditions.

Pretreatment method	Initial		Final		Hydrogen yield (mL/g-VS)
	VS (%)	DOC (mg/L)	VS (%)	DOC (mg/L)	
Unfrozen control	1.8±0.2	1400±20.0	1.3±0.2	997±20.0	39.1
Frozen dry sub	6.2±0.3	6196±15.0	5.5±0.2	4571±10.0	217.2
Boiled dry sub	6.3±0.5	5479±30.0	4.8±0.2	4302±10.0	110.1
Pretreated Sub (NaOH)	4.1±0.2	2171±35.0	3.1±0.2	1812±30.0	15.1

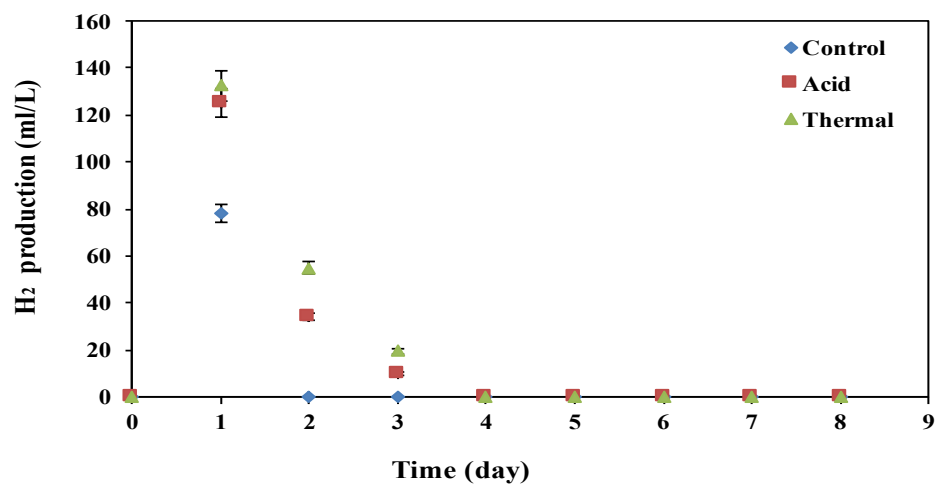
(Note: The ± values indicate standard deviation in the triplicate experiments. The H<sub>2</sub> yield is calculated from the average values without showing the standard deviation.)

**Table 2.4** Comparison of hydrogen yields with other works.

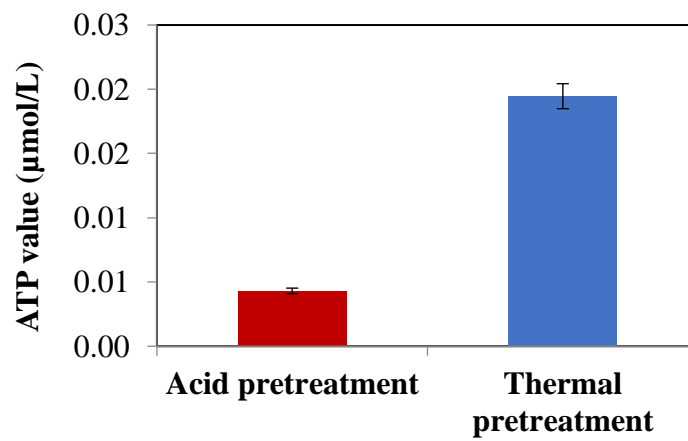
Inoculum	Substrate	Pretreatment	Temp. (°C)	Max. H <sub>2</sub> yield (mL/g- VS)	Ref.
Mix culture – cow dung compost	Wheat straw	HCl	36	62.8	[77]
Pure culture – <i>Clostridium sp.</i>	Corn stalk	Enzyme	55	132.0	[78]
Pure culture	Corn stalk	0.2% HCl, boiled 30 min	36	149.7	[79]
Pure culture – <i>Clostridium sp.</i>	Corn stalk	Bio-pretreatment	36	176.0	[80]
Mix culture – dairy manure	Corn cob	1% HCl + 100 °C, 30 min	36	107.9	[81]
Mix culture – cow dung compost	Wheat straw	No pretreatment	36	1.0	[77]
Pure culture – <i>Clostridium sp.</i>	Corn straw	No pretreatment	35	9.0	[82]
Pure culture – <i>C. saccharolyticus</i>	Maize leaves	130 °C 30 min	70	42.0	[83]
Pure culture – <i>C. saccharolyticus</i>	Sweet sorghum plant	130 °C 30 min	70	32.4	[83]
Pure culture – <i>C. saccharolyticus</i>	Sugarcane bagasse	130 °C 30 min	70	19.6	[83]
Pure culture – <i>C. saccharolyticus</i>	Wheat straw	130 °C 30 min	70	49.0	[83]
Optimized sludge	<i>I. aquatica</i>	Boiled 1 min + dried	35	110.1	This study
Optimized sludge	<i>I. aquatica</i>	1% NaOH + 1 min heating	35	15.1	This study
Optimized sludge	<i>I. aquatica</i>	Frozen + dried	35	217.2	This study



**Fig. 2.1** Effect of different inoculum pretreatment methods on H<sub>2</sub> concentration (%) with frozen *I. aquatica* as substrate (Acid: 2 M HCl - pH 3, Thermal: 100 °C, 30 min).



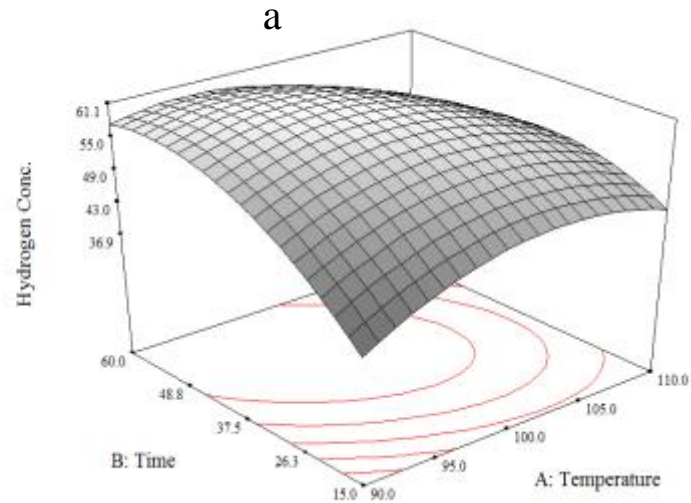
**Fig. 2.2** Effect of different inoculum pretreatment methods on H<sub>2</sub> production (mL/L) with frozen *I. aquatica* as substrate (Acid: 2 M HCl - pH 3, Thermal: 100 °C, 30 min).



**Fig. 2.3** ATP value of the HPB after one-day fermentation at different pretreatment methods.

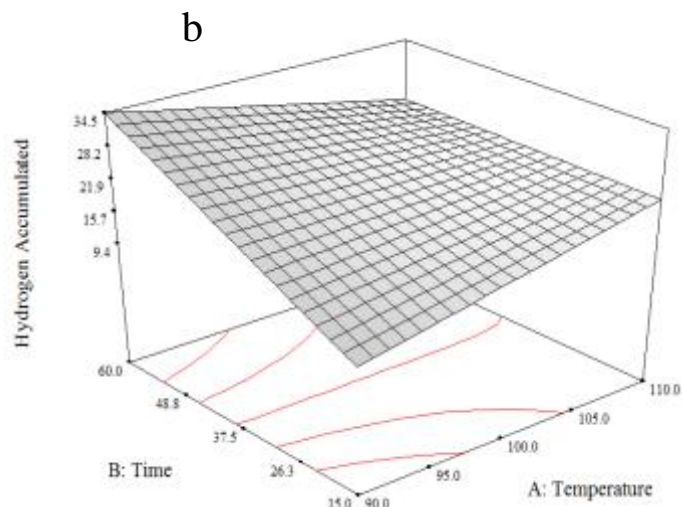
DESIGN-EXPERT Plot

Hydrogen Conc.  
X = A: Temperature  
Y = B: Time



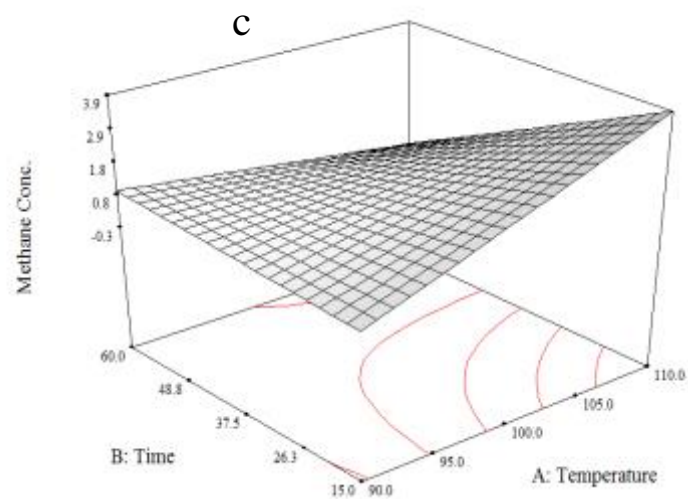
DESIGN-EXPERT Plot

Hydrogen Accumulated  
X = A: Temperature  
Y = B: Time



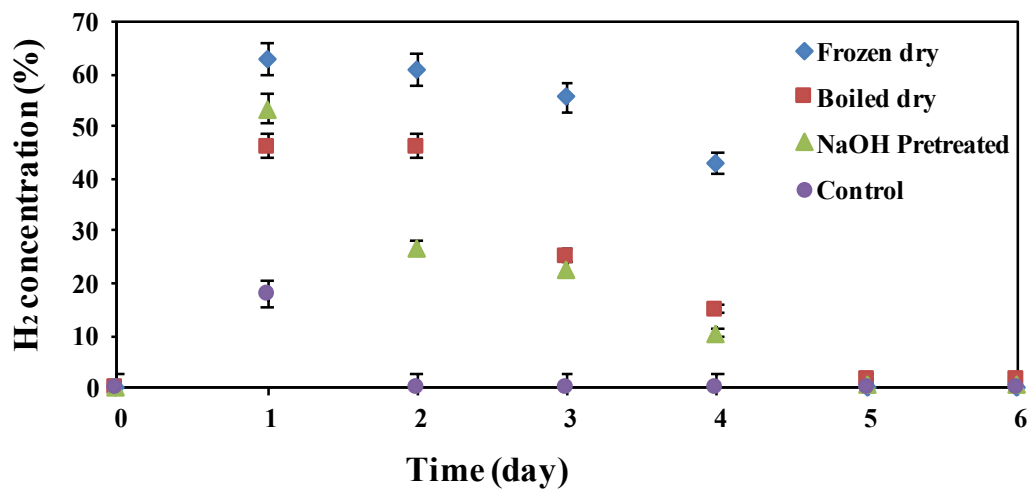
DESIGN-EXPERT Plot

Methane Conc.  
X = A: Temperature  
Y = B: Time

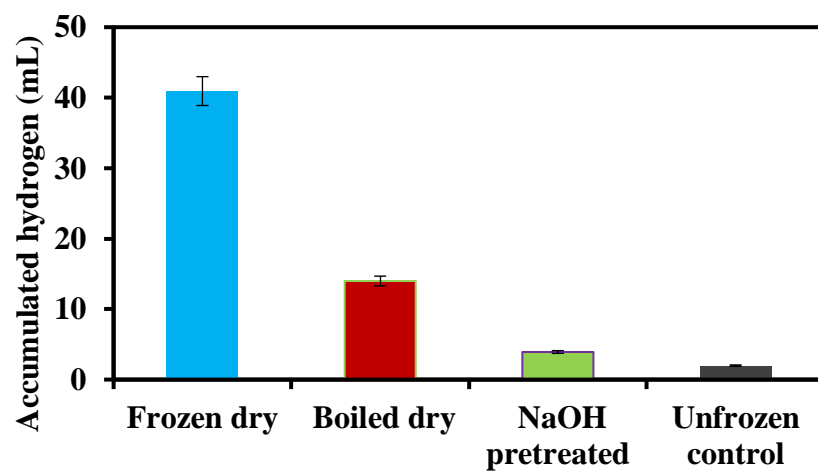


**Fig. 2.4** 3-D Model and 2-D contour plot demonstrating the effect of different temperature ( $^{\circ}\text{C}$ ) and residence times (min) of thermal pretreatment on (a)  $\text{H}_2$  concentration (%), (b) accumulated  $\text{H}_2$  (ml) and (c)  $\text{CH}_4$  concentration (%).

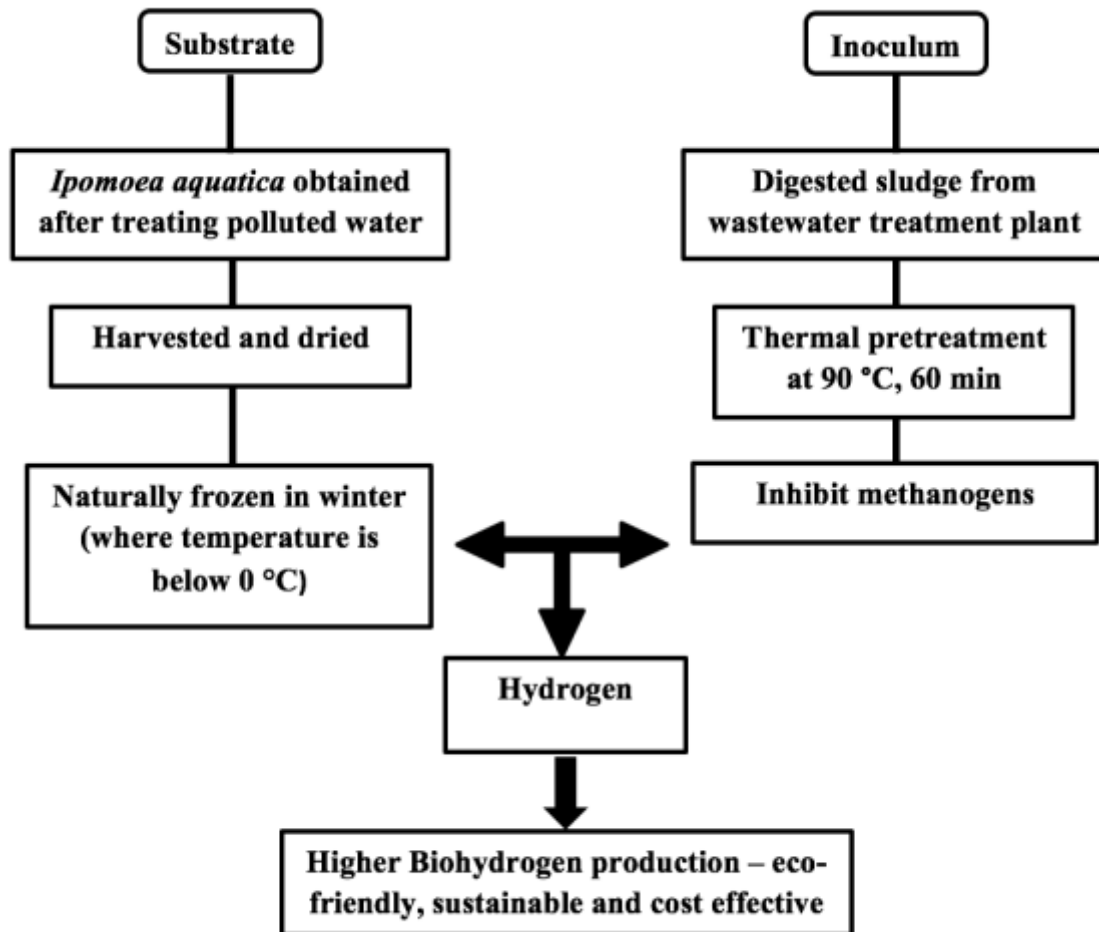




**Fig. 2.5** Effect of optimized inoculum on the H<sub>2</sub> concentration (%) of frozen and boiled dry substrate and NaOH pretreated substrate of *I. aquatica*.



**Fig. 2.6** Effect of optimized inoculum on the accumulated H<sub>2</sub> (mL) of frozen dry, boiled dry, NaOH pretreated substrate and unfrozen control of *I. aquatica*.



**Fig. 2.7.** Process flow diagram demonstrating the practical use of *I. aquatica* and digested sludge for biohydrogen production.

## **Chapter 3 Optimization of *C. vulgaris* as a model of microalgal biomass for enhanced bioenergy production**

### **3.1 Introduction**

From Chapter 2, it was identified that *I. aquatica* was a suitable substrate for biohydrogen production with high energy outcome. However, *I. aquatica* poses some limitation as an annual crop in colder countries, thus cannot be used for practical application in these countries. Therefore, alternative substrate such as microalgal biomass which is widely distributed, was investigated for biohydrogen production. Microalgae has similar characteristic with *I. aquatica* such as water purification ability and contain similar constituents like carbohydrates, proteins and lipids, making comparison between them possible. Recently, microalgal biomass has drawn worldwide attention due to its characteristics such as rapid aquatic growth, wide distribution, high bioenergy productivity, continuous supply and so on [33 - 34]. Among the various microalgal biomass, *Chlorella* is a typical type of microalgal biomass composed of 10 - 70% carbohydrates and 15 - 70% proteins, indicating great potential to be used as feedstock [34, 36]. However, some previous literatures indicated that the intact and strong cell membranes of microalgae would result in a low biogas yield, limiting the efficient digestion in the fermentation process. To help disrupt the cell walls, pretreatment or disintegration of the microalgal biomass is needed [38]. Until now, some different pretreatment methods on microalgal biomass have been investigated. Baccay and Hashimoto investigated that acid pretreatment can bring about swelling of organic structure at low pH, thus making the substrate easier to be hydrolyzed [40]. Furthermore, it has the characteristics such as low cost and simple operation. Thermal pretreatment has also been accepted as the optimum pretreatment method especially for agricultural wastes as it is effective in increasing biohydrogen production by thoroughly destroying the cell membrane [41]. Nevertheless, the optimum pretreatment

method for microalgal biomass is still subject to much debate. Therefore, the acid and thermal pretreatment methods were further investigated in this chapter.

DS was chosen as a source of inoculum in this experiment due to its availability in abundance and demonstration of positive results from previous experiments as demonstrated in chapter 2.

Consecutively, the substrate to inoculum ratio (S/I) was evaluated which is another key factor in dark hydrogen fermentation. It was reported that as the substrate concentration increased the hydrogen production increased as well until a certain threshold. But beyond the threshold value it caused bioreactor upset leading to a decline in the hydrogen production [84]. Also, the higher concentration of inoculum could cause increased nutrient consumption and waste production which would inhibit the hydrogen production itself [85]. Therefore, an optimum S/I is important for enhancing the overall efficiency of dark hydrogen fermentation process. Pakarinen *et al.*, (2008) reported that the substrate to inoculum ratio of 2:1 increased H<sub>2</sub> production efficiently [86]. However, there is no report on the effect of S/I higher than 2:1 on H<sub>2</sub> production from *C. vulgaris* and digested sludge and the same was investigated in this chapter.

In the light of the above research background, this chapter aimed at optimization of the overall hydrogen fermentation process for biohydrogen production from *C. vulgaris*. Acid and thermal pretreatment of *Chlorella* was carried out to investigate the optimum pretreatment method. Furthermore, RSM with a CCD was used to find the optimum thermal pretreatment conditions and analyze the data statistically. Also, the S/I of *Chlorella* and DS were investigated for the optimization of the overall fermentation process. Along with the optimization of the process, the energy from the process was evaluated for practical application and prospects.

### **3.2 Materials and Methods**

### 3.2.1 Inoculum preparation from digested sludge

The DS was obtained from a wastewater treatment plant in Ibaraki prefecture, Japan. After sub packaging in plastic bottles, the digested sludge was stored in the refrigerator at 4 °C before using. The pH, total solid (TS), volatile solid (VS) and DOC (dissolved organic carbon) of the DS were 6.8, 11.4 g/L, 7.8 g/L, and 808 mg/L respectively. DS was acclimatized by incubating them at 35 °C in 500 mL serum bottles containing trace mineral solution (200 mL/L) for 15 days. The composition of trace mineral solution is as follows: FeSO<sub>4</sub>·7H<sub>2</sub>O (1.1 g/L), MgSO<sub>4</sub>·7H<sub>2</sub>O (24.6 g/L), CaCl<sub>2</sub>·2H<sub>2</sub>O (2.9 g/L), NaCl (23.4 g/L), MnSO<sub>4</sub>·4H<sub>2</sub>O (111 mg/L), ZnSO<sub>4</sub>·7H<sub>2</sub>O (28.8 mg/L), Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O (29.2 mg/L), CuSO<sub>4</sub>·5H<sub>2</sub>O (25.2 mg/L), Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O (24.2 mg/L) and H<sub>3</sub>BO<sub>3</sub> (31 mg/L) [20]. In addition, 0.5 g glucose was added every alternate day to enable the acclimatization of DS.

The acclimatized DS was then thermally pretreated by using a hot air oven (WFO-600PD, EYELA, Japan) at 90 °C for 60 min to inhibit the hydrogen consuming bacteria (HCB). Thermal pretreatment at 90 °C for 60 min was obtained as the optimum pretreatment condition of inoculum from the previous chapter.

### 3.2.2 Substrate preparation from *C. vulgaris*

*C. vulgaris* biomass used in this study was bought from the company (CHLORELLA INDUSTRY co. ltd, Japan). Centrifugation was chosen as a method to harvest microalgae as it is the most widely used method [87]. The centrifugation was carried out at 10,000 rpm for 5 min using a centrifugal machine (6800, KUBOTA, Japan) and the residue was used in the further experiments.

Different pretreatment methods were employed on *C. vulgaris* biomass. For acid pretreatment, the pH of *C. vulgaris* biomass was adjusted to 3 using 3% HCl, and then stored in refrigerator at 4 °C for 24 h. After 24 hours, the pretreated microalgal biomass was adjusted

to room temperature. Finally, the pH was set to 5.5 using 3% NaOH for hydrogen fermentation. In case of thermal pretreatment, the *C. vulgaris* was subjected to heating using a hot air oven (WFO-600PD, EYELA, Japan) at the corresponding temperature and residence time. Initially to identify the best pretreatment method among acid and thermal pretreatment, 100°C for 60 min was used as thermal pretreatment condition. Later the *C. vulgaris* was pretreated thermally according to the temperature and corresponding residence time as designed by RSM software.

RSM, including two factors and a CCD was used in this research to study the effect of independent variables on dependent variables. The maximum hydrogen concentration, cumulative hydrogen production and HY were chosen as the response or dependent variables, while temperature ( $X_1$ : 100°C-140°C) and pretreatment time ( $X_2$ : 20-60 min) were chosen as independent variables. Design expert version 6.0.6 was used as the software. The response was fitted using a polynomial quadratic equation to correlate it to the independent variables. The general form of the predictive polynomial quadratic equation used to code variables is as shown in Eq. (2) [88].

$$Y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i=1}^k \sum_{i<j=2}^k b_{ij} x_i x_j \quad (2)$$

Where  $X_i$  are the input variables, which influence the response variable  $Y$ ,  $b_0$  the offset term,  $b_i$  the  $i^{\text{th}}$  linear coefficient,  $b_{ii}$  the quadratic coefficient and  $b_{ij}$  is the  $ij^{\text{th}}$  interaction coefficient.

### 3.2.3 Hydrogen fermentation experiment

During the initial experiments to identify the optimum pretreatment condition 2.5 g of *C. vulgaris* and 25 mL of the heat treated DS was used. For the experiments to determine the optimum S/I, appropriate amounts of substrate and inoculum were added. S/I was defined to be the initial ratio of VS contained in the substrate to the VS contained in the inoculum in each

reactor. The inoculum volume in each reactor contributed to 0.24-g VS. Consecutively, different amounts of harvested and pretreated *C. vulgaris* were added to the reactors to get the desired SIRs of 2, 3, 5, 8, 11 and 14 corresponding to 0.5-g VS, 0.7-g VS, 1.2-g VS, 1.9-g VS, 2.6-g VS and 3.4-g VS, respectively.

All batch experiment were carried out in 50 mL serum bottles. The pH of each bioreactor was adjusted to 5.5 using 2 M HCl or 2 M NaOH prior to dark fermentation. All the bottles were sealed with butyl rubber seals and aluminum caps. In addition, to create anaerobic conditions the bottles were purged with nitrogen gas (SHIMADZU, Japan). Then dark fermentation was carried out at 35°C with constant shaking until no biogas was produced.

### 3.2.4 Analytical methods

The biogas yield and composition was measured every day. Biogas was collected using 20 mL plastic syringes which were connected to the bioreactor using plastic tubes as connectors. The volume of the biogas was read directly using the scale on the syringe. The gas composition was detected via gas chromatography (GC-8A, SHIMADZU, Japan) using a machine equipped with a thermal conductivity detector (80°C) and a Porapak Q column (60°C). Nitrogen was used as the carrier gas. DOC (TOC – 5000A, SHIMADZU, Japan), VS and HY were determined in accordance with standard methods, and pH was detected using a pH meter (TES1380, Japan). The morphology and structure of the microalgal cells were observed using a scanning electron microscope (SEM, DS-720, Topcon, Tokyo, Japan).

### 3.2.5 Energy balance calculation

In order to demonstrate the efficiency of biohydrogen production from *C. vulgaris*, in terms of energy obtained at the end of the process, the bioenergy was calculated using Eq. 3 [64].

$$EH_2 = F \cdot PH_2 (T_w) \cdot HH_2 \quad (3)$$



Where  $E_{H_2}$  is the energy produced from hydrogen,  $PH_2 (T_w)$  is the production of hydrogen per unit volume at the working temperature  $T_w$  and  $HH_2$  is the lower heating value of hydrogen which is around 119.96 mJ/Kg.

### 3.3 Results and Discussion

#### 3.3.1 Effect of different pretreatment methods on hydrogen production from *C. vulgaris*

The results of various pretreatment methods on the daily hydrogen concentration are shown in Fig. 3.1. From the results, it is clear that the heat pretreated *C. vulgaris* showed the highest hydrogen concentration as compared to acid pretreatment and control. Thermal pretreatment had the highest concentration of 33.2% on Day 1 and continued to have the highest concentration throughout the fermentation experiment. On the other hand, the acid pretreatment and control showed hydrogen concentration of 21.6% and 18.9% respectively. These results indicate that pretreatment is a necessary factor. Fig. 3.2 showed the accumulated hydrogen production (mL/L) from different pretreatment methods. The results, clearly showed that hydrogen production from heat pretreated *C. vulgaris* biomass achieved highest volume after Day 1. And the values of accumulated hydrogen production from control, acid pretreatment and thermal pretreatment were 65.7 mL/L, 167.9 mL/L, and 213.3 mL/L, respectively.

Table 3.1 shows the HY, DOC and pH variation from *C. vulgaris* for different pretreatment methods. Thermal pretreatment showed a hydrogen yield of 76.6 mL  $H_2$ /g-VS which was 3 times higher than that of acid pretreatment (25.1 mL  $H_2$ /g-VS) and 6 times higher than the control (13.3 mL  $H_2$ /g-VS). From these results, it can be noted that pretreatment is a necessity and showed enhanced hydrogen yield. Several studies demonstrated that the biohydrogen production is related to the amount of soluble sugars available, which depends on the effective hydrolysis of the substrate [89]. Also, thermal pretreatment showed better results as a

pretreatment method as compared to acid pretreatment which is in accordance with other studies where microalgae was used as the substrate [89]. This can be attributed to the fact that thermal pretreatment is more efficient in the hydrolysis of *C. vulgaris* as indicated by the high DOC value of  $2120\pm 30.0$  mg/L (Table 3.1). DOC is an indicator of the organic content usually composed of soluble sugars and other lower weight components [90]. Therefore, thermal pretreatment was effective in breaking down the cell wall of the algae and releasing the organics enabling efficient hydrolysis. In addition, the thermal pretreatment showed a sharp increase in the final pH which may have resulted from the large ions produced during the hydrolysis of algal biomass (Table 3.1). Reports have also indicated that the efficient hydrolysis of microalgal biomass leads to the formation of large amount of alkali anions leading to a variation in the pH [91].

On the other hand, the acid pretreatment also demonstrated higher HY compared to the control and correspondingly a high DOC value of  $1989\pm 35.0$  mg/L. However, in comparison with thermal pretreatment, the acid pretreatment was not efficient. The reason could be the formation of potent inhibitory compounds such as furfural and hydroxyl methyl furfural (HMF). These compounds are known to have negative effects on the cell membrane function and cell growth of the microorganisms which might be a limiting factor in the fermentation process [92]. Previous studies have shown that furfural and HMF are toxic by-products originated by degradation of pentose and hexose due to strong pretreatment conditions such as acid pretreatment [89]. Therefore, although pretreatment is a necessary factor, the type of pretreatment and its conditions play an important role which also depends on the type of substrate being used.

In order to further establish the above results, SEM images were taken to study the morphology and cell wall structure of the microalgal biomass under different pretreatment conditions. As indicated in Fig. 3.3a, the control without any pretreatment showed a spherical

shape, which is a typical structure of microalgal biomass and *C. vulgaris* in particular [93 - 94]. The cell wall had a smooth surface with no crevices. But, in case of acid pretreatment (Fig. 3.3b), I could see a slight change in the volume of the microalgal cells as indicated by the elongation of the cells, leading to an irregular surface. However, acid pretreatment was not effective enough in disrupting the cell wall completely to release the organics. This can also be supported by the lower DOC value of acid pretreatment as compared to thermal pretreatment (Table 3.1). Fig. 3.3c demonstrates the effect of thermal pretreatment on the microalgal cell wall. It is clearly seen that the thermal pretreatment was effective in breaking down the cell wall completely. There was a huge reduction in the volume of the cell and the cell wall was completely distorted releasing the organics into the solution. This can be further verified with the high DOC content ( $2120 \pm 30.0$  mg/L) of thermal pretreatment. Conclusively, from the  $H_2$  concentration, HY, DOC, pH and SEM results thermal pretreatment was obtained as the optimum pretreatment method for *C. vulgaris*. Further investigations were made to identify the optimum thermal pretreatment conditions.

### **3.3.2 Identification of the optimum thermal pretreatment condition of *C. vulgaris* for higher HY by using RSM analysis**

In order to investigate the optimum thermal pretreatment conditions for *C. vulgaris*, 13 experiments were carried out as described in Section 3.2.2. The coded and actual values of the independent variables along with actual and predicted values of hydrogen concentration and hydrogen yield for each run is indicated in Table 3.2.

Analysis of variance (ANOVA) was carried out and the results are shown in Table 3.3. The model F- value of 30.40 implies that the model was significant, because model terms with value of 'Prob > F' less than 0.05 shows that they are significant [95]. Therefore, the model terms  $X_1$ ,  $X_2$ ,  $X_2^2$  and  $X_1 X_2$  were significant for hydrogen concentration in this study. Though

the model term  $X_1^2$  was insignificant ( $P > 0.05$ ), it cannot be eliminated, because the co-efficient of determination ( $R^2 = 0.96$ ) which indicates that this model can justify 96% variability of the response. The mathematical equation of regression model in terms of actual variables is as shown in Eq. 4. This equation was generated by ANOVA as presented in Table 3.3.

$$Y = +122.47666 - 0.78543 * X_1 + 0.35688 * X_2 + 3.5937E - 003 * X_1^2 + 0.014844 * X_2^2 - 0.014375 * X_1 * X_2 \quad (4)$$

Where Y,  $X_1$  and  $X_2$  are the hydrogen concentration (%), heating temperature ( $^{\circ}\text{C}$ ) and reaction time (min), respectively.

The experimental values versus predicted values for hydrogen concentration (%) are shown in Fig. 3.4. It clearly demonstrates that the experimental values are well distributed near the predicted values (straight line) and correspondingly a notable correlation exists between these values. This signifies that the central composite design model was effective in optimization of the thermal pretreatment condition of *C. vulgaris*. The maximum hydrogen concentration as predicted by the model was 68.5 % at the optimum condition of  $100^{\circ}\text{C}$  for 60 min.

In order to further demonstrate the variation in hydrogen concentration and hydrogen yield with the changing thermal pretreatment conditions 3-D model and 2-D contour plots were generated. Fig. 3.5 presents the 3-D response surface and 2-D contour plots for hydrogen concentration at different thermal pretreatment conditions based on the predicted values by RSM. The 3-D model clearly demonstrates that the hydrogen concentration decreased with the increasing temperature but did not show any significant change with the changing time. The maximum hydrogen concentration was around 68.5% and was obtained at a pretreatment condition of  $100^{\circ}\text{C}$  for 60 min. On the contrary the lowest value of hydrogen concentration was obtained at  $140^{\circ}\text{C}$  for 60 min and these results showed great correlation with actual values from the experiment (Table 3.2). From the 3-D response surface we can arrive at the conclusion that thermal pretreatment of *C. vulgaris* was more temperature dependent rather than time. The

reason for reduced hydrogen concentration at higher temperatures can be attributed to the severity of the treatment condition. Reports have suggested that severe pretreatment conditions leads to the formation of inhibitory compounds such as furfurals which limit the activity of the microorganisms [92]. The 2-D contour plots demonstrated an elliptical fold running diagonally, indicating the slight interdependence between the variables, temperature and time. Results of hydrogen yield showed a similar tendency and thermal pretreatment at 100°C for 60 min achieved the highest HY of 190.9 mL H<sub>2</sub>/g-VS (Table 3.2). These results correlated with the previous experiments to identify the optimum pretreatment method, where again thermal pretreatment at 100°C for 60 min showed better results than acid pretreatment. It is interesting to note that thermal pretreatment at all conditions demonstrated better results than acid pretreatment and the control.

A similar tendency was obtained from the accumulated hydrogen production. The 3-D response surface and 2-D contour lines to estimate the cumulative H<sub>2</sub> production (mL) over independent variables that are temperature and time is as shown in Fig. 3.6. As a result, the cumulative H<sub>2</sub> production (mL) increased with decreasing temperature and increasing residence time. The highest volume of H<sub>2</sub> production was obtained at a temperature of 100°C for 60 min. However, the lowest value of H<sub>2</sub> production was 11.1 mL and 10.9 mL at temperature of 148.28°C for 40 min and 140°C for 60 min, respectively.

Conclusively, thermal pretreatment at 100°C for 60 min was effective in the hydrolysis of *C. vulgaris* on enhancing hydrogen production.

### **3.3.3 Effect of S/I on hydrogen production from *C. vulgaris***

To study the effect of different S/I on the hydrogen production from *C. vulgaris*, experiments were carried out at different substrate concentrations with the inoculum concentration being kept constant. The results of which are depicted in Fig. 3.7 and Table 3.4.

On increasing the S/I from 2 to 8 the H<sub>2</sub> concentration increased from 23.4% to 69.6%. But on further increasing the S/I from 8 to 14 the H<sub>2</sub> concentration decreased to 59.3% (Fig. 3.7). Fig. 3.8 showed accumulated H<sub>2</sub> production (mL/L), obtained from different S/I. Accumulated H<sub>2</sub> increased from 84.8 to 213.3, 595.8 and 1335.9 mL/L with corresponding SIR of 2, 3, 5 and 8. However, when S/I was further increased, the accumulated H<sub>2</sub> decreased. A similar tendency was observed with HY. S/I of 8 demonstrated the highest HY of 190.9 mL H<sub>2</sub>/g-VS which reduced to 21.8 mL H<sub>2</sub>/g-VS on increasing the S/I to 14 (Table 3.4). From these results it is clear that the hydrogen production increases with increasing substrate concentration until a certain threshold, any further increase beyond the threshold would cause severe substrate inhibition [96].

These results can be further validated by the DOC, VS and pH analysis as shown in Table 3.4. Increasing the S/I from 2 to 14 caused an obvious increase in the initial DOC and VS (%) from 1964±28.0 mg/L and 2.4 to 3968±40.0 mg/L and 11.4 respectively. An increase in the S/I from 2 to 8 brought about a 2 times increase in the DOC content and correspondingly a 7 times increase in the HY. This indicates that increasing the substrate concentration increases the overall dissolved organics which in turn increases the HY. However, high levels of DOC can cause substrate inhibition. High levels of DOC and VS can be unfavourable as an overload of substrate makes it difficult for the microorganisms to convert them to hydrogen [97]. Moreover, lack of microorganisms leads to VFA accumulation and bioreactor upset due to acidification of the reactor. This can be supported by the pH results at an S/I of 14. While other S/I showed an increase in the final pH, the S/I of 11 and 14 showed a decrease which is can be attributed to the acidification of the reactor at high substrate concentrations. The pH plays a key role in suppressing the activity of the microorganism in fermentation [98 - 99]. Therefore, an optimum S/I is required to provide optimum substrate for the microorganisms and to also maintain the pH of the reactor. In this study, an optimum S/I of 8 was obtained for enhanced biohydrogen

production from *C. vulgaris*. The energy produced per unit volume of the reactor under optimum conditions was calculated as 6261 kJ, where the heating value of H<sub>2</sub> was 119.96 mJ/Kg (Eq. 3) and was 1.3 times higher than *I. aquatica*.

### 3.3.4 Comparison of HY with other research works

In order to further validate the optimum pretreatment condition and the use of *C. vulgaris* as substrate for hydrogen production, a comparison with other works were made as shown in Table 3.5. It can be noted that *C. vulgaris* was used as a model of microalgae and demonstrated high HY along with digested sludge as source of inoculum in many researches. *Scenedesmus obliquus* demonstrated a comparatively high HY of 90.3 mL H<sub>2</sub>/g- VS which was higher than *C. vulgaris* used as substrate in other researches. The reason for this high HY can be attributed to the use of pure culture (*C. butyricum*) as the source of inoculum. Although, *Scenedesmus* demonstrated a HY which was 3 times higher when pure culture was used as compared to DS, it has disadvantages in practical and large scale applications.

The acid pretreatment of *C. vulgaris* in this study showed much lower HY value compared to other researches which could be attributed to the severity of acid pretreatment as it was carried out for 24 hr. Other intensive pretreatments like ultrasonic and combined pretreatment showed a HY of about 37.9 mL H<sub>2</sub>/g- VS and 41.6 mL H<sub>2</sub>/g- VS, respectively. But these pretreatment conditions are not very favourable for large scale applications. From the comparative analysis with other researches we can say that most of the pretreatment methods are either energy intensive or ineffective in producing high HY's. Therefore, thermal pretreatment of *C. vulgaris* at 100°C for 60 min and at a S/I of 8:1 demonstrating the highest high HY of 190.9 mL H<sub>2</sub>/ g- VS showed the best results. Conclusively, the optimization of pretreatment methods and S/I in this study was successful for *C. vulgaris* as a model of

microalgae and can be applied to other microalgal biomass in the future. Furthermore, it can be agreed upon that microalgal biomass is an attractive substrate for H<sub>2</sub> production.

### **3.4 Summary**

*C. vulgaris* and digested sludge was used as a suitable substrate and inoculum for biohydrogen production. Thermal pretreatment of *C. vulgaris* showed better results than acid pretreatment. Furthermore, RSM results indicated 100°C for 60 min as the optimum thermal pretreatment condition of *C. vulgaris*. S/I of 8 was obtained as the optimum condition for obtaining the highest HY of 190.9 mL/g-VS. Also, energy of 6261 kJ was achieved from the process. Conclusively, a sustainable process by using *C. vulgaris* as substrate and digested sludge as inoculum to produce clean H<sub>2</sub> energy was developed. This process could be of great importance to developing countries in particular due to its cost effectiveness, eco-friendly nature and easy operation.



**Table 3.1** Variation of DOC, final pH and HY according to the different pretreatment methods of *C. vulgaris* biomass.

Pretreatment methods	DOC (mg/L)	Final pH	H <sub>2</sub> yield (mL H <sub>2</sub> /g- VS)
Control	1775±20.0	5.5±0.1	13.3
Acid	1989±35.0	5.5±0.2	25.1
Thermal	2120±30.0	5.9±0.1	76.6

(Note: The ± values indicate standard deviation in the triplicate experiments. The H<sub>2</sub> yield is calculated from the average values without showing the standard deviation)

**Table 3.2** CCD for thermal pretreatment of *C. vulgaris*.

Run no.	Coded values		Real values		H <sub>2</sub> %		HY (mL H <sub>2</sub> /g-VS)
	x <sub>1</sub>	x <sub>2</sub>	X <sub>1</sub>	X <sub>2</sub>	Predicted values	Actual values	
1	-1.000	-1.000	100.00	20.00	64.20	65.00	70.20
2	0.000	0.000	120.00	40.00	49.00	49.00	54.44
3	1.000	1.000	140.00	60.00	37.05	39.00	34.83
4	0.000	0.000	120.00	40.00	49.00	49.00	54.44
5	0.000	-1.414	120.00	11.72	65.98	63.00	124.66
6	0.000	0.000	120.00	40.00	49.00	49.00	54.44
7	0.000	0.000	120.00	40.00	49.00	49.00	54.44
8	-1.414	0.000	91.72	40.00	65.96	67.00	87.56
9	-1.000	1.000	100.00	60.00	68.47	67.00	190.90
10	1.000	-1.000	140.00	20.00	55.78	60.00	90.08
11	1.414	0.000	148.28	40.00	37.79	34.00	40.12
12	0.000	0.000	120.00	40.00	49.00	49.00	54.44
13	0.000	1.414	120.00	68.28	55.77	56.00	47.60

X<sub>1</sub>: Temperature, °CX<sub>2</sub>: Time, min

**Table 3.3** ANOVA for H<sub>2</sub> concentration in thermal pretreatment of *C. vulgaris*.

<b>Source</b>	<b>Sum of squares</b>	<b>Degree of freedom</b>	<b>Mean square</b>	<b>F- Value</b>	<b>Prob &gt; F</b>
Model	1278.4	5	255.7	36.6	< 0.0001
X <sub>1</sub>	793.4	1	793.4	113.7	< 0.0001
X <sub>2</sub>	104.4	1	104.4	14.9	0.0061
X <sub>1</sub> <sup>2</sup>	14.4	1	14.4	2.1	0.1943
X <sub>2</sub> <sup>2</sup>	245.2	1	245.2	35.1	0.0006
X <sub>1</sub> X <sub>2</sub>	132.2	1	132.2	18.9	0.0033

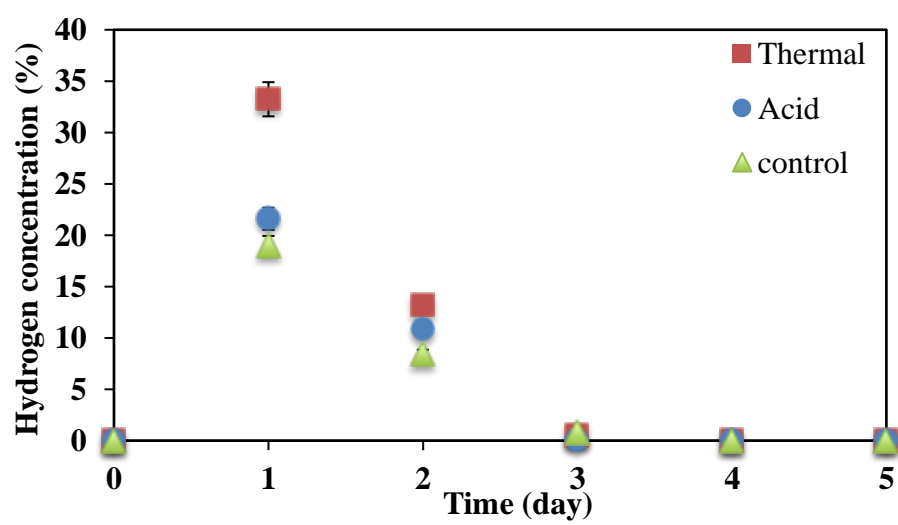
**Table 3.4** Variation of VS, DOC, final pH and HY according to the different SIR 's of *C. vulgaris* and DS.

S/I ratios	VS (%)	DOC (mg/L)	Final pH	HY (mL H <sub>2</sub> /g-VS)
2:1	2.4±0.2	1964±28.0	6.0±0.2	27.6
3:1	3.5±0.0	2120±30.0	5.9±0.1	76.6
5:1	4.8±0.1	2591±22.0	5.9±0.0	97.9
8:1	6.8±0.3	3462±35.0	5.8±0.0	190.9
11:1	8.4±0.2	3612±37.0	5.6±0.1	104.5
14:1	11.4±0.1	3968±40.0	5.3±0.1	21.8

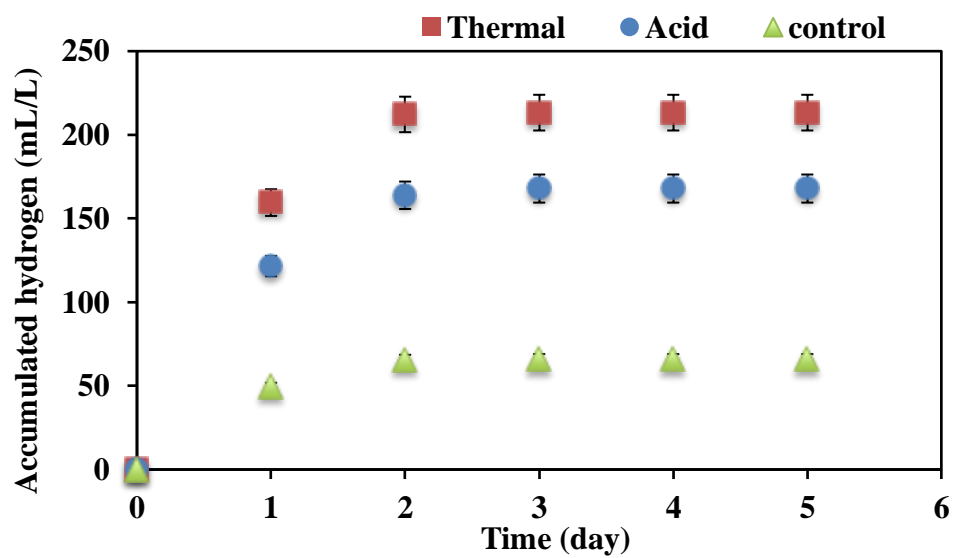
(Note: The ± values indicate standard deviation in the triplicate experiments. The H<sub>2</sub> yield is calculated from the average values without showing the standard deviation)

**Table 3.5** Comparison of HY with other works

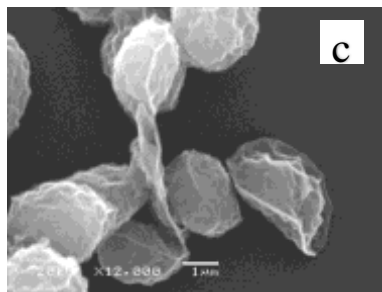
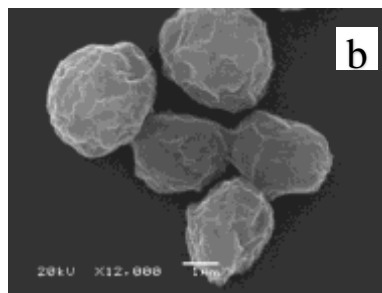
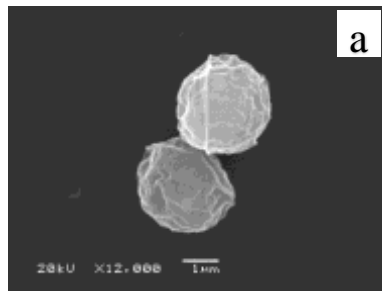
Pretreatment method	Algal biomass	Pretreatment condition	Inoculum	HY (mLH <sub>2</sub> / g-VS)	Ref.
Ultrasonic	<i>C. vulgaris</i>	T = n.d, F=20 kHz, P = 150 W, SEI levels= 10,000–100,000 kJ/Kg	DS	31.9 – 37.9	[95]
Microwave heating	<i>C. pyrenoidosa</i>	T = 140°C, t = 15 min,	DS	12.6	[95]
Acidic HCl	<i>C. vulgaris</i>	t = 10, 35, 60 min, HCl dosage = 0.1, 1.6, 3% (v/w)	DS	13.6 – 36.5	[95]
Acidic HCl+ ultrasonic	<i>C. vulgaris</i>	t = 30 min, F = 20 kHz, P =150 W, SEI levels = 10,000, 55,000, 100,000 KJ/Kg, HCl = 0.10, 1.6, 3% (v/w)	DS	24.2 – 41.6	[95]
Thermal	Lipid-extracted <i>Scenedesmus</i>	T = 100, 121°C, t = 4, 8 h	DS	31.7–31.8	[95]
Thermal	<i>S. obliquus</i>	T = 121°C, t = 15 min	<i>Clostridium butyricum</i>	90.3	[95]
Acidic HCl	<i>C. vulgaris</i>	t = 24 hr, HCl dosage = 3% (v/w)	DS	25.1	This study
Thermal	<i>C. vulgaris</i>	T = 100°C, t = 60 min	DS	190.9	This study



**Fig. 3.1** Effect of different pretreatment methods of *C. vulgaris* biomass on H<sub>2</sub> concentration (%) with DS as inoculum (Acid: 3% HCl – pH 3, Thermal: 100°C, 60 min).

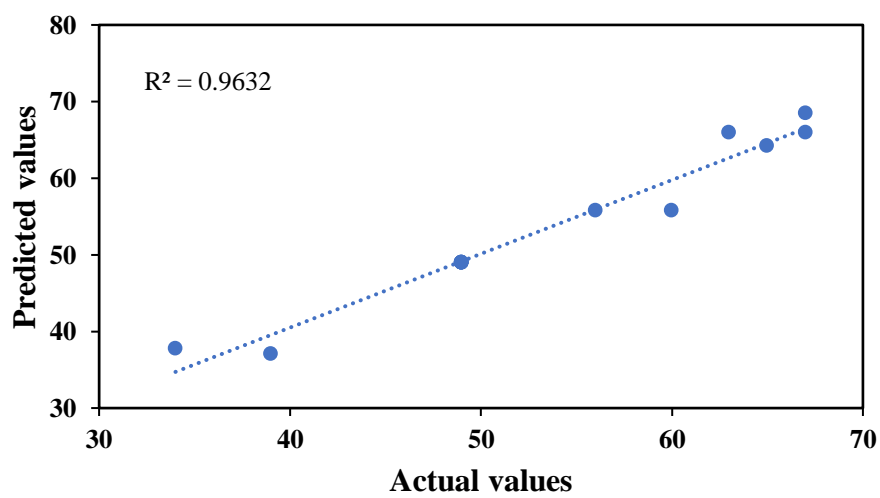


**Fig. 3.2** Effect of different pretreatment methods of *C. vulgaris* biomass on accumulated H<sub>2</sub> production (mL/L) with digested sludge as inoculum (Acid: 2M HCl - pH 3, Thermal: 100°C, 60 min).



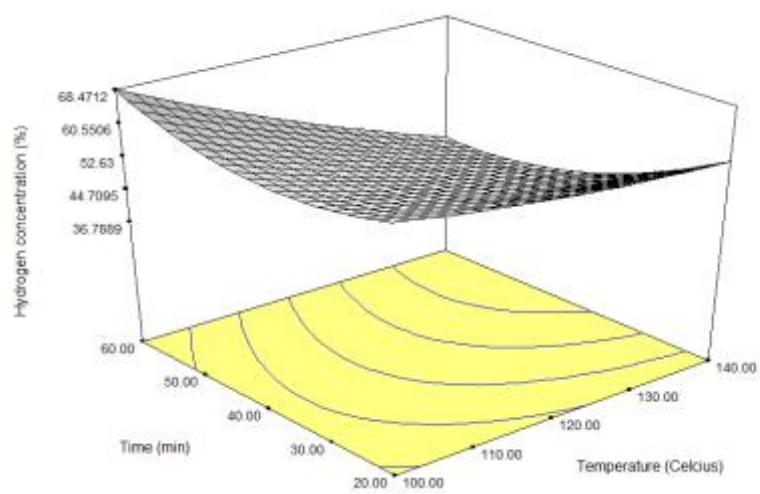
**Fig. 3.3** SEM observations of *C. vulgaris* under different conditions, (a) control, (b) acid pretreatment (3% HCl - pH 3) and (c) thermal pretreatment (100 °C, 60 min).





**Fig 3.4** Predicted values versus experimental values of H<sub>2</sub> concentration.

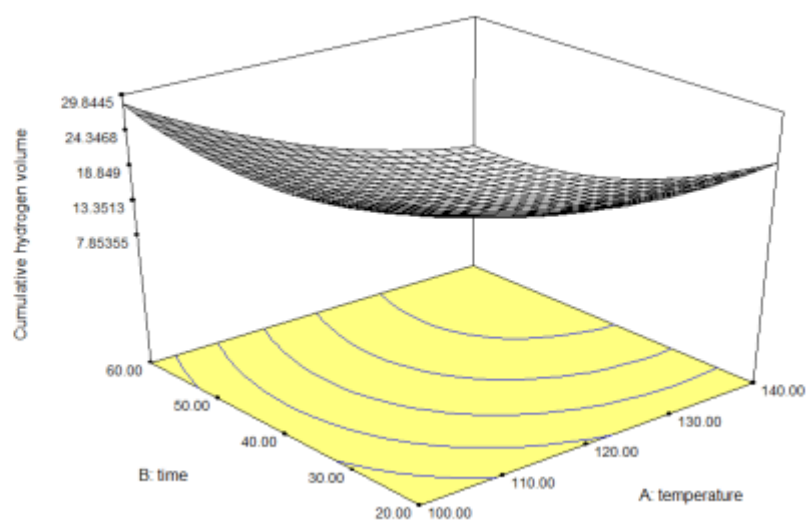
DESIGN-EXPERT Plot  
Hydrogen Concentration  
X = A: Temperature  
Y = B: Time



**Fig. 3.5** 3-D Model and 2-D contour plot demonstrating the effect of different temperature (°C) and residence times (min) of thermal pretreatment of *C. vulgaris* on H<sub>2</sub> concentration (%).

DESIGN-EXPERT Plot

Cumulative hydrogen volume  
X = A: temperature  
Y = B: time



**Fig. 3.6** 3-D model and 2-D contour plot demonstrating the effect of different temperature (°C) and residence times (min) of thermal pretreatment of *C. vulgaris* on cumulative H<sub>2</sub> production.

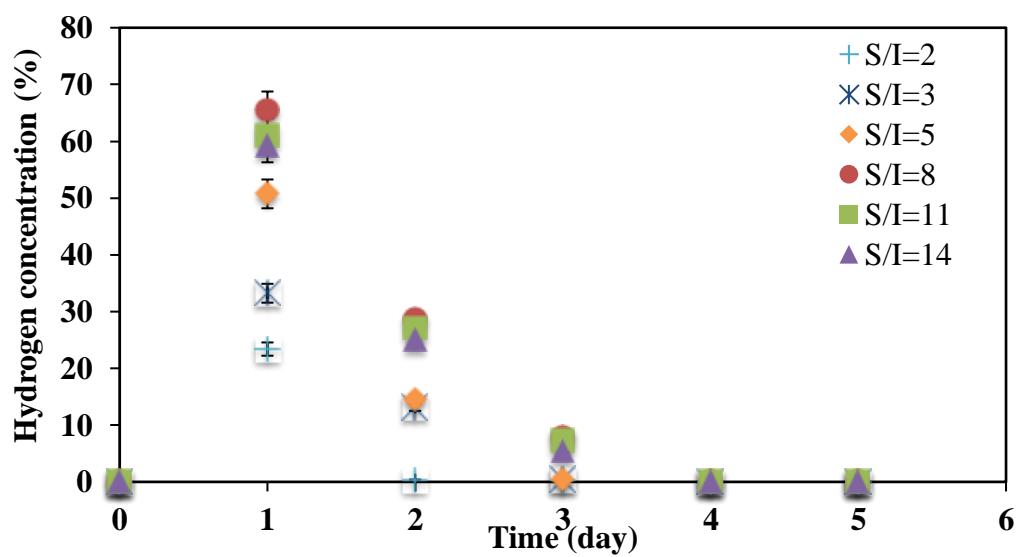
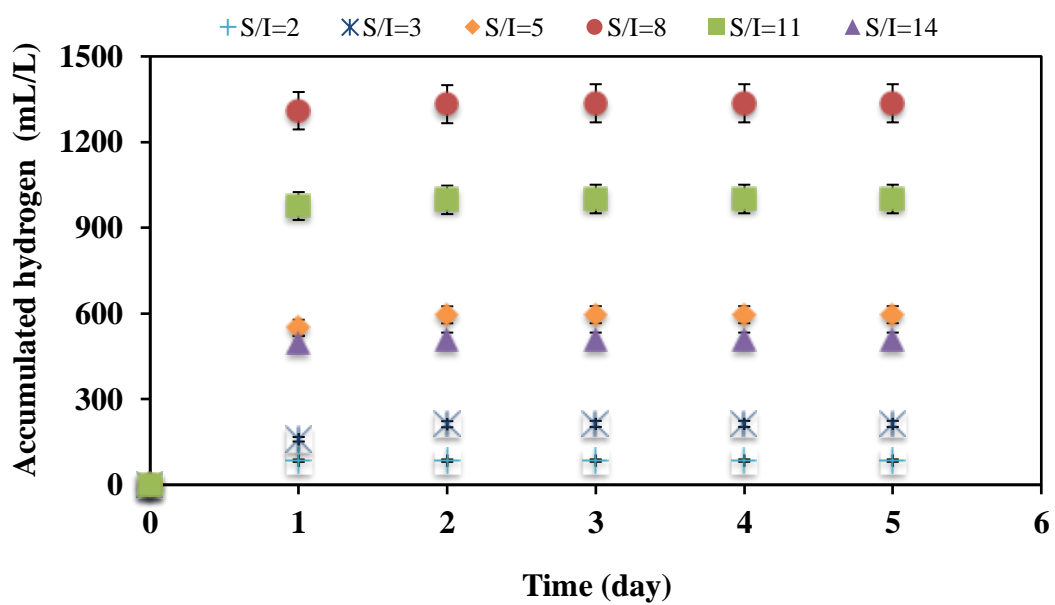


Fig. 3.7 Effect of different S/I on H<sub>2</sub> concentration (%) from *C. vulgaris* and DS.



**Fig. 3.8** Effect of different S/I on accumulated hydrogen (mL/L) from *C. vulgaris* and DS.

## Chapter 5 Conclusions

In this research, two aquatic biomass, namely *I. aquatica* and *C. vulgaris* was investigated for high bioenergy generation. Different pretreatment methods were used and their effects on bioenergy generation was analyzed. Also, a novel pretreatment method for *C. vulgaris* was identified through photocatalysis using PEGm - P/Ag/Ag<sub>2</sub>O/Ag<sub>3</sub>PO<sub>4</sub>/TiO<sub>2</sub> photocatalyst under simulated solar light. The following conclusions can be drawn from the previous chapters.

### 5.1 Optimization of biohydrogen production from digested sludge and *I. aquatica*

*I. aquatica* for the first time, was used as a suitable substrate for biohydrogen production. Along with, the optimization of DS, used as inoculum was evaluated. Thermal pretreatment of DS showed better results than acid pretreatment and control. Furthermore, RSM results indicated 90°C for 60 min as the optimum pretreatment condition of inoculum with a HY of 1.02 mol H<sub>2</sub>/mol-glucose. Also, frozen dry *I. aquatica* demonstrated the highest HY (217.16 mL/g-VS) among all the other substrate pretreatment conditions. A comparison was made with HY from other substrates which clearly showed that *I. aquatica* demonstrated a HY manifold higher than wheat straw, corn stalk, corncob and maize leaves. The energy obtained from the process was around 4797 kJ. Conclusively, a sustainable process that favours water purification and clean bioenergy production was developed.

### 5.2 Optimization of *C. vulgaris* as a model of microalgal biomass for enhanced energy production

*C. vulgaris* and digested sludge was used as a suitable substrate and inoculum for biohydrogen production. Thermal pretreatment of *C. vulgaris* showed better results than acid pretreatment. Furthermore, RSM results indicated 100°C for 60 min as the optimum thermal

pretreatment condition of *C. vulgaris*. S/I of 8 was obtained as the optimum condition for obtaining the highest HY of 190.9 mL/g-VS. Also, energy of 6261 kJ was achieved for the overall process. Conclusively, a sustainable process by using *C. vulgaris* as substrate and digested sludge as inoculum to produce clean H<sub>2</sub> energy was developed. This process could be of great importance to developing countries in particular due to its cost effectiveness, eco-friendly nature and easy operation.

#### **5.4 Future research**

Pretreatment method for lipid separation and simultaneous biogas generation from *Chlorella vulgaris* will be investigated in the future research. Also, to further enhance the biohydrogen generation and complete utilization of the substrate, co-digestion of carbohydrate rich substrate (like *I. aquatica*) with protein rich substrate (like *C. vulgaris*) will be investigated.

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## List of publications and awards

### Publications:

1. Stanislaus, M. S., Zhang, N., Zhao, C., Zhu, Q., Li, D., Yang, Y., *Ipomoea aquatica* as a new substrate for enhanced biohydrogen production by using digested sludge as inoculum, *Energy*, No.118.1, pp 264-271,2017 (IF: 4.292)
2. Stanislaus, M. S., Yue, Y., Zhang, N., Zhao, C., Hu, X., Zhu, Q., Li, D., Yang, Y., Improvement of Biohydrogen Production by Optimization of Pretreatment Method and Substrate to Inoculum Ratio from Microalgal Biomass and Digested Sludge, *International Journal of Hydrogen Energy*. Accepted (IF: 3.205)
3. Zheng, H., Li, D., Stanislaus, M. S., Zhang, N., Zhu, Q., Hu, X., Yang, Y., Development of a bio-zeolite fixed-bed bioreactor for mitigating ammonia inhibition of anaerobic digestion with extremely high ammonium concentration livestock waste, *Chemical Engineering Journal*, No. 280, pp106–114, 2015 (IF: 5.310)
4. Zhang, N., Stanislaus, M. S., Hu, X., Zhao, C., Zhu, Q., Li, D., Yang, Y., Strategy of mitigating ammonium-rich waste inhibition on anaerobic digestion by using illuminated bio-zeolite fixed-bed process, *Bioresource Technology* No. 222, pp 59-65, 2016 (IF: 4.917)
5. Hu, X., Zhu, Q., Gu, Z., Zhang, N., Liu, N., Stanislaus, M. S., Li, D., Yang, Y., Wastewater treatment by sonophotocatalysis using PEG modified TiO<sub>2</sub> film in a circular Photocatalytic-Ultrasonic system, *Ultrasonics Sonochemistry*, No. 36, pp 301–308, 2017 (IF:4.556)
6. Zhu, Q., Hu, X., Stanislaus, M. S., Zhang, N., Xiao, R., Liu, N., Yang, Y., P/Ag/Ag<sub>2</sub>O/Ag<sub>3</sub>PO<sub>4</sub>/TiO<sub>2</sub> composite film for water purification and antibacterial application under solar light irradiation, *A novel Science of The Total Environment*, No.577.15, pp 236–244, 2017 (IF: 3.916)

## Awards:

1. **Outstanding presentation award:** Stanislaus, M.S., Zheng, H., Li, D. W., Zhang, Z. Y., Yang, Y. N. Development of Higher Biohydrogen Production Process from *Ipomoea aquatica*, an Aquatic Purification Plant. The 7th Korea -China- Japan Graduate Student Forum, Life Environment and Resource, Beijing, China. 26-29, September 2014.
2. **Outstanding presentation award:** Stanislaus Mishma Silvia, Nan Zhang, Zhu Qi, Yingnan Yang. Study of the Effects of Photocatalytic Pretreatment on Various Macromolecular Components and *Chlorella vulgaris* to Enhance Biohydrogen Production. International Symposium on Agriculture Education for Sustainable Development, Tsukuba, Japan. November 2015.
3. **Outstanding presentation award:** Stanislaus Mishma Silvia, Yue Yuan, Zhang Nan, Zhao Chenyu, Yang Yingnan, Fermentative Biohydrogen production from *Chlorella vulgaris*, a Microalgal Biomass. The 8th Korea -China- Japan Graduate Student Forum (2015).
4. Recognition of excellence: Stanislaus, M.S., Zhu, Q., Zhang, N., Zhao, C. Y., Yang, Y. N.: Photocatalytic Pretreatment of *Chlorella vulgaris* for Improving Biohydrogen Production The 9th Korea-China-Japan Graduate Student Forum, Daejeon, Japan, September 20-23, (2016).

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