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Ipomoea aquatica as a new substrate for enhanced biohydrogen production by using digested sludge as inoculum

Mishma S. Stanislaus, Nan Zhang, Chenyu Zhao, Qi Zhu, Dawei Li, Yingnan Yang*

Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

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ABSTRACT

Ipomoea aquatica, a tropical plant was used as a new substrate, and the digested sludge (DS) was used as inoculum for biohydrogen production. In order to inhibit the hydrogen consuming bacteria (HCB), the DS was subjected to thermal and acid pretreatment to identify the optimum method. The results showed that thermal pretreatment was better than acid pretreatment. To further investigate the best thermal pretreatment condition of DS, response surface methodology (RSM) was employed. Consecutively, thermal pretreatment at 90 °C for 60 min was identified as the optimum pretreatment condition for inoculum. Further, *Ipomoea aquatica* used as substrate was also optimized under conditions like freezing, boiling, and alkali pretreatment to attain high hydrogen yield (HY). Frozen and dried *I. aquatica* demonstrated the highest HY of 217.16 mL/g-VS, which was manifold higher than control and other treatment conditions. The energy consumed in the fermentation process was evaluated which was lesser than energy produced in the process. Furthermore, a practical process was proposed. To the best of our knowledge, it's the first time that *I. aquatica* was used as substrate to produce hydrogen through an attractive process that could not only benefit the environment by water purification but also contributes to clean energy production.

1. Introduction

Currently global consumption of fossil fuels is equivalent to more than 11 billion tonnes of oil. Crude oil reserves are vanishing at the rate of 4 billion tonnes a year, and at this rate all known accessible conventional oil deposits will be gone by 2042 [1]. Hydrogen can provide an alternative source of energy to meet this rising global demand. It is a clean fuel with no combustion by-products other than water. The high energy density (122 kJ/g) makes it especially attractive as a mobility fuel to replace gasoline [2,3].

Conventional methods of hydrogen production like steam reforming, electrolysis and thermolysis have major drawbacks due to their hazardous nature and high energy consumption [4]. One option to overcome these problems would be to use biological means such as waste biomass to produce hydrogen through fermentation. The annual global yield of biomass residue exceeds 220 billion tonnes which potentially equals the energy of 60–80 billion tonnes of crude oil [5].

Ipomoea aquatica, commonly known as 'water spinach' a free floating semi-aquatic plant plays an important role in pollutant removal from lakes and ponds in order to overcome eutrophication [6]. However, the rapidly growing *I. aquatica* needs to be harvested as a resource to restrain secondary pollution in aquatic environments. Its high carbohydrate content of 54.2% and presence of mineral elements like K, Na, Ca, Mg, Fe and Mn could be a viable source for biohydrogen production [6]. 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 this research, for the first time *Ipomoea aquatica* was used to produce biohydrogen. Furthermore, for the plant substrate to be readily hydrolysed by bacteria, pretreatment is necessary [7–11]. In the present research, commonly used pretreatment methods for substrates with easy operation such as freezing, boiling and alkali pretreatment were examined. On the other hand, digested sludge (DS) is present in abundance all over the world and it's 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 like

* Corresponding author.
E-mail address: yo.innan.fu@u.tsukuba.ac.jp (Y. Yang).

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agricultural waste, food residues, plant residues, waste water and so on [8–12]. However, in a mixed culture system, under anaerobic condition some hydrogen consuming bacteria (HCB) existing in the DS, such as methanogens, homoacetogens and Archaea [13–16], 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 [17]. Acid pretreatment using HCl [18–22] and thermal pretreatment [7,12,23,24] are the most widely accepted pretreatment methods for inoculum. However, the most efficient pretreatment for higher hydrogen production is still subject to much debate.

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 [7,12–14]. Response surface methodology (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 [25]. In this research the parameters (temperature and time) of thermal pretreatment of DS was optimized using RSM to enhance hydrogen production.

The objective of this research 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 water purification plants. Additionally, an overall energy balance of the entire process was calculated and comparison of the hydrogen yield with other researches was carried out.

2. Materials and methods

2.1. Inoculum preparation from digested sludge

Digested sludge obtained from a waste water treatment plant in Japan, was acclimatized for a week at 35 °C using a trace mineral solution and then used as inoculum. The inoculum preparation is explained in detail in our previous work [26]. 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 (SHIMAZU, 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 central composite design (CCD) was used in this research to study the effect of independent variables on dependent variables. The maximum H_2 concentration, accumulated H_2 and CH_4 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. Substrate preparation from Ipomoea 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 in order to prevent decomposition and also 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 (SHIMAZU, 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.

2.3. 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 (SHIMAZU, 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) (WAKO, Japan) 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 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 previous experiments.

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, SHIMAZU, 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 [26] was evaluated on Day 2 using a Bac Titer-Glo™ Micro-bial Cell Viability Assay (Promega, USA).

2.5. Energy balance calculation

In order to demonstrate the efficiency of biohydrogen production from *I. aquatica,* in terms of overall energy obtained at the end of the process, the energy balance was evaluated. The energy consumed and produced for the overall process was calculated

using equations (1)–(3) [27].

$$Q = Cp\ m\ dT \quad (1)$$

Where Q is the amount of heat (KJ), Cp is specific heat capacity (kJ/Kg-K), m is mass (Kg) and dT is the difference in temperature.

$$Ew = [\rho \cdot Cp\ (Tw - Ta) \cdot F]/\eta \quad (2)$$

Where Ew is the energy required to warm the reactor (KJ), ρ is the biomass density, Tw is the working temperature, Ta is the ambient (outdoor) temperature and F is the working volume.

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

Where EH_2 is the energy produced from hydrogen per unit volume of the reactor and PH_2 (Tw) is the specific production of hydrogen.

The specific heat capacity of the digested sludge and *I. aquatica* was similar and around 4.18 KJ/Kg-K [28].

3. Results and discussion

3.1. Effect of thermal and acid pretreatment methods of inoculum on H_2 production from I. aquatica

Hydrogen fermentation experiment was conducted using different inoculum pretreatment methods and the results are as shown in Fig. 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_2 concentration. In addition to higher H_2 concentration, thermal pretreatment also demonstrated the highest H_2 production of 132.5 mL/L followed by acid pretreatment (125.3 mL/L) and control (78.1 mL/L).

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 [7]. Therefore, the concentration of H_2 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 anion concentration inhibited the growth of HPB and resulted in reduced hydrogen producing ability [29]. These results were in accordance with results reported in various literature where thermal pretreatment was used as an appropriate pretreatment method for inoculum [7,9,12,23,30].

Fig. 2 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_2 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 cell's ability to maintain internal pH [31]. 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 [32]. Thus, ATP value could be an effective indicator of the activity of microorganism during H_2 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 1. Larger 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 [33]. Thermal pretreatment showed the highest $^{\delta}$DOC (DOC difference between initial and final conditions) value of 23.79 mg/L and correspondingly the highest HY of 75.09 mL/g −VS (Table 1). Acid pretreatment also showed a comparable $^{\delta}$DOC value with a HY of 52.15 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 186.12 ± 0.00 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_2 concentration, 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.

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 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. 3a uses three-dimensional (3-D) response surfaces and two-dimensional (2-D) contour lines to estimate the H_2 concentration over independent factors such as time and temperature. The H_2 concentration

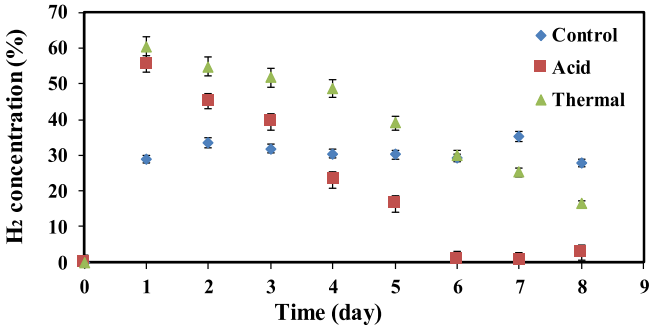

Fig. 1. Effect of different inoculum pretreatment methods on H_2 concentration (%) with frozen *I. aquatica* as substrate (Acid: 2 M HCl - pH 3, Thermal: 100 °C, 30 min).

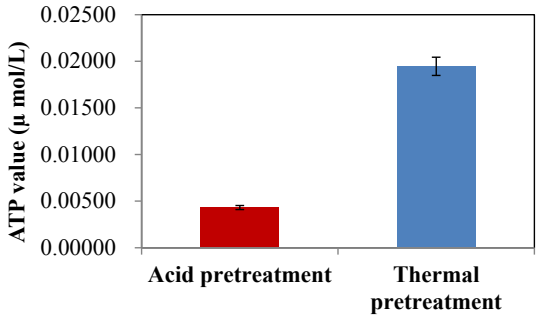

Fig. 2. ATP value of the HPB after one-day fermentation at different pretreatment methods.

Table 1
Variation of VS (%), DOC (mg/L) and HY (mL/g–VS) 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 ± 0.60	154.28 ± 0.00	1.4 ± 0.20	152.51 ± 0.00	26.00
Acid Pretreatment[a]	2.0 ± 0.00	163.83 ± 2.00	1.3 ± 0.20	143.04 ± 1.00	52.15
Thermal Pretreatment[b]	2.0 ± 0.20	186.12 ± 0.00	1.2 ± 0.20	162.33 ± 0.00	75.09

(Note: The + - values indicate standard deviation in the triplicate experiments. The H_2 yield is calculated from the average values without showing the standard deviation.).
[a] Acid pretreatment: 2 M HCl, pH - 3.
[b] Thermal pretreatment: 100 °C, 30 min.

Table 2
Variation of VS (%), DOC (mg/L) and HY (mL/g–VS) according to different thermal pretreatment conditions of inoculum.

Run	Factor 1A: Temperature (°C)	Factor 2B: Time (min)	Initial		Final		Hydrogen yield (mol H_2/mol-Glucose)
			VS (%)	DOC (mg/L)	VS (%)	DOC (mg/L)	
1	85.85	37.5	2.4 ± 0.20	187.36 ± 4.60	1.8 ± 0.30	169.30 ± 3.00	0.56
2	90	15	2.5 ± 0.10	197.41 ± 2.00	2.0 ± 0.10	148.15 ± 1.00	0.25
3	90	60	2.4 ± 0.20	185.06 ± 6.20	1.7 ± 0.20	117.61 ± 1.00	1.02
4	100	5	2.5 ± 0.10	192.54 ± 3.20	2.0 ± 0.20	159.03 ± 2.00	0.14
5	100	37.5	2.6 ± 0.10	190.09 ± 3.00	2.0 ± 0.10	163.31 ± 0.00	0.46
6	100	69.31	2.6 ± 0.10	198.02 ± 2.00	2.0 ± 0.10	157.70 ± 3.00	0.51
7	110	15	2.2 ± 0.30	200.32 ± 3.70	1.6 ± 0.20	157.01 ± 1.00	0.65
8	110	60	2.4 ± 0.20	202.21 ± 5.00	1.7 ± 0.20	172.33 ± 1.50	0.65
9	114.14	37.5	2.5 ± 0.10	204.35 ± 6.00	1.9 ± 0.00	169.14 ± 2.00	0.45

(Note: The + - values indicate standard deviation in the triplicate experiments. The H_2 yield is calculated from the average values without showing the standard deviation.).

increased significantly with the increasing residence time of heating but did not show significant increase with increasing temperature. The value of H_2 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_2 concentration of 36.45%, as denoted by the lowest point in the 3-D response surface model (Fig. 3a).

The effect of different thermal pretreatment condition on accumulated H_2 is shown in Fig. 3b. As seen from the 3-D model the accumulated H_2 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_2. The lowest point on the 3-D model depicts the lowest accumulated H_2 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. 3c demonstrates the 3-D model showing the relative effects of temperature and residence time of thermal pretreatment on CH_4 concentration. The methane concentration showed significant increase on increasing temperature and decreasing residence time, contrary to H_2 concentration (Fig. 3a). This is due to the poor inhibition of methanogens at low residence times which favoured methane production. The lowest CH_4 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. Pretreatment at 90 °C for 60 min showed the highest HY of 1.02 mol H_2/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_2 [33]. Accordingly, the HY reduces to 0.65 mol H_2/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_4 (Fig. 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 [34]. On further reduction of temperature and residence times (100 °C for 37.5 min and 5 min), the HY drastically reduced to 0.46 mol H_2/mol-glucose and 0.14 mol H_2/mol-glucose respectively. This was due to low degree of degradation as demonstrated by the low $^{\delta}$DOC value and was also inefficient in inhibiting the HCB as indicated in Fig. 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.

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 [7]. 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 an H_2 concentration of over 60.0% (data not shown) in the first three days of fermentation, whereas the boiled dry substrate showed a concentration of less than 46.0%. The NaOH pretreated substrate showed a high concentration of H_2 (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_2 for various substrate conditions of *I. aquatica* are shown in Fig. 4. Frozen dry substrate showed the highest accumulated H_2 of 40.9 mL which was 20 times higher than

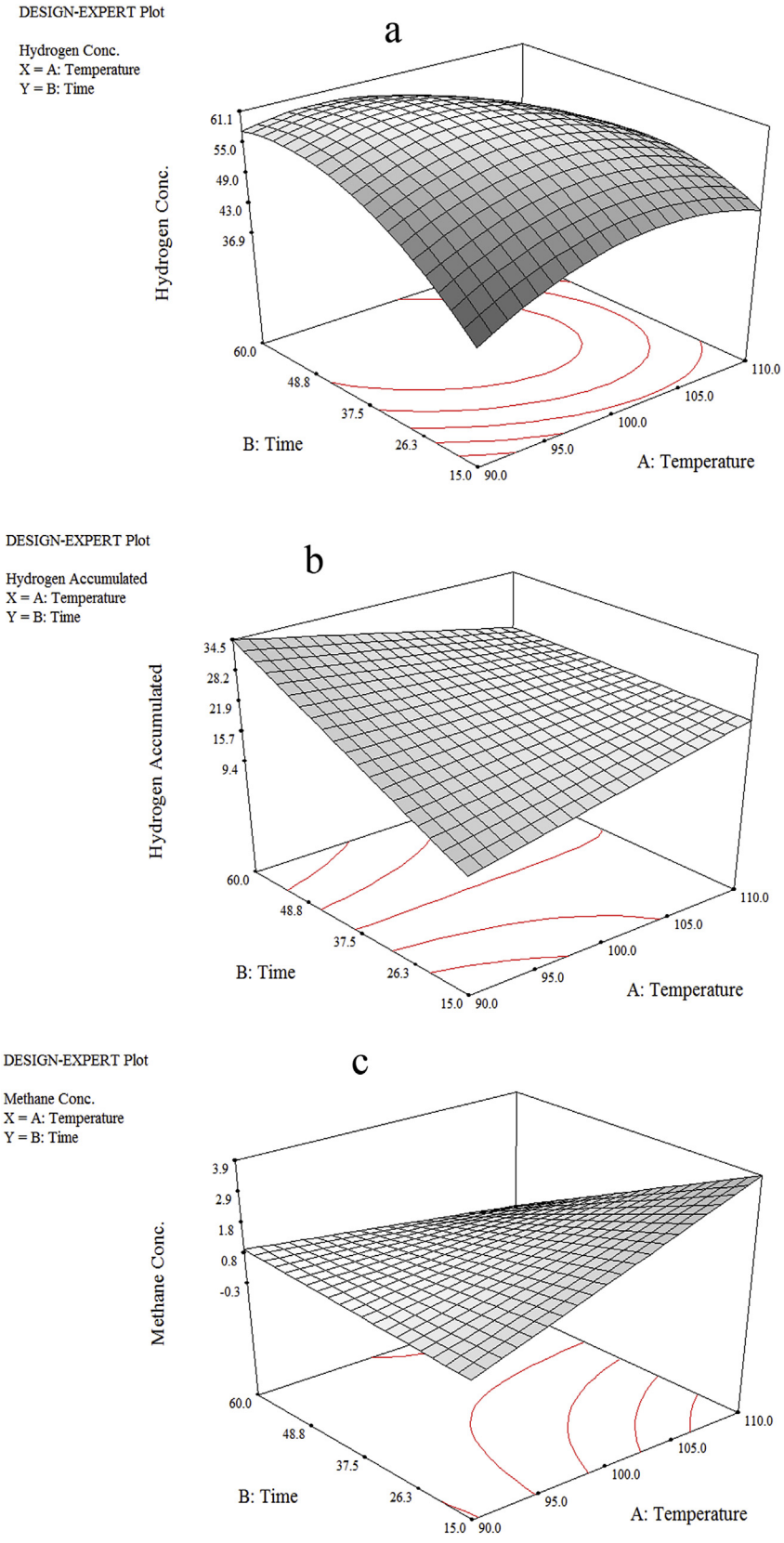

Fig. 3. 3-D Model and 2-D contour plot demonstrating the effect of different temperature (°C) and residence times (min) of thermal pretreatment on (a) H_2 concentration (%), (b) Accumulated H_2 (ml) and (c) CH_4 concentration (%).

unfrozen control, 10 times higher than NaOH pretreated substrate and 2.8 times higher than boiled dry substrate. Higher accumulated H_2 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 [7]. 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_2, 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 hydroxymethylfurfural (HMF). These by-products of sugar negatively affect fermentation efficiency because they are toxic to fermentative microorganisms and inhibit their metabolism [35]. 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 3.

To substantiate the above results analyses of VS, DOC and HY are defined in Table 3. Here again the frozen dry substrate of *I. aquatica* illustrated the highest HY of 217.16 mL/g-VS and initial DOC value of 247.86 ± 1.50 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_2 [32]. On the other hand, the unfrozen control showed a very low initial DOC of 56.00 ± 2.00 mg/L and HY of 39.08 mL/g-VS. The HY and initial DOC value were 110.09 mL/g-VS and 219.18 ± 3.00 mg/L respectively for boiled dry substrate; which further decreased to 15.15 mL/g-VS and 86.84 ± 3.55 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 [36]. This accounts for the comparatively low HY in case of boiled dry substrate.

Jun Cheng et al. reported that alkali pretreatment was feasible in pretreating rice straw and demonstrated a maximum HY of 155.00 mL/g-VS in dark-fermentation [37]. 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 that *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 (56.00 ± 2.00 mg/L), 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 (217.16 mL/g-VS). The very 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 [6,38]. Therefore, *I. aquatica* shows interesting characteristics that can enhance both hydrogen production as well as the activity of microorganisms especially under optimum pretreatment conditions.

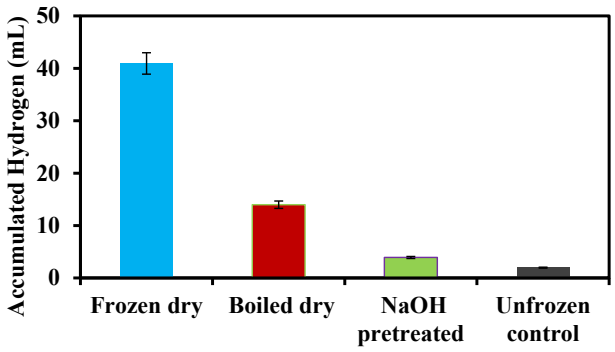

Fig. 4. Effect of optimized inoculum on the accumulated H_2 (ml) of frozen dry, boiled dry, NaOH pretreated substrate and unfrozen control of *I. aquatica.*

3.4. Comparison of the hydrogen yield

Table 4 shows a comparison of HY from *I. aquatica* with other substrates obtained from various studies. The highest HY (217.16 mL/g-VS) was obtained in this study from frozen dry *I. aquatica* as substrate, which is manifold higher than wheat straw (1.00 mL/g-VS), corncob (107.90 mL/g-VS), maize leaves (42.00 mL/g-VS) and corn stalk (149.70 mL/g-VS). 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 [39–41]; 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) and therefore corresponded to high hydrogen yield as well.

Some researchers used thermophilic conditions to enhance the fermentation process [42], 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.

3.5. Energy evaluation and proposal of a practical process for biohydrogen production from I. aquatica

The energy consumed for the pretreatment of digested sludge and *I. aquatica* from equation (1) were 564.3 KJ and 1580.04 KJ respectively. Energy utilized for warming the bioreactor and maintaining the temperature at 35 °C was 2287 KJ (Equation (2)), where the biomass density (ρ) was 0.955 mg/L and the global efficiency of the warming system was $\eta \approx 0.48$ [27]. Therefore, the overall energy consumed in the process was 4431.34 KJ. The energy produced per unit volume of the reactor was calculated as 4797 kJ/kg, where the heating value of HH_2 was 119.96 MJ/kg. Conclusively, the overall energy obtained (Energy produced − Energy consumed) was 365.66 kJ/kg. From the lab scale experiment results, the energy produced was higher than the energy consumed. However, 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. 5). *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

Table 3
Variation of VS (%), DOC (mg/L) and HY (mL/g–VS) 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 ± 0.20	56.00 ± 2.00	1.3 ± 0.00	39.90 ± 2.00	39.08
Frozen dry sub	6.2 ± 0.30	247.86 ± 1.50	5.5 ± 0.20	182.86 ± 0.00	217.16
Boiled dry sub	6.3 ± 0.50	219.18 ± 3.00	4.8 ± 0.20	172.09 ± 0.00	110.09
Pretreated Sub (NaOH)	4.1 ± 0.20	86.84 ± 3.55	3.1 ± 0.20	72.48 ± 3.00	15.15

(Note: The + - values indicate standard deviation in the triplicate experiments. The H_2 yield is calculated from the average values without showing the standard deviation.).

Table 4
Comparison of hydrogen yields with other works.

Inoculum	Substrate	Pretreatment	Temp. (°C)	Max. H_2 yield (mL/g- VS)	Ref.
Mix culture – cow dung compost	Wheat straw	HCl	36	62.80	[5]
Pure culture – *Clostridium* sp.	Corn stalk	Enzyme	55	132.00	[39]
Pure culture	Corn stalk	0.2% HCl, boiled 30 min	36	149.70	[40]
Pure culture – *Clostridium* sp.	Corn stalk	Bio-pretreatment	36	176.00	[41]
Mix culture – Dairy manure	Corncob	1%HCl +100 °C, 30 min	36	107.90	[43]
Mix culture – cow dung compost	Wheat straw	No pretreatment	36	1.00	[5]
Pure culture – *Clostridium* sp.	Corn straw	No pretreatment	35	9.00	[44]
Pure culture – *C.saccharolyticus*	Maize leaves	130 °C 30 min	70	42.00	[42]
Pure culture – *C.saccharolyticus*	Sweet sorghum plant	130 °C 30 min	70	32.40	[42]
Pure culture – *C.saccharolyticus*	Sugarcane bagasse	130 °C 30 min	70	19.60	[42]
Pure culture – *C.saccharolyticus*	Wheat straw	130 °C 30 min	70	49.00	[42]
Optimized sludge	*I. aquatica*	Boiled 1 min + dried	35	110.09	This study
Optimized sludge	*I. aquatica*	1% NaOH +1 min heating	35	15.15	This study
Optimized sludge	*I. aquatica*	Frozen + dried	35	217.16	This study

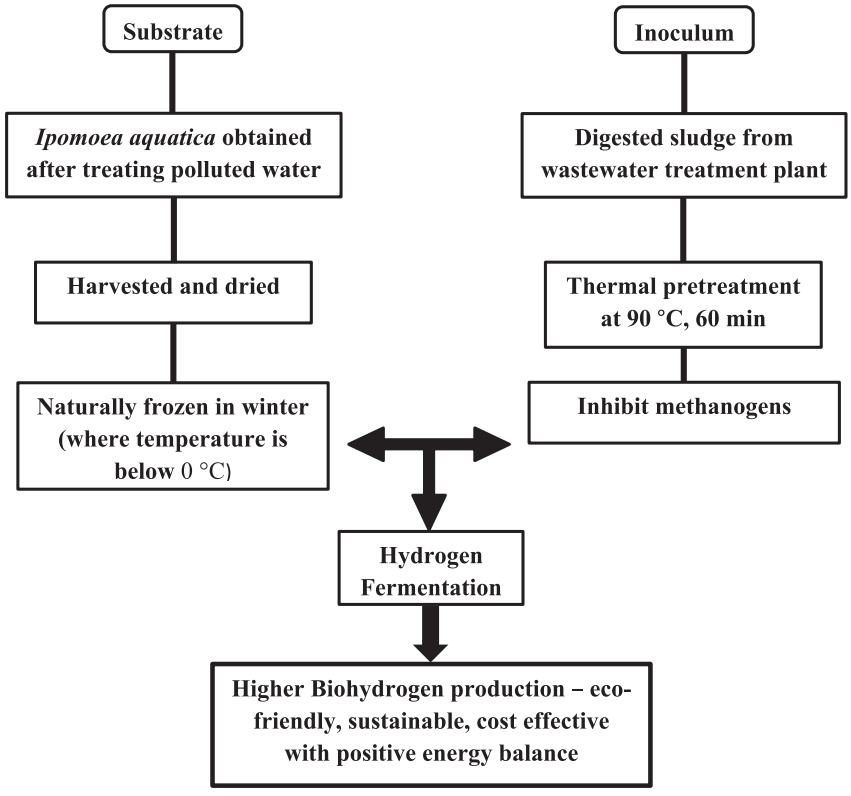

Fig. 5. Process flow diagram demonstrating the practical use of *I. aquatica* and digested sludge for biohydrogen production.

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. Also, in order to further increase the

overall energy production we will conduct two stage fermentation involving combined hydrogen and methane production in the future. 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.

4. Conclusions

I. aquatica for the first time, was used as a suitable substrate for biohydrogen production. Along with, the optimization of digested sludge (DS), used as inoculum was evaluated. Thermal pretreatment of DS showed better results than acid pretreatment. Furthermore, RSM results indicated 90 °C for 60 min as the optimum pretreatment condition of inoculum. Also, frozen dry *I. aquatica* demonstrated the highest HY among all the other substrate pretreatment conditions along with positive energy production. Conclusively, a sustainable process that favours water purification and clean energy production was developed. This process could be of great importance to developing countries in particular due to its cost effectiveness and easy operation. In the future, scale up of this system will be carried out for practical applications.

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