Sacrificial Hydrogen Production from Enzymatic Hydrolyzed Chlorella over a Pt-loaded TiO₂ Photocatalyst

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Chlorella is single-cell green algae which has the photosynthetic pigments chlorophylls. Through photosynthesis, it multiplies rapidly, requiring only carbon dioxide, water, sunlight, and a small amount of minerals. Since dried chlorella is composed of about 45% protein, 20% fat, 20% carbohydrate, and others, ethanol yield is low through saccharification and fermentation of chlorella. Therefore, hydrogen production from chlorella was examined through enzymatic hydrolysis and photocatalytic reaction over a Pt-loaded TiO₂ photocatalyst (Pt/TiO₂). Enzymatic hydrolysis of chlorella (10.0 g) was performed in phosphate buffer (60 mL) using hydrolytic enzyme (1.0 g) such as protease, cellulase, and xylanase. After hydrolysis, the precipitates were removed by centrifugation to give the supernatant aqueous enzymatic hydrolyzed solution (EH). It was found that the EH solution obtained from protease-hydrolysis contained large amounts of hydrolyzed materials as a consequence of freeze-drying. Main component in EH solution was found to be amino acids by colorimetric analysis using ninhydrin. The EH solution was subjected to the sacrificial hydrogen production over a Pt/TiO₂ under UV irradiation by a high-pressure Hg lamp. Hydrogen (57.9 mg) was obtained from 1.0 g of dried chlorella through protease-hydrolysis and photocatalytic hydrogen production over Pt/TiO₂. This is the first report on sacrificial H₂ production from chlorella.

Key words

Sacrificial H₂ production, Photocatalyst, Chlorella, Protease, Pt/TiO₂
1. Introduction

Biofuels such as bioethanol, biodiesel, and biogas have gained much attention as new sustainable energy alternatives to petroleum-based fuels \(^1\). Bioethanol production has been recognized as one of the promising approaches. Recently bio-ethanol resources shifted from starch to cellulosic materials and algae to avoid direct competition with food sources \(^2\).

However, ethanol concentration is usually too low (<50 g L\(^{-1}\)) to separate by distillation in low energy cost \(^3\). On the other hand, gaseous H\(_2\) can be spontaneously isolated from reaction mixtures without operation to separate. Therefore, hydrogen production from biomass is one of the economical approaches to biofuels.

On these backgrounds, much attention has been paid to photo-catalytic H\(_2\) production over a Pt-loaded TiO\(_2\) (Pt/TiO\(_2\)), which is initiated by the charge-separation on TiO\(_2\) under photoexcitation \(^5\). Electron reduces water to generate H\(_2\) on Pt while hole oxidizes hydroxide to hydroxyl radicals \(^6\). It is well known that the use of electron-donating sacrificial agents remarkably accelerates the TiO\(_2\)- photocatalyzed H\(_2\) production since the hydroxyl radical is consumed by the sacrificial agents \(^7\). We have applied sacrificial H\(_2\) production over Pt/TiO\(_2\) to produce H\(_2\) from saccharides derived from lignocelluloses \(^8\) ~ \(^10\) and glycerol which is a byproduct in biodiesel production \(^11\).

Chlorella is single-cell green algae with 2-10 \(\mu\)m of diameter and is composed of protein, lipids, saccharides, mineral, and others \(^12\). Since the content of saccharides is low, ethanol production is inefficient. Here, we examined the possibility for photocatalytic hydrogen production over Pt/TiO\(_2\) using enzymatic hydrolyzed chlorella as sacrificial agents.

2. Experimental

2.1 Materials

Frozen chlorella which is bred as feed for rotifer was purchased from Seibutsu Kogaku Kenkyusho (Yaizu, Shizuoka, Japan). The frozen chlorella was thawed and dried in a drying machine and then ground by a Wonder Blender WB-1 (Osaka Chemical Co. Ltd, Osaka) to produce powdered dry chlorella. Three kinds of enzymes, protease (protease A AmanoSD, Amano enzyme, Nagoya), cellulase (Acremozyme KM, Kyowa Kasei, Osaka), and xylanase (Sumizyme X, Shin Nikon Chemicals, Anjo), were used. A phosphate buffer (0.1 M, pH 7.6) was prepared by dissolving Na\(_2\)HPO\(_4\) (2.469 g) and NaH\(_2\)PO\(_4\) (0.312 g) in 100 mL of water.

2.2 Analytical method

The amounts of amino acids in enzymatic hydrolyzed solution (EH solution) were determined by colorimetric analysis as follows. Aqueous solution of ninhydrin (20 g/L) was prepared and the pH was adjusted to 3.0 by the addition of dilute aqueous HCl solution. Aqueous solution of ninhydrin (300 \(\mu\)L) was mixed with aqueous solution of glycine (15 g/L, 0-250 \(\mu\)L) and the volume was adjusted to 600 \(\mu\)L by pure water. After standing at 40 °C for 30 min, the solution was subjected to colorimetric analysis. Absorbance at 570 nm was measured in UV-visible spectra to make the calibration line between the concentrations of glycine between the absorbance. The EH solution (50 \(\mu\)L), the aqueous ninhydrin solution (300 \(\mu\)L), and water (250 \(\mu\)L) were mixed to stand at 40 °C for 30 min. The solution was subjected to measurement of absorbance at 570 nm. By assuming that all amino acids were glycine, the amount of amino acid was roughly determined using the calibration line.

Saccharides in the EH solution were measured by a high-performance liquid chromatography (HPLC) on a LC-20AD system (Shimadzu, Kyoto) equipped with a RI detector (RID-10A) using an anion exchange column (NH2P-50 4E, Shodex Asahipak, 250 mm in length and 4.6 mm in ID, Yokohama, Japan). Acetonitrile-water (82 v/v) was flowed at 1.0 mL/min as a mobile phase.

The evolved gas from the photocatalytic H\(_2\) production was analyzed by gas-liquid chromatography (GLC) on a Shimadzu GC-8A equipped with a TCD detector at temperatures increasing from 40 to 180 °C using a stainless column (3 mm\(\Phi\) × 6 m) packed with a SHINCARBON ST (Shimadzu).

2.3 Strategy for hydrogen production from chlorella

Hydrogen production from chlorella was performed through enzymatic hydrolysis followed by photocatalytic H\(_2\) production over Pt/TiO\(_2\) following the procedure shown in Fig. 1. Chlorella includes colored materials such as chlorophyll which may disturb the light-absorption by the catalyst. Therefore, dried chlorella was subjected to the refluxing in ethanol to remove the colored materials as follows. The powdered chlorella (20 g) was subject to treatment with ethanol (100 mL) under refluxing temperature (78 °C) for 6 h. After cooling, the precipitate was collected by filtration and dried at 60 °C for 24 h. Almost all amount of colored materials remained in the ethanol solution. The treated or non-treated chlorella was turned into water-soluble materials by enzymatic hydrolysis using protease, cellulase, and xylanase. The resulting water-soluble materials (EH solution) were subjected to...
photoncatalytic hydrogen production using Pt/TiO₂.

During our investigations on sacrificial H₂ production over Pt/TiO₂ catalyst, we found that sacrificial agents with all of the carbon attached oxygen atoms such as saccharides (e.g. glucose and xylose) and polyalcohols (e.g. 1,2-ethandiol, glycerol, arabitol) continued to serve as an electron source until their sacrificial ability was exhausted in the photocatalyzed H₂ evolution over Pt/TiO₂ catalyst. Here, we applied the sacrificial H₂ production to transformation from chlorella to H₂.

2.4 Enzymatic hydrolysis

Decolored or untreated chlorella powder (10 g) and hydrolytic enzyme (protease, cellulase, or xylanase, 1.0 g) were dispersed into a phosphate buffer (0.1 M, pH 7.6, 60 mL) in the reaction vessel. The enzymatic hydrolysis were performed using protease, cellulase, and xylanase under magnetic stirring at 50 °C, 45 °C, and 45 °C which were the optimized temperatures, respectively. After reaction for 48 h, the reaction mixture was subjected to centrifugation to remove the precipitate which was thought to be un-hydrolyzed chlorella. The supernatant of EH solution was collected. As shown in Table 1, the EH solutions were named as 1a-1d by whether they were decolorized or not, and the kinds of hydrolytic enzyme.

In order to estimate the progress of hydrolysis of chlorella, the EH solution was subjected to freeze-drying on an Eyela EDM-1200 (Tokyo, Japan) where water was removed from EH solutions to give the solid.

2.5 Preparation of photocatalyst

Anatase-type TiO₂ (ST01) was purchased from Ishihara Sangyo (Japan). Following the previous literature, an aqueous solution (400 mL) containing TiO₂ (4.0 g), K₂PtCl₆ (200 mg), and 2-propanol (306 mL) was introduced reaction vessel. The reaction vessel was cylindrical with 30 cm of height and 7.5 cm of diameter. A high-pressure mercury lamp (100 W, UVL-100HA, Riko, Japan) was inserted into the reaction vessel. The reaction temperature was controlled by running water in lamp jacket and water bath during the irradiation. After the oxygen was purged by N₂ gas, the solution was irradiated for 24 h by stirring to produce a Pt-loaded TiO₂ catalyst (Pt/TiO₂). After irradiation, the water was entirely removed from the photolysates by an evaporator. The resulting precipitate was washed with water in a filter and then dried under reduced pressure to produce Pt/TiO₂ where the optimized amount of Pt (2.0 wt%) was loaded.

### Table 1

| EH sol | DC | Enzyme | Solid component (g/L) | Amino acids (g/L) | Glucose (g/L) | H₂ (mL/g) | H₂ (mg) |
|--------|----|--------|-----------------------|------------------|---------------|-----------|---------|
| 1a     | Yes| Protease | 126                   | 50.1             | 13.8          | 115       | 574     |
| 1b     | No | Protease | 117                   | 98.0             | 18.3          | 119       | 579     |
| 1c     | Yes| Cellulase | 57                    | 52.2             | 37            | 67        | 334     |
| 1d     | Yes| Xylanase | 72                    | 62.9             | 8.7           | 51        | 255     |

- a) Enzymatic hydrolysis was performed for a phosphate buffer (60 mL) containing dried chlorella (10.0 g) and enzyme (1.0 g)
- b) DC means the decolorization to remove the colored materials by refluxing with ethanol.
- c) Weight of solid components obtained from the enzymatic hydrolysis of chlorella in aqueous solution was determined by freeze-drying to remove water. The solution before enzymatic hydrolysis contained 167 g/L of chlorella.
- d) The amounts of amino acids were determined by colorimetric analysis using ninhydrin. The amounts of amino acids were determined by assuming that all amino acids were glycine.
- e) Limiting H₂ volume (mL) evolved from 1 mL of the EH solution at 20 °C.
- f) Volume of H₂ evolved from 1 g of solid component = \( \frac{H₂^{max}}{\text{weight of solid component}} \)
- g) The amounts of H₂ obtained from 10.0 g of dried chlorella. H₂= \( \frac{H₂^{max} \times 60 \times 2}{24.04} \)
2.6 Photocatalytic hydrogen production

Typical procedure of photocatalytic H₂ production is shown as follows. The Pt/TiO₂ powder whose weight was 100 mg (1.25 mmol of TiO₂) was set in a reaction vessel (Fig. 2). The EH solution (V₁ = 0.10 - 0.50 mL) were introduced into a reaction vessel. The volume of the solution was adjusted to 150 mL by adding water. A high-pressure mercury lamp (100 W) was inserted into the cylindrical reaction vessel (30 cm of height and 6.0 cm of diameter), which was attached to a measuring cylinder with a gas-impermeable tube to collect the evolved gas. After the O₂ was purged from the reaction vessel by N₂ gas, irradiation was performed at room temperature (ca. 20 °C) under vigorous stirring by a magnetic stirrer for 30–170 h until the gas evolution ceased. The evolved gas was collected by a measuring cylinder to measure the volume of the evolved gas and analyzed on a GLC. H₂ and CO₂ were detected in the gas and analyzed on a GLC. H₂ and CO₂ were detected in the evolved gas.

3. Results and Discussion

3.1 Enzymatic hydrolysis

Table 1 shows the weight of the solids in the EH solutions. In the cases of 1a and 1b where protease was used as an enzyme, the weights of the solids were larger (126 and 117 g/L), showing that enzymatic hydrolysis proceeded well compared with the cases using cellulase and xylanase. Since the weight of the solid was 167 g/L before hydrolysis, more than 70% of the solid was hydrolyzed into water-soluble components. The amounts of amino acid and glucose are listed in Table 1. It was found that components of the solids of the EH solutions were mostly consisted of the amino acids. In general, chlorella is composed of protein (45%), lipids (20%), saccharides (20%), mineral (10%), and others 16. Therefore, these analyses were in accord with the reported values. Thus, the EH solution was used as sacrificial agent for the following sacrificial H₂ production over Pt/TiO₂ photocatalyst.

3.2 Photocatalytic hydrogen production

At first, it was confirmed that gas evolution was very inefficient from the non-enzymatic hydrolyzed solution which was prepared by magnetic stirring of the chlorella powder (10 g) in a phosphate buffer (60 mL) for 48 h at 50 °C. The amounts of the evolved H₂ and CO₂ from the photocatalytic H₂ generation in the presence of various volumes of EH solution (V₁ in mL) were summarized in Table 2. Fig. 3(A) shows an example of the H₂ and CO₂ evolutions in the photocatalytic H₂ generation using solution 1a. The evolved H₂ volume (V₁) increased as the volume of 1a (V₁) used increased. However, the volume ratio of V₁ to V₅ (V₁/V₅) was dependent on the Vi used. As the ratio of V₁ to weight of catalyst (V₁/catalyst) decreased, the V₁/V₅ increased. The colored material in EH solution and the carboxylic acids formed during the photocatalytic reaction may lower the reactivity of photocatalyst.

Therefore, we plotted the V₁/V₅ against the V₁/catalyst, as shown in Fig. 3(B). The intercept of the plots represented H₂_{max}, which was the limiting H₂ volume obtained from 1 mL of 1a at an infinite amount of the catalyst. In the case of 1a, the H₂_{max} value was 115 mL/mL.

Table 2 shows the H₂_{max} values using 1a-1d as sacrificial agents. In the case of 1a and 1b, which was produced by protease-hydrolysis of chlorella, the H₂_{max} were the larger values. The decolorization did not affect the H₂_{max}, but shortened the irradiation time, as shown in Table 2. The volume ratio of H₂ to CO₂ was 1.39-1.57:1, which was close to the ratio for the photo-degradation of glycine (eq. (1)) rather than glycine (eq. (2)).

As has been reported so far 14-15, the sacrificial agents which have hetero-atoms such as oxygen and nitrogen atoms at all of carbons were entirely decomposed into CO₂ and water in sacrificial photocatalytic hydrogen production. Therefore, glycine (molecular weight 75 g/mol) is the most efficient sacrificial agent among amino acids and is decomposed entirely and has theoretical hydrogen amounts of 1/75×3×24,040 = 962 mL/g from 1 g of glycine at 20 °C (eq. (1)). Also, glucose (molecular weight 180 g/mol) has theoretical hydrogen amounts of 1/180×12×24,040 = 1,600 mL/g from 1 g of glucose (eq. (2)). In the cases of 1a and 1b, the hydrogen amounts were 1,021 mL/g for 1a and 916 mL/g for 1b which exceeded the theoretical amounts of glycine in the case of 1a. This showed that the EH solution involved water-soluble oligosaccharides other than glycine and glucose.
According to the above results, mass balance is estimated, as shown in Fig. 4. The hydrolysis of dry chlorella (1.0 g) in 6.0 mL of a phosphate buffer solution using protease (100 mg) gave an aqueous EH solution (\(V_1 = 0.10 \sim 0.50\) mL) under irradiation with a high-pressure Hg lamp.

Table 2  Photocatalytic \(\text{H}_2\) production from the enzymatic hydrolyzed (EH) solution of chlorella \(^a\)

| EH solution | Irradn. time | Gas | \(\text{H}_2\) | \(\text{CO}_2\) | \(\text{H}_2^{\max}\) |
|-------------|--------------|-----|-------------|-------------|-----------------|
| No | \(V_1\) (mL) | (h) | (mL) | (mL, \(V_0\)) | (mL) | (mL/mL) |
| 1a | 0.10 | 70 | 18 | 11 | 7 | 115 |
| | 0.20 | 70 | 27 | 16 | 11 |
| | 0.30 | 70 | 40 | 24 | 16 |
| | 0.40 | 150 | 42 | 25 | 18 |
| | 0.50 | 48 | 45 | 27 | 18 |
| 1b | 0.10 | 30 | 20 | 12 | 8 | 119 |
| | 0.20 | 67 | 21 | 13 | 8 |
| | 0.30 | 170 | 28 | 17 | 11 |
| | 0.40 | 170 | 30 | 18 | 12 |
| | 0.50 | 120 | 32 | 19 | 13 |
| 1c | 0.10 | 45 | 11 | 7 | 4 | 674 |
| | 0.20 | 48 | 18 | 11 | 7 |
| | 0.30 | 50 | 27 | 16 | 11 |
| | 0.40 | 140 | 44 | 28 | 16 |
| | 0.50 | 70 | 35 | 21 | 14 |
| 1d | 0.10 | 47 | 8 | 5 | 3 | 51.1 |
| | 0.20 | 50 | 17 | 10 | 7 |
| | 0.30 | 47 | 28 | 17 | 11 |
| | 0.40 | 40 | 30 | 18 | 12 |
| | 0.50 | 69 | 41 | 25 | 16 |

\(^a\) Sacrificial \(\text{H}_2\) production was performed for an aqueous solution (150 mL) containing Pt/TiO\(_2\) (100 mg) and enzymatic hydrolyzed chlorella (EH) solution (\(V_1 = 0.10 \sim 0.50\) mL) under irradiation with a high-pressure Hg lamp.

\[\text{H}_2\text{N-CH}2\text{-CO}_2\text{H} + 2\text{H}_2\text{O} \xrightarrow{\text{hv}} \text{Pt/TiO}_2 \] 2\(\text{CO}_2 + \text{NH}_3 + 3\text{H}_2\)  
(glycine (MW 75))

\[\text{C}_6\text{H}_12\text{O}_6 + 6\text{H}_2\text{O} \xrightarrow{\text{hv}} \text{Pt/TiO}_2 \] 6\(\text{CO}_2 + 12\text{H}_2\)  
(glucose (MW 180))

According to the above results, mass balance is estimated, as shown in Fig. 4. The hydrolysis of dry chlorella (1.0 g) in 6.0 mL of a phosphate buffer solution using protease (100 mg) gave an aqueous EH solution (\(V_1 = 0.10 \sim 0.50\) mL) under irradiation with a high-pressure Hg lamp. The EH solution was subjected to photo-catalytic \(\text{H}_2\) production to evolve \(\text{H}_2\) (57.9 mg). We previously reported that \(\text{H}_2\) production from lignocelluloses which proceeded through alkali-pretreatment, saccharification with cellulase and xylanase, and sacrificial \(\text{H}_2\) production over Pt/TiO\(_2\) produced 39.4 mg and 37.1 mg.
4. Conclusion

Hydrolyzed solution of chlorella contained large amounts of amino acids but few amounts of saccharide. Enzymatically hydrolyzed solution of chlorella was successfully used for the sacrificial H$_2$ evolution. The evolved H$_2$ volume ($V_H$) increased as the volume of enzymatic hydrolyzed solution ($V_1$) increased. As the ratio of $V_1$ to weight of catalyst decreased, the $V_H/V_1$ increased. The limiting value of $V_H/V_1$, which corresponds to H$_2$ volume at infinite amount of the catalyst, was the largest when protease was used. This is the first report on sacrificial H$_2$ production from chlorella.

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