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Improvement of biohydrogen production by optimization of pretreatment method and substrate to inoculum ratio from microalgal biomass and digested sludge

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ABSTRACT

Biohydrogen from microalgal biomass has shown particular advantage due to its high growth rate and high bioenergy production. As a representative of microalgae, *Chlorella vulgaris* was chosen as substrate along with digested sludge (DS) as inoculum in this research. In order to improve the hydrolysis of algal biomass and enhance biohydrogen production, pretreatment methods like acid and thermal pretreatment were employed. Thermal pretreatment showed better results than acid pretreatment of microalgal biomass. 100 °C for 60 min was identified as the optimum condition for the thermal pretreatment of *C. vulgaris* by response surface methodology (RSM) analysis. Experiments were also carried out to identify the optimum substrate to inoculum ratio (SIR) for the process. SIR of 8 generated the highest hydrogen yield of 190.90 mL H_2/g-VS. Moreover, the overall energy balance of the process was evaluated and the results showed a positive energy balance of 1790.13 kJ/kg. The results indicated that optimization of pretreatment methods and substrate to inoculum ratio was effective in enhancing biohydrogen production from microalgal biomass and digested sludge.

1. Introduction

Hydrogen is widely acknowledged as an efficient and clean energy carrier among the various alternative forms of renewable energy since it has a high energy content of 122 kJ/g which is 2.75 times higher than fossil fuels [1]. By 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 across the globe [2]. However, they are energy intensive, expensive and eco-destructive processes. Owing to these issues, biological processes is an alternative method of biohydrogen production as it can be operated under ambient conditions, are less energy intensive and more eco-friendly [3]. Among the various biological hydrogen production methods, dark fermentative hydrogen production (DFHP) is widely recognized due to its high rate of cell growth, non-requirement of light energy and the potential for cost-effective hydrogen production [4–6].

The availability and cost of feedstock for fermentative hydrogen production is a major bottleneck. 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 [7]. Yet only few studies have been conducted on the use of microalgal biomass as a feedstock for DFHP. 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 [7]. However, some previous literature indicated that the intact and strong cell membranes of microalgae would result in a low biogas yield, limiting the efficient digestion in the DFHP process. To help disrupt the cell walls, pretreatment or disintegration of the microalgal biomass is needed [8,9]. 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 [10]. Although ultrasonic pretreatment is a good choice for microalgal biomass, it is energy-intensive for large scale applications and difficult to be used for practical application. Baccay and Hashimoto (1984) [11] investigated that

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acid pretreatment can bring about swelling of organic structure at low pH, thus making the substrate easier to be hydrolyzed. 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 [12]. 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.

Digested sludge (DS) was chosen as a source of inoculum in this research due to its availability in abundance and demonstration of positive results from previous researches [13,14]. However, in a mixed culture system, under anaerobic condition some hydrogen consuming bacteria (HCB) existing in the DS, such as methanogens, homoacetogens and Archaea [15,16], often readily consume the hydrogen produced by HPB. Therefore, in order to harness hydrogen from a mixed culture system the HCB were restrained by thermal pretreatment as it was obtained as the optimum pretreatment method from previous research.

Consecutively, the substrate to inoculum ratio (SIR) was evaluated which is another key factor in DFHP. 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 [17]. Also, the higher concentration of inoculum could cause increased nutrient consumption and waste production which would inhibit the hydrogen production itself [18]. Therefore, an optimum SIR is important for enhancing the overall efficiency of DFHP. Pakarinen (2008) reported that the substrate to inoculum ratio of 2:1 increased H_2 production efficiently [19]. However, there is no report on the effect of SIRs higher than 2:1 on H_2 production from *Chlorella vulgaris* and digested sludge.

In the light of the above research background, this study aimed at optimization of the overall DFHP process for biohydrogen production from *Chlorella vulgaris.* Acid and thermal pretreatment of *Chlorella* was carried out in order to investigate the optimum pretreatment method. Furthermore, a response surface methodology (RSM) with a central composite design (CCD) was used to find the optimum thermal pretreatment conditions and analyze the data statistically. Also, the SIR of *Chlorella* and DS were investigated for the optimization of the overall DFHP process. Along with the optimization of the process, the overall energy balance of the process was evaluated for practical application and future prospects.

2. Materials and methods

2.1. Inoculum and substrate preparation

The digested sludge 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, 11.40 g/L, 7.80 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). The composition of trace mineral solution is as follows: $FeSO_4{\cdot}7H_2O$ (1000 mg/L), $CaCl_2{\cdot}2H_2O$ (125 mg/L), $MgCl_2{\cdot}6H_2O$ (125 mg/L), $CoCl_2{\cdot}6H_2O$ (25 mg/L), $MnSO_4$ (25 mg/L), $ZnSO_4{\cdot}7H_2O$ (25 mg/L), $NiCl_2{\cdot}6H_2O$ (25 mg/L), $CuSO_4{\cdot}5H_2O$ (25 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (25 mg/L) and H_3BO_3 (25.0 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) 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 our previous researches.

Chlorella 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 its the most widely used method [7]. The centrifugation was carried out at 100 × 100 rpm for 5 min using a centrifugal machine (6800, KUBOTA) and the residue was used in the further experiments.

2.2. Pretreatment of Chlorella vulgaris

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

2.3. Experimental design using RSM

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 hydrogen concentration, cumulative hydrogen production and hydrogen yield (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. (1) [21].

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

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

2.4. Batch fermentation

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 SIR, appropriate amounts of substrate and inoculum were added. SIR was defined to be the initial ratio of volatile solids (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 0.48-g VS, 0.72-g VS, 1.20-g VS, 1.92-g VS, 2.64-g VS and 3.36-g VS respectively.

All batch experiments 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 (SHIMAZU, Japan). Then dark fermentation was carried out at 35 °C with constant shaking until no biogas was produced.

2.5. 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) (TOC – 5000 A, SHIMAZU, Japan), Volatile Solids (VS) and hydrogen yield (HY) were determined in accordance with standard methods, and pH was detected using a pH meter (TES1380). The morphology and structure of the microalgal cells were observed using a Scanning Electron Microscope (SEM, DS-720, Topcon, Tokyo, Japan).

3. Results and discussion

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. 1. From the results, it's 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.

Table 1shows the cumulative hydrogen yield (HY) (mL H_2/g-VS), 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 [22]. Also, thermal

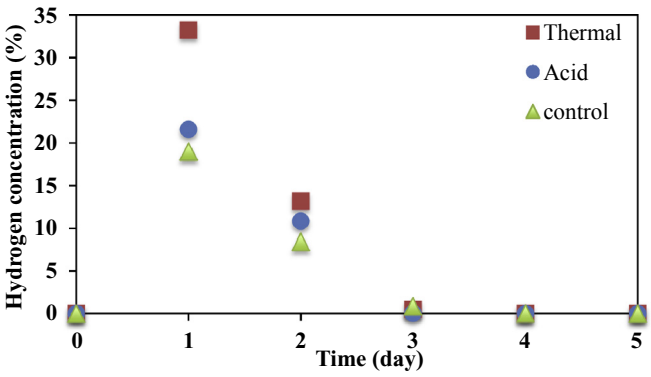

Fig. 1. Effect of different pretreatment methods of *C. vulgaris* biomass on H_2 concentration (%) with DS as inoculum (Acid: 3% HCl - pH 3, Thermal: 100 °C, 60 min).

Table 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_2 yield (mL H_2/g- VS)
Control	1775.0 ± 20.0	5.5 ± 0.1	13.3 ± 5.1
Acid	1989.0 ± 35.0	5.5 ± 0.2	25.1 ± 5.0
Thermal	2120.0 ± 30.0	5.9 ± 0.1	76.6 ± 8.5

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 [22]. This can be attributed to the fact that thermal pretreatment is more efficient in the hydrolysis of *Chlorella vulgaris* as indicated by the high DOC value of 2120.0 mg/L (Table 1). DOC is an indicator of the organic content usually composed of soluble sugars and other lower weight components, which could represent the total amount of carbon during the hydrolysis phase [23]. 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 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 [24].

On the other hand, the acid pretreatment also demonstrated higher HY compared to the control. 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 Hydroxymethyl furfural (HMF). Researches have stated that furfural and Hydroxymethyl furfural (HMF) are formed as result of thermo or thermo-acidic pretreatment [25]. 5-HMF were found in the slurry of *Chlorella Vulgaris* after thermal or dilute acid pretreatment [26,27]. 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 [28]. 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 [22]. 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. 2a, the control without any pretreatment showed a spherical shape, which is a typical structure of microalgal biomass and *Chlorella vulgaris* in particular [29,30]. The cell wall had a smooth surface with no crevices. But in case of acid pretreatment (Fig. 2b) we 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 1). Fig. 2c demonstrates the effect of thermal pretreatment on the microalgal cell wall. It's 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 (2120.0 mg/L) of thermal pretreatment. Conclusively, from the H_2 concentration, HY, DOC, pH and SEM results thermal pretreatment was obtained as the

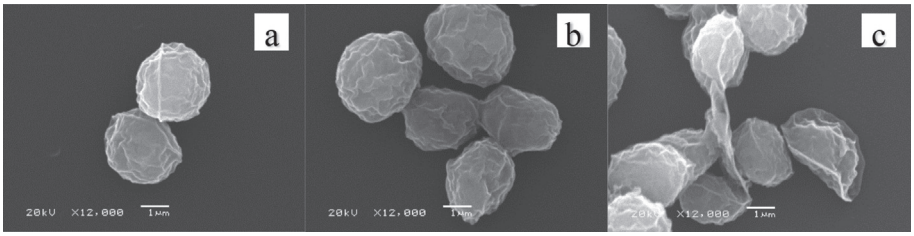

Fig. 2. SEM observations of *Chlorella vulgaris* under different conditions, (a) control, (b) acid pretreatment (3% HCl – pH 3) and (c) thermal pretreatment (100 °C, 60 min).

optimum pretreatment method for *Chlorella vulgaris*. Further investigations were made to identify the optimum thermal pretreatment conditions.

3.2. Identification of the optimum thermal pretreatment condition of Chlorella vulgaris for higher HY by using RSM analysis

In order to investigate the optimum thermal pretreatment conditions for *Chlorella vulgaris*, 13 experiments were carried out as described in Section 2.3. The coded and actual values of the independent variables along with actual and predicted values of hydrogen concentration and hydrogen yield (HY) for each run is indicated in Table 2.

Analysis of variance (ANOVA) was carried out and the results are shown in Table 3. The model F- value of 36.65 implies that the model was significant, because model terms with value of 'Prob > F' less than 0.05 shows that they are significant [27]. 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. (2). This equation was generated by analysis of variance (ANOVA) as presented in Table 3.

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

where Y, X_1 and X_2 are the hydrogen concentration (%), heating temperature (C) and reaction time (min) respectively.

The experimental values versus predicted values for hydrogen concentration (%) are shown in Fig. 3. 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 *Chlorella vulgaris*. The maximum hydrogen concentration as predicted by the model was 68.47% at the optimum condition of 100 °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. 4 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 68.47% and was obtained at a pretreatment condition of 100 °C for 60 min. On the contrary the lowest value of hydrogen concentration was obtained at 140 °C for 60 min and these results showed great correlation with actual values from the experiment (Table 2). From the 3-D response surface we can arrive at the conclusion that thermal pretreatment of *Chlorella 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 [28]. It has been reported that the higher temperature could attribute to more severe accumulation of HMF during the thermal pretreatment, leading to a poor performance during the fermentation process [31]. As a result, due to the serious accumulation of the inhibitory component, the higher temperature failed to achieve a good performance during the fermentation process. The 2-D contour plots demonstrated an elliptical fold running diagonally, indicating the slight interdependence between the variables, temperature and time. Results of

Table 2
CCD for thermal pretreatment of *Chlorella vulgaris*.

Run no.	Coded values		Real values		H_2%		HY
	x_1	x_2	X_1	X_2	Predicted values	Actual values	(mL H_2/g-VS)
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_1: Temperature, °CX_2: Time, min.

Table 3
ANOVA for H_2 concentration in thermal pretreatment of *Chlorella vulgaris*.

Source	Sum of Squares	Degree of freedom	Mean Square	F-= Value	Prob > F
Model	1278.40	5	255.68	36.65	<0.0001
X_1	793.39	1	793.39	113.73	<0.0001
X_2	104.40	1	104.40	14.97	0.0061
X_1^2	14.38	1	14.38	2.06	0.1943
X_2^2	245.24	1	245.24	35.15	0.0006
X_1 X_2	132.25	1	132.25	18.96	0.0033

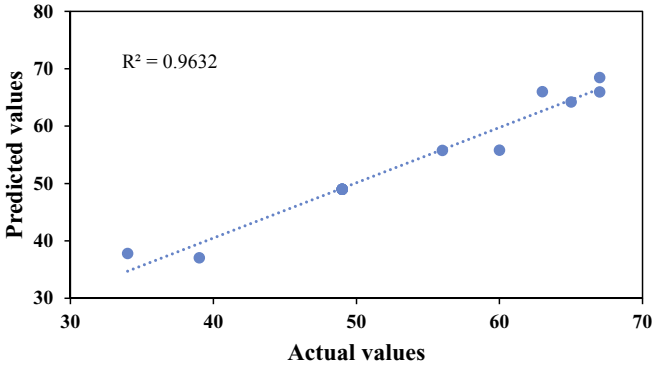

Fig. 3. Predicted values versus experimental values of H_2 concentration.

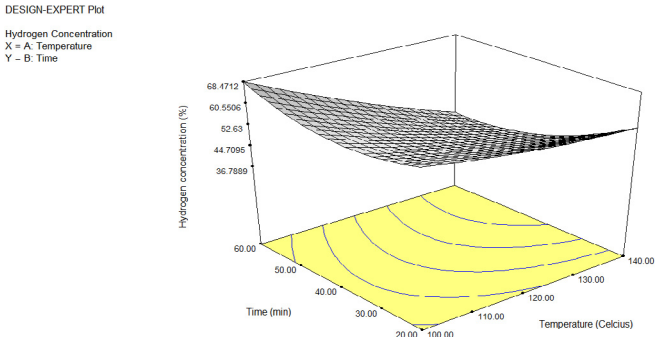

Fig. 4. 3-D Model and 2-D contour plot demonstrating the effect of different temperature (°C) and residence times (min) of thermal pretreatment of *Chlorella vulgaris* on H_2 concentration (%).

hydrogen yield showed a similar tendency and thermal pretreatment at 100 °C for 60 min achieved the highest HY of 190.9 mL H_2/g-VS (Table 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's interesting to note that thermal pretreatment at all conditions demonstrated better results than acid pretreatment and the control. Conclusively, thermal pretreatment at 100 °C for 60 min was effective in the hydrolysis of *Chlorella vulgaris* on enhancing hydrogen production.

3.3. Effect of SIR on hydrogen production from Chlorella vulgaris

To study the effect of different SIR's on the hydrogen production from *Chlorella vulgaris,* experiments were carried out at different substrate concentrations with the inoculum concentration being kept constant. The results of which are depicted in Fig. 5 and Table 4. On increasing the SIR from 2 to 8 the H_2 concentration increased from 23.4% to 69.6%. But on further increasing the SIR from 8 to 14 the H_2 concentration decreased to 59.3% (Fig. 5). A

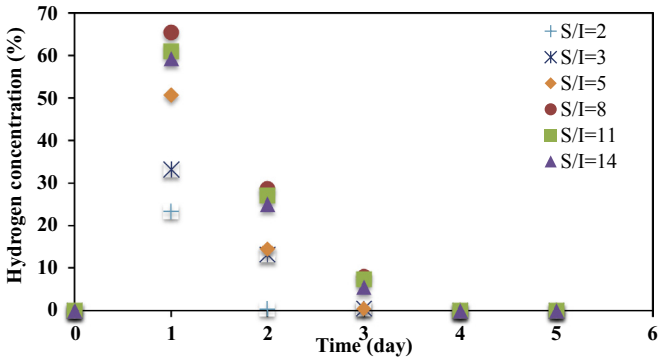

Fig. 5. Effect of different SIR's on H_2 concentration (%) from *C. vulgaris* and DS.

Table 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_2/g-VS)
2:1	2.4 ± 0.2	1964.0 ± 28.0	6.0 ± 0.2	27.6 ± 9.4
3:1	3.5 ± 0.0	2120.0 ± 30.0	5.9 ± 0.1	76.6 ± 8.5
5:1	4.8 ± 0.1	2591.0 ± 22.0	5.9 ± 0.0	97.9 ± 22.0
8:1	6.8 ± 0.3	3462.0 ± 35.0	5.8 ± 0.0	190.9 ± 28.3
11:1	8.4 ± 0.2	3612.0 ± 37.0	5.6 ± 0.1	104.5 ± 22.7
14:1	11.4 ± 0.1	3968.0 ± 40.0	5.3 ± 0.1	21.8 ± 10.4

similar tendency was observed with HY. SIR of 8 demonstrated the highest HY of 190.90 mL H_2/g-VS which reduced to 21.80 mL H_2/g-VS on increasing the SIR to 14 (Table 4). From these results it's clear that the hydrogen production increases with increasing the substrate concentration until a certain threshold, any further increase beyond the threshold would cause severe substrate inhibition [32].

These results can be further validated by the DOC, VS and pH analysis as shown in Table 4. Increasing the SIR from 2 to 14 caused an obvious increase in the initial DOC and VS (%) from 1964.0 mg/L and 2.4–3968.0 mg/L and 11.4 respectively. An increase in the SIR from 2 to 8 bought 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 [33]. 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 SIR of 14. While other SIR's showed an increase in the final pH, the SIR 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 [34,35]. Therefore, an optimum SIR is required to provide optimum substrate for the microorganisms and to also maintain the pH of the reactor. In this study, an optimum SIR of 8 was obtained for

Table 5
Energy evaluation for different thermal pretreatment conditions.

Expt. no.	Temperature (C)	Time (min)	*E_{CV} (kJ)	H_2%	HY (mL H_2/g-VS)	**EP (kJ/kg)
1	100.00	20.00	1000	65.00	70.20	2273
2	120.00	40.00	2400	49.00	54.44	1762
3	140.00	60.00	4200	39.00	34.83	1307
4	120.00	11.72	703.2	63.00	124.66	4037
5	91.72	40.00	1834.4	67.00	87.56	2835
6	100.00	60.00	3000	67.00	190.90	6200
7	140.00	20.00	1400	60.00	90.08	2771
8	148.28	40.00	2965.6	34.00	40.12	1331
9	120.00	68.28	4096.8	56.00	47.60	1485

* E_{CV} – Energy consumed for pretreatment of *C. vulgaris*.
** EP – Energy produced.

enhanced biohydrogen production from *Chlorella vulgaris.*

3.4. Energy evaluation for overall biohydrogen production from *Chlorella vulgaris*

In order to demonstrate the efficiency of biohydrogen production from *Chlorella vulgaris,* 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 (3)–(5) [36].

$$Q = C_p m\, dT \tag{3}$$

where Q is the amount of heat (kJ), C_p is specific heat capacity (kJ/kg-K), m is mass (kg) and dT is the difference in temperature.

$$E_w = \left[\rho \bullet C_p(T_w - T_a) \bullet F\right]/\eta \tag{4}$$

where E_w is the energy required to warm the reactor (kJ), ρ is the biomass density, T_w is the working temperature, T_a is the ambient (outdoor) temperature and F is the working volume.

$$EH_2 = F \bullet PH_2(T_w) \bullet HH_2 \tag{5}$$

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

$$EC = E_{DS} + E_{CV} + E_w \tag{6}$$

where EC is the energy consumed (kJ), E_{DS} is the energy required for pretreatment of DS and E_{CV} is the energy required for pretreatment of *C. vulgaris.*

The specific heat capacity of the digested sludge and *Chlorella vulgaris* was around 4.18 kJ/kg-K and 1.25 kJ/kg-K respectively [37]. The energy consumed for the pretreatment of digested sludge and *C. vulgaris* from equation (3) were 564.3 kJ and 3000 kJ respectively. Energy utilized for warming the bioreactor and maintaining the temperature at 35 °C was 845.57 kJ (Eq. (4)), where the biomass density (ρ) was 0.955 mg/L and the global efficiency of the warming system was $\eta \approx 0.48$ [36]. Therefore, the overall energy consumed in the process was 4409.87 kJ. The energy produced per unit volume of the reactor was calculated as 6200 kJ/kg, where the heating value of HH_2 was 119.96 MJ/kg (Eq. (5)). Conclusively, the overall energy obtained (Energy produced (EP) – Energy consumed (EC)) was 1790.13 kJ/kg.

Table 5 shows the energy consumed at different thermal pretreatment conditions and corresponding EP from microalgal biomass. The optimum pretreatment condition of 100 °C for 60 min was chosen after considering various factors such as $E_{CV,}$ HY, H % and EP. The energy consumed at 100 °C for 60 min was comparatively higher (3000 kJ). However, in order to attain a higher HY (190.9 mL H_2/g-VS), H_2% (67) and EP (6200 kJ), the energy consumed for pretreatment cannot be avoided. Pretreatment conditions at 100 °C, 20 min, 140 °C, 20 min and 91.72 °C, 40 min consumed lower energy and demonstrated a high H % and HY. However, their EP was lower and therefore the overall energy obtained at these conditions were very low. Other pretreatment conditions at 120 °C, 40 min, 140 °C, 60 min, 148.28 °C, 40 min and 120 °C, 68.28 min were energy intensive. The H% and HY were low at these conditions as the pretreatment might have resulted in the formation of inhibitory compounds due to severe pretreatment conditions [28]. Also, the overall energy obtained was negative, further proving that these conditions were inappropriate for pretreatment. On the other hand, pretreatment at 120 °C, 11.72 min had a high HY, H%, EP and low energy consumption. Therefore, this condition might have been more favourable as optimum pretreatment condition, but in comparison with pretreatment at 100 °C for 60 min, factors such as HY, H% and EP are comparatively lower. Also, as this a basic energy balance calculation, the optimum pretreatment condition cannot be decided solely based on it. Conclusively, in order to obtain an overall better performance in terms of HY, H%,

Table 6
Comparison of hydrogen yield (HY) with other works.

Pretreatment method	Algal biomass	Pretreatment condition	Inoculum	H_2 Yield (mLH_2/g- VS)	Ref.
Ultrasonic	*Chlorella vulgaris*	T = n.d, F = 20 kHz,P = 150 W, SEI levels = 10,000–100,000 kJ/kg	Anaerobic digested sludge	31.9–37.9	[27]
Microwave heating	*C. pyrenoidosa*	T = 140 °C, t = 15 min,	Anaerobic digested sludge	12.6	[27]
Acidic HCl	*Chlorella vulgaris*	t = 10,35, 60 min, HCl dosage = 0.1, 1.6, 3%(v/w)	Anaerobic digested sludge	13.6–36.5	[27]
Acidic HCl + ultrasonic	*Chlorella vulgaris*	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)	Anaerobic digested sludge	24.2–41.6	[27]
Thermal	Lipid-extracted *Scenedesmus*	T = 100, 121 °C, t = 4, 8 h	Anaerobic digested sludge	31.7–31.8	[27]
Thermal	*Scenedesmus obliquus*	T = 121 °C, t = 15 min	*Clostridium butyricum*	90.3	[27]
Acidic HCl	*Chlorella vulgaris*	t = 24 h, HCl dosage = 3% (v/w)	Anaerobic digested sludge	25.1	This study
Thermal	*Chlorella vulgaris*	T = 100 °C, t = 60 min	Anaerobic digested sludge	190.9	This study

EP and positive energy balance pretreatment at 100 °C for 60 min was considered as the optimum pretreatment condition.

3.5. Comparative analysis of the hydrogen yield 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 6. It can be noted that *Chlorella 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_2/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 (*Clostridium 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 as compared to other researches which could be attributed to the severity of acid pretreatment as it was carried out for 24 h. Other intensive pretreatments like ultrasonic and combined pretreatment showed a HY of about 37.9 mL H_2/g- VS and 41.6 mL H_2/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 SIR of 8:1 demonstrating the highest high HY of 190.9 mL H_2/g- VS and with a positive energy balance showed the best results. Conclusively, the optimization of pretreatment methods and SIR 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_2 production.

4. Conclusions

Chlorella 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*. SIR of 8 was obtained as the optimum condition for obtaining the highest HY of 190.9 mL/g-VS. Also, a positive energy balance of 1790.13 kJ/kg 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_2 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. In the future, scale up of this system and two-stage fermentation system will be carried out for practical applications.

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