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Original Research Paper

# Biogenic $H_2$ production from mixed microalgae biomass: impact of pH control and methanogenic inhibitor (BESA) addition

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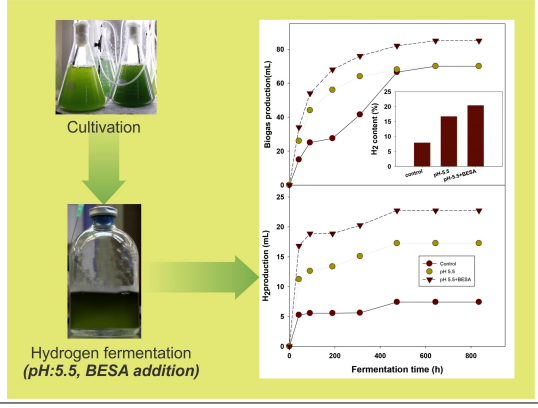
## HIGHLIGHTS

- Mixed microalgal biomass was evaluated for fermentative biohydrogen production.
- pH control at 5.5 was necessary for enhancement of production performances.
- Methanogenic inhibitor (BESA) addition enhanced hydrogen production by 3 folds.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Hydrogen production from mixed microalgae biomass, predominantly containing *Scendesmus* and *chlorella* species, was investigated with a focus on enhancement strategies, in particular (i) pH control (at 5.5) and (ii) methanogenic inhibitor (BESA) addition along with pH control at 5.5. The results obtained showed that the later condition remarkably increased the performances. This was mainly ascribed to the occurrence of a suitable environment for the hydrogen producers to perform actively. Hydrogen production under these conditions (i.e., both pH 5.5 and pH 5.5+BESA) was significantly higher than that of the control experiment. Using the pH control at 5.5 and BESA addition, peak hydrogen production rate (HPR) and hydrogen yield (HY) were attained as 210 mL/L/d and 29.5 mL/g $VS_{added}$, respectively. This improvement was nearly 3-folds higher compared with the control experiment with an HPR of 62 mL/L/d and an HY of 9.5 mL/g $VS_{added}$.



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**Abbreviations**

| | |
|---|---|
| BESA | 2-bromoethanesulfonic Acid |
| COD | Chemical Oxygen Demand |
| EPR | Energy Production Rate |
| EY | Energy Yield |
| GC | Gas Chromatography |
| HPR | Hydrogen Production Rate |
| HY | Hydrogen Yield |
| MJ | Mega Joule |
| TS | Total Solids |
| VFA | Volatile Fatty Acid |
| VS | Volatile Solids |

## 1. Introduction

Over many years, use of fossil fuel resources has resulted in serious environmental pollutions. In this context, producing clean energy carriers such as hydrogen ($H_2$) from organic biomass has attracted the attention of environmental and energy scientists. $H_2$ has a high energy content (142 MJ $kg^{-1}$) and only water is produced during its combustion, making hydrogen a green and potential fuel for the future (Kumar et al., 2015; Sivagurunathan et al., 2016; Kumar et al., 2016a).

Algae biomasses (micro and macro) have recently gained much attention because of their unique characteristics. An example is the presence of higher titers of protein, carbohydrate (mainly glucose, galactose, xylose, and other sugars in lower quantities), and lipids in comparison with terrestrial, lignocellulosic biomass (Cea-Barcia et al., 2014; Salam et al., 2016). Besides, bio$H_2$ production from algae biomass – by means of dark fermentation – approximates $CO_2$ neutrality because of its high carbon fixation ability. Furthermore, relatively easy cultivation methods and the diversity of the species make algae-bio$H_2$ as a promising approach. Other interesting properties include high growth rates and less sterilization necessity (Chen et al., 2011; Park et al., 2015; Sambusiti et al., 2015).

Dark fermentative $H_2$ generation relies mostly on complex bacterial sources as inoculum, where suppressing $H_2$-scavenging ($CH_4$-formation activity) is of high concern. To accomplish this task, various strategies have been developed, among which pH control is an effective way. Additionally, pH regulates $H_2$ metabolism and drifts between solventogenesis and acidogenesis (Kumar and Lin, 2013). Besides, pH would play a main role in suppressing the methanogenic activity, which is very crucial for biohydrogen fermentation. Another method of depressing methanogenic archaea is the application the chemical inhibitors, e.g., 2-bromoethanesulfonic acid (BESA) (Wang et al., 2003).

In this study, experiments were dedicated to the combined effects of these two techniques, i.e., pH control and BESA addition towards more reliable inoculum pretreatments for more efficient $H_2$ production using mixed microalgae biomass as feedstock. The processes were investigated by monitoring organic matter degradation, in terms of total solids (TS) and volatile solids (VS) as well as chemical oxygen demand (COD).

## 2. Materials and methods

### *2.1. Collection and cultivation of microalgae consortia and seed inoculum for $H_2$ production*

Microalgae consortia were collected in small plastic containers by a mesh net (pore size of 10-20 μm,) from aquatic niches located in the vicinity of the wastewater treatment plant facility of bio-eco engineering lab (National Institute of Environmental Studies, Tsukuba, Japan) and were then stored in vials prior to inoculation at room temperature. Subsequently, the collected microalgae consortia were sieved using a commercial mesh filter in order to remove the impurities such as dust particles, sand, insect larvae, etc. Then, the consortia were grown in Bold's basal medium as reported earlier (Cea-Barcia et al., 2014; Kumar et al., 2016b).

Seed inoculum for $H_2$ production was collected from a lab-scale reactor, which was used to produce methane. Inoculum was used as it was obtained from the reactor with TS and VS values of 20.1 g/L and 12.5 g/L, respectively.

### *2.2. Biohydrogen fermentation and pretreatment methods*

Hydrogen fermentation was conducted in 100 mL batch serum vials with 80 mL working volume (Kumar et al., 2016b). Pretreatment method for the seed inoculum included BESA addition (1 g/L) and adjustment of initial pH to 5.5. Control experiments with untreated sludge inoculum were performed for comparison. Initial 1.43 g/L VS of wet algal biomass was added into the reactor vials. All the measurements were run in duplicate to ensure the reproducibility of the experiments and the data were reported as mean values.

### *2.3. Analytical methods*

A glass syringe with hypodermic needles and adjustable volume was used to measure the biogas produced in the reactors. Gas composition (hydrogen, carbon dioxide, and methane) was determined with a gas chromatograph (GC-8A, Shimadzu) equipped with a thermal conductivity detector and a stainless steel column packed with Shincarbon ST (Shimadzu GLC). pH was measured by using a pH meter equipped with a composite electrode (GST-5721C, DKK-DOA). Protein and carbohydrate analyses were performed following the corrected Lowry and phenol-sulfuric standard methods (Dubois et al., 1956; Frølund et al, 1996). Other measurements such as volatile fatty acids (VFA), TS, VS were conducted by the procedure reported in our previous study (Kumar et al., 2016b).

### *2.4. Kinetics and energy evaluation*

Modified Gompertz equation (**Eq. 1**) was used for data analysis, based on the cumulative $H_2$ production for each experiment (Sivagurunathan et al., 2014).

$$H = P \times \exp\left[-\exp\left\{\frac{R_H}{p} \times (\lambda - t) \times e\right\} + 1\right] \quad (1)$$

where H (t) is the cumulative hydrogen production (mL) at culture time t (h); P is the maximal (cumulative) hydrogen production (mL); $R_H$ is the maximum hydrogen production rate (mL/h); λ is the lag phase time (h), and t is the cultivation time (h). Analysis of variance (ANOVA) for the results obtained during different conditions was performed using Microsoft Excel 2013.

The energy production rate of biohydrogen (*EPR*, J/L/d) was calculated using **Equation 2**:

$$EPR = (\mathrm{HPR}/22.4) \times HV_{H_2} \quad (2)$$

where HPR is the hydrogen production rate (mL/L/d), and $HV_{H2}$ is the heating value of hydrogen (286 J/mmol), and 22.4 is the mol conversion factor.

The energy yield of biohydrogen (*EY*, J/g VS) was calculated as follows (**Eq. 3**):

$$EY = \mathrm{HY}/22.4 \times HV_{H_2} \quad (3)$$

where HY is hydrogen yield (mL/g $VS_{added}$), $HV_{H2}$ is heating value of hydrogen (286 J/mmol). 22.4 is the mol conversion factor.

## 3. Results and discussion

### *3.1. Influence of pH control and BESA addition on hydrogen production*

The threshold factor that determines the hydrogenase enzyme activity and balances between acid and solventogenesis in anaerobic fermentation is pH. Besides, pH control has positive effects on the hydrogen production performances as reported (Sivagurunathan et al., 2014). In this study, experiments (i) without pH control (blank) and (ii) with pH adjusted at 5.5 (a favorable pH range for maximal production) together with the addition of methanogenic inhibitor (BESA) were executed. Hydrogen production rate (HPR, mL/L/d) and hydrogen yield (HY, mL/g $VS_{added}$) were monitored.

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In agreement with the above considerations, the results obtained indicated that pH control had an influence on HPR. Whereas the control experiment began to produce methane (not significant amount though, less than 4 mL) after a period of 18 d (data not shown). This revealed that controlling pH was a necessary step in $H_2$ formation. HPR and HY values increased by more than 2 folds in case of the pH control. More specifically, the control experiment resulted in the HPR and HY of 62 mL/L-d and 9.5 mL/g $VS_{added}$, respectively, whereas the pH control at 5.5 turned led to the HPR and HY of 106 mL/L/d and 22.2 mL/g $VS_{added}$, respectively. The results are shown in Table 1.

**Table 1.**
Kinetic parameters of the modified Gompertz model and statistical analysis.

| Parameters | Control | pH 5.5 | pH 5.5 + BESA |
|---|---|---|---|
| $P$ (mL) | 6.5 | 15.7 | 21.1 |
| $R_m$ (mL/h) | 0.16 | 0.27 | 0.53 |
| λ (h) | 5.7 | 2.4 | 6.40 |
| $R^2$ | 0.88 | 0.92 | 0.96 |
| Adjusted $R^2$ | 0.82 | 0.88 | 0.94 |
| S.E.E. | 1.00 | 2.02 | 1.92 |
| SS | 36 | 1564 | 2939 |
| *MS* | 18 | 521 | 979 |
| P (significance %) | 0.0052 | 0.0023 | 0.0004 |
| DF | 2 | 3 | 2 |
| F value | 18 | 26 | 53 |

Addition of methanogenic inhibitors such as BESA has been previously proved to terminate the growth of hydrogen consumers and support favorable conditions for improved production performances (Scholten et al., 2000). In this research, the addition of BESA significantly influenced the production performances. Daily variations in the production performances of the control and treatments are depicted in Figure 1. As presented, the biogas and cumulative hydrogen production were remarkably enhanced through the pH adjustment and BESA addition. The biogas production was increased dramatically until the end of the fermentation, whereas the hydrogen formation stopped at about 500 h under all conditions.

The cumulative hydrogen production observed for the control experiment stood at 7.4 mL, whereas controlled pH of 5.5 resulted in 17.3 mL and pH control with the addition of BESA led to a cumulative hydrogen production of 22.8 mL. Interestingly, biogas production values at the end of the experiment were comparable under all conditions, i.e., 68, 71 and 85 mL for the control, pH 5.5, and pH 5.5. plus BESA addition, respectively. However, the hydrogen content was significantly different among treatments (Fig. 1; inset). More specifically, the hydrogen content was measured at 8% for the control experiment while pH control and pH control with BESA addition led to 17 and 21% hydrogen contents, respectively. This proves the efficacy of pH control and BESA addition in suppress methanogens.

### *3.2. Kinetic and energy production assessment*

The kinetic assessment of HPR and HY is beneficial to determine the important parameters of fermentation such as hydrogen production potential (P), production rate ($R_m$) and the lag phase values, which can be helpful in describing the batch process. According to the parameters determined from the model simulation (Table 1), it can be observed that a peak $R_m$ value of 0.53 ml/h was attained under pH5.5+BESA condition. Corresponding P, $R_m$, and λ values for the control, pH 5.5, and pH 5.5+BESA are provided in Table 2. The accuracy of data is strongly supported by the high correlation coefficients ($R^2$ and adjusted $R^2$, ranging from 0.88–0.98, and 0.82–0.97, respectively). The results were assessed statistically by ANOVA. Additionally, very low standard error of estimate (S.E.E., i.e., 1.0–2.85) was observed. ANOVA provided the Pearson's correlation coefficients (p-value), sum of squares (SS), mean squares (MS), degree of freedom (DF), and F-values for all the experimental conditions and the values are provided in Table 2. pH 5.5+BESA pretreatment condition showed very high coefficient values of SS (2939), MS (979), F value (53), p value (<0.0004), and DF (2) in comparison with the other experimental conditions. Peak energy production rate (EPR) and energy yield (EY) were obtained when pH 5.5+BESA condition was used and the corresponding values were 2378 J/L/d and 330 J/g $VS_{added}$, respectively. Compared with the control, 3-fold increments were achieved in these values by using the pH 5.5+BESA treatment.

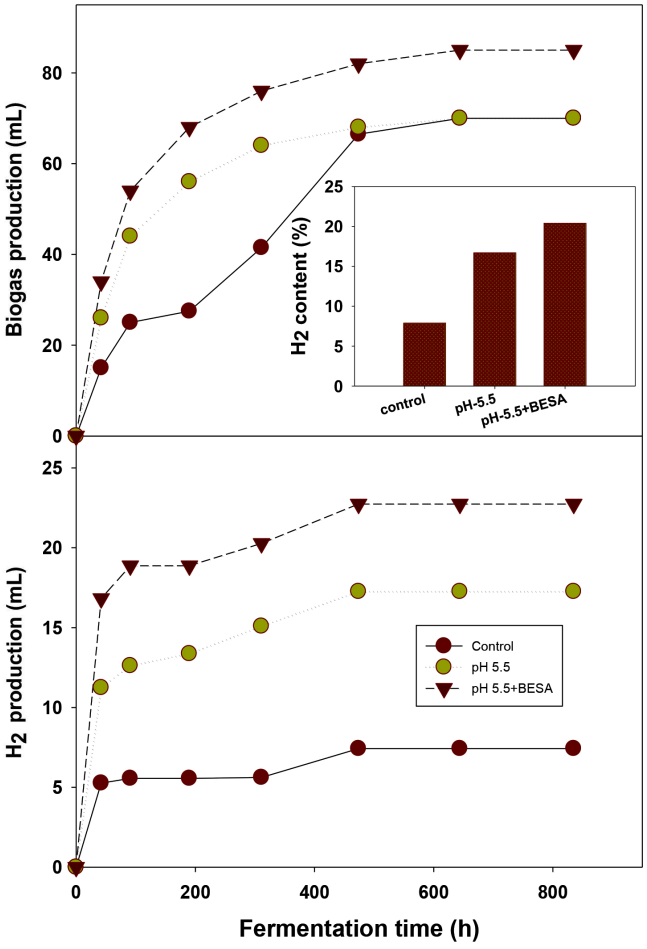


**Fig.1.** Cumulative hydrogen and biogas production under various conditions (inset: $H_2$ content).

**Table 2.**
Production performance under various conditions.

| Conditions | Biogas production (mL) | $H_2$ production (mL) | HPR (mL/L/d) | HY (mL/g $VS_{added}$) | EPR (J/L/d) | EY (J/g $VS_{added}$) |
|---|---|---|---|---|---|---|
| **Control** | 68.0 | 7.4 | 62.0 | 9.5 | 717.0 | 106.0 |
| **pH 5.5** | 71.0 | 17.3 | 106.0 | 22.2 | 1212.0 | 248.0 |
| **pH 5.5+ BESA** | 85.0 | 22.8 | 210.0 | 29.5 | 2378.0 | 330.0 |

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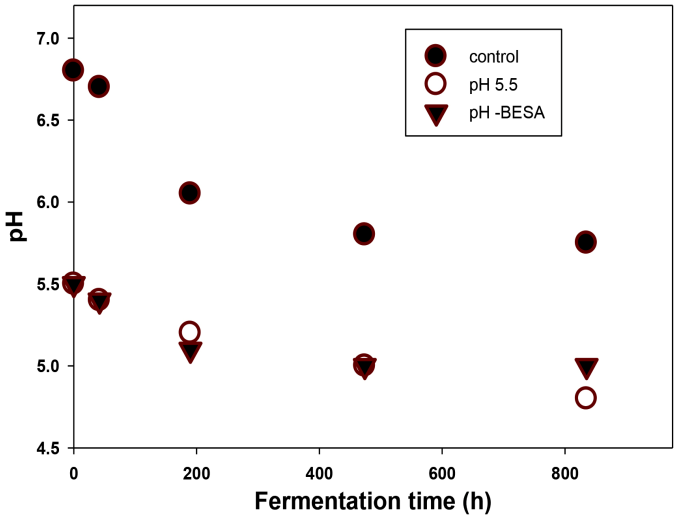


**Fig.2.** pH variations under various experimental conditions during 800 h fermentation period.

### 3.3. pH variation and volatile fatty acids (VFA) production

Changes in pH and the associated VFA production are critical parameters to evaluate the behavior of a biohydrogen production system. This is attributed to the fact that $H_2$ production is accompanied by the generation of VFAs that are able to reduce the pH of the system, influencing the activity of $H_2$-producing bacteria (by shifting between acid formation and hydrogen production) as explained in various reports published previously (Park et al., 2011; Kumar and Lin, 2013; Durán-Padilla et al., 2014). Changes in pH during 800 h fermentation are depicted in **Figure 2**. As can be seen, gradual decreases of pH were observed under pH 5.5 conditions (both with and without BESA) to 4.8 - 5 (ΔpH 0.7-0.5). In the case of the control experiment, the drop of pH was only 0.9 (from 6.7 to 5.8 from), probably due to the little amount of methane production (data not shown). Total VFA production for the control, pH 5.5, and pH 5.5+BESA were found at 1020, 1182, and 1460 mg/L, respectively (**Fig. 3**). These concentrations are more or less similar to the values reported earlier using microalgae biomass (Kumar et al., 2016b).

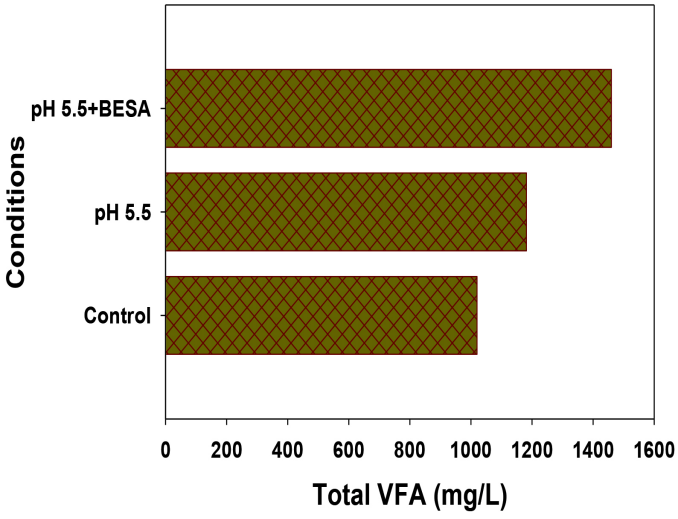


**Fig.3.** Total VFAs production under various experimental conditions.

### 3.4. TS, VS, and other organic matter removal

The solubilization and degradation of organic matters during fermentation is a requisite step to knowing the background information on reaction mechanisms. Besides, the products turnover is depended on the degradation of carbonaceous matters. This is usually evaluated by TS, VS, and COD removals during digestion/fermentation (Zhen et al., 2015; Zhen et al., 2016).

**Figure 4** illustrates the organic matter removal efficiencies, i.e., initial and final values of the components during 800 h of hydrogen fermentation under various conditions. As anticipated, peak removal rates were achieved while the production performances were higher. Peak COD removal efficiency of 28% was achieved with the pH 5.5+BESA experiment, followed by pH 5.5, and the control conditions with 24% and 16%, respectively. These values are in close agreement with the removal rates obtained during the conversion of de-oiled jatropha waste to hydrogen (Kumar and Lin, 2013). The order of removal efficiency for VS and TS was shown to be similar (pH 5.5+BESA > pH 5.5 > control) with values of 55%, 48%, and 32% for TS and 62%, 52%, 39% for VS, respectively.

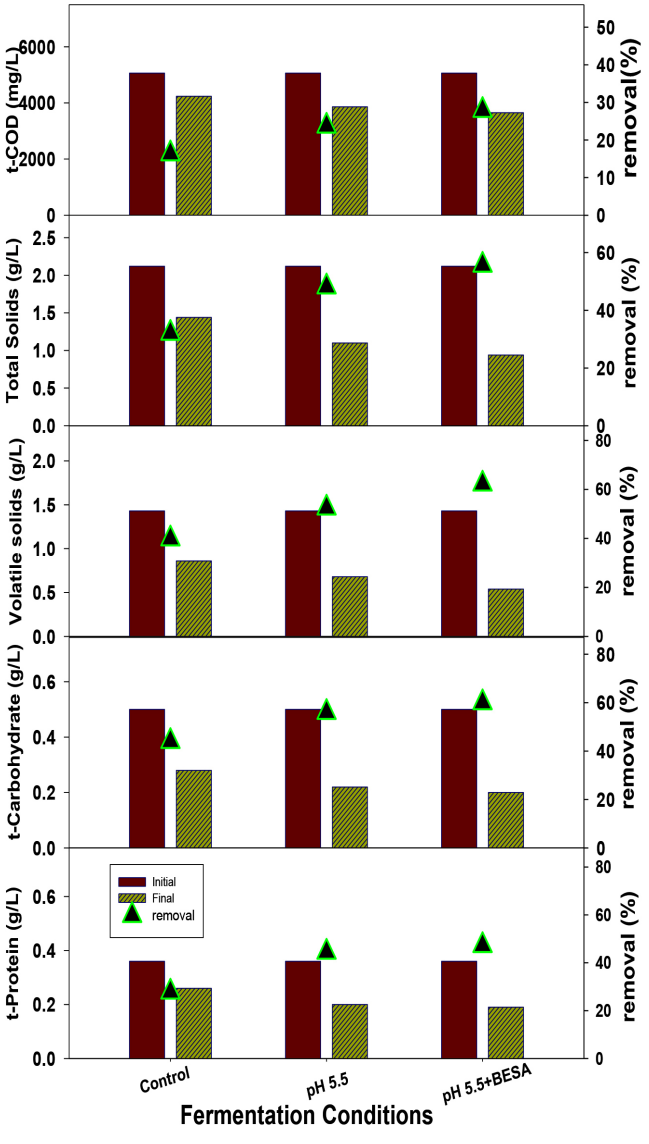


**Fig.4.** Organic fraction degradation (initial and final including removal rates) during 800 h fermentation period.

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Initial total carbohydrate and protein were also measured at 0.5 and 0.36 g/L, respectively. The final carbohydrate values were recorded at 0.28, 0.22, and 0.20 g/L while the final protein values stood at 0.26. 0.20, and 0.19 g/L for the control, pH 5.5, and pH 5.5+BESA conditions, respectively (**Fig. 4**). The carbohydrate values and the removal rates obtained were approximately similar to those reported by Ortigueira et al. (2015) who investigated hydrogen fermentation of *Spirogyra* hydrolyzate by *Clostridium buytricum*. It is worth noting that the intermediate products such as VFAs could be converted to various biopolymers (poly hydroxy butyrate) or undergo anaerobic digestion for the production of additional energy source, i.e., methane. In fact, integration of other methodologies could create circular bio-refinery and energy production technologies that might lead to more sustainability energy generation in the future.

## 4. Conclusion

Biogenic $H_2$ formation from microalgae consortia was demonstrated. The EPR and EY for the control, pH 5.5, and pH 5.5+BESA were 717, 1212, and 2378 J/L/d and 106, 248, and 330 J/g$VS_{added}$, respectively. Controlling pH at 5.5 and the addition of the methanogenic inhibitor, i.e., BESA could be an effective strategy to enhance the biohydrogen production performances.

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