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Optimization of conditions for direct bio-hydrogen production from water hyacinth by *Clostridium diolis* C32-KKU

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Abstract

Water hyacinth contains cellulose and hemicellulose which can be used as a substrate for bio-hydrogen production. *Clostridium diolis* C32-KKU, a cellulolytic bacterium, was employed to directly ferment water hyacinth to bio-hydrogen. The objective of this study was to optimize the direct bio-hydrogen production from water hyacinth by *C. diolis* C32-KKU. Two operation modes for bio-hydrogen production i.e. static and shaking modes were investigated. The results showed that the shaking mode was more effective than the static mode for hydrogen production. The shaking mode was then used to optimize bio-hydrogen production by variation of initial pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) and initial water hyacinth concentration (5, 10, 15, 20, 25 and 30 g-dry weight (dw)/L). The maximum hydrogen production of 19 mL/L was obtained at the initial pH of 5.5 and water hyacinth concentration of 10 g-dw/L. The cellulase activity of 0.0081 unit/mL was obtained under the optimal condition. The results of this study showed that direct bio-hydrogen production from lignocellulosic materials could be feasible.
Keywords: Bio-hydrogen, Anaerobic fermentation, *Clostridium diolis,* Water hyacinth

1. Introduction

Currently, the demand for energy is rising continuously, while the supply of fossil fuel is decreasing, this causes fossil fuel to increase in price. Therefore, scientists worldwide are trying to solve this problem by searching for a source of renewable energy in order to respond to the energy requirements. Hydrogen is an interesting alternative energy because its combustion generates only water and heat and has high energy yield of 122 kJ/g[1]. Traditionally, hydrogen can be produced by chemical processes such as electrolysis of water and steam reforming process but these processes require high energy input and high reaction temperature (>850°C)[2] while biological processes require mild conditions. Thus, biological hydrogen production is environmentally friendly. Mixed culture or pure culture can be used as biocatalyst for hydrogen production.

Water hyacinth is an unwanted flora which is a free floating aquatic plant. It is well known that water hyacinth can grow and spread very fast, leading to problems in navigation, irrigation and power generation[2]. However, water hyacinth mainly consists of cellulose (18%-31%), hemicellulose (18%-43%), and lignin (7%-26%)[3] which can be easily hydrolyzed to fermentable sugar. Therefore, it is suitable to be used as raw materials for hydrogen production.

Most research has focused on conversion of lignocellulosic materials to hydrogen by mixed cultures or pure cultures which are isolated from soil, sludge of waste water treatment and animal manure. Conversion of lignocellulosic materials to hydrogen in most research consists of two steps. Firstly, lignocellulosic materials are hydrolyzed to monosaccharides such as glucose, xylose and arabinose by enzyme or alkaline-acid, followed by fermentation of sugar to hydrogen by microorganism. However, the cost of pretreatment is an expensive aspect to industrial application. *C. diolis* C32-KKU is a cellulolytic bacterium isolated from cow dung. It can produce cellulase to hydrolyze lignocellulosic materials to sugar and then convert sugar to hydrogen. Therefore, the objective of this study was to optimize direct bio-hydrogen production from water hyacinth by variation of initial pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) and initial water hyacinth concentration (5, 10, 15, 20, 25 and 30 g-dw/L). The results from this study would provide the important information on developing the direct bio-hydrogen production process from lignocellulosic materials.

2. Materials and Methods

2.1 Feedstock and Microorganism

Water hyacinth was collected from a pond in Khon Kaen University, Khon Kaen, Thailand. Colons of water hyacinth were dried at 80°C in a hot air oven, milled to pass a 0.5 mm screen by pin disc mill. Finally, dried water hyacinth was stored in plastic bag and kept in a dry place at room temperature prior to usage. Basic anaerobic (BA) medium comprised of solution A; NH_4Cl 100 g/L; NaCl 10 g/L; $MgCl_2$ 10 g/L; $CaCl_2$ 5 g/L, solution B; $K_2HPO_4 \cdot 3H_2O$ 200 g/L and solution D; $FeCl_2 \cdot 4H_2O$ 2 g/L; H_3BO_3 0.05 g/L; $ZnCl_2$ 0.05 g/L; $CuCl_2 \cdot 2H_2O$ 0.038 g/L; $MnCl_2 \cdot 4H_2O$ 0.05 g/L; $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ 0.05 g/L; $AlCl_3$ 0.05 g/L; EDTA 0.5 g/L; $CoCl \cdot 6H_2O$ 0.05 g/L; $NiCl_2 \cdot 6H_2O$ 0.092 g/L; Conc. HCl 1 mL; $Na_2SeO_3 \cdot 5H_2O$ 0.1 g/L; $NaHCO_3$-solution 52 mL; Yeast extract 100 g/L.

C. diolis C32-KKU, a cellulolytic bacterium, isolated from cow dung was employed in this study. For inoculum preparation, *C. diolis* C32-KKU was cultured in BA medium and 5 g/L of carboxymethylcellulose (CMC) was used as a carbon source.

2.2 Experimental procedure

The batch test was conducted to investigate the optimum operation mode, initial pH and initial dry weight concentration of water hyacinth for direct hydrogen production by *C. diolis* C32-KKU. Three series of batch experiments were conducted. First, the effects of two operation modes (static and shaking at 150 rpm) on hydrogen production were investigated at the initial pH and initial concentration of water hyacinth of 6.0 and 10 g-dw/L each,

respectively. The incubating temperature was 37ºC. The obtained optimal operation mode which gave the maximum hydrogen production was further used to study the effects of initial pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) on the hydrogen production efficiency. The control experiment was without pH adjustment. The initial concentrations of water hyacinth were fixed at 10 g-dw/L each. Then, the effects of initial concentrations of water hyacinth (5, 10, 15, 20, 25 and 30 g-dw/L each) were studied by using the obtained optimum operation mode and the initial pH. The control experiment was without water hyacinth addition.

The experiments were performed in 120-mL serum bottles with a working volume of 70 mL comprising of 7 mL of inoculums and 63 mL of BA medium. All batches were inoculated from freshly growing culture on BA medium at exponential phase. Water hyacinth was used as the carbon source. Water hyacinth at the designated concentration was added to the substrate solution prior the pH adjustment to the designated value by using 1 N HCl or 1 N NaOH. The serum bottles were capped with rubber stoppers and aluminum caps before purging with nitrogen gas to create the anaerobic environment. The serum bottles were incubated in an incubator at 37ºC for 120 h. All treatments were conducted in triplicate.

2.3 Analytical methods

The volume of biogas was determined using wetted glass syringes of 20 mL. The hydrogen content in the gas phase was determined by gas chromatography (GC-Shimadzu 17-A, Japan) with a thermal conductivity detector (TCD). Nitrogen was used as the carrier gas with the flow rate of 54 mL/min. The column was a 2 m x 4 mm diameter Molecular sieve 5A column. The temperatures of injector port, detector and column oven were 100, 100 and 50ºC, respectively.

Hydrogen gas production was calculated from the bioreactor headspace measurements of gas composition and the total volume of hydrogen produced at each time interval using the mass balance equation[4]:

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V_{H,0}(C_{H,i} - C_{H,i-1}) \quad (1)$$

where $V_{H,i}$ and $V_{H,i-1}$ are the cumulative hydrogen gas volume at the current (i) and previous time interval (i-1), respectively; $V_{G,i}$ and $V_{G,i-1}$ are the total biogas volume at the current and previous time interval; $C_{H,i}$ and $C_{H,i-1}$ are the fraction of hydrogen gas in the headspace at the current and previous time interval; V_H is the volume of headspace of serum bottle (30 mL).

Hydrogen yield was calculated as the total molaric amount of hydrogen divided by gram-dry weight of water hyacinth added (mol H_2/g-dw of water hyacinth). The total molaric amount of hydrogen was calculated using the ideal gas law as: molar hydrogen production (mmol H_2/L) = volumetric hydrogen production (ml H_2/L)/(RT), where R = 0.08205784 L×atm/K×mol, and T = 310 K[5].

The liquid samples were centrifuged at 10,000 rpm for 5 min, acidified by 0.2 N oxalic acid and filtered through 0.45 mm cellulose acetate membrane. The HPLC (Shimadzu LC-10AD, Japan) with an ultraviolet detector (UV) and Aminex HPX-87H column was used for volatile fatty acids (VFAs) and ethanol analysis. The oven temperature was 45ºC, and 5 mM H_2SO_4 was used as a mobile phase at the flow rate of 0.6 mL/min. For sugar

concentrations in the samples (glucose, xylose and arabinose) were determined by HPLC (Shimadzu LC-10AD, Japan) with a refractive index detector (RID). The oven temperature was 45ºC, and 5 mM H_2SO_4 was used as a mobile phase at the flow rate of 0.6 mL/min. Determination of cellulase activity was modified from Obi and Okeke (1995)[11].

2.4 Kinetic analysis

The cumulative hydrogen production in the batch experiment followed the modified Gompertz equation[7]:

$$H = P\exp\left\{-\exp\left[\frac{R_m e}{P}(\lambda - t)+1\right]\right\} \qquad (2)$$

where H is the cumulative volume of hydrogen produced (mL), P is the hydrogen production potential (mL), R_m is the maximum production rate (mL/h), λ is the lag-phase time (h), t is the incubation time (h), and e is 2.718. In this study P is expressed as mL of hydrogen/L of medium and R_m is expressed as mL of hydrogen/(L of medium×h).

3. Results and Discussion

3.1 Effects of operation mode on direct hydrogen production

Two operation modes for direct hydrogen production i.e. static and shaking were investigated. After 20 h of fermentation time, bacteria in shaking mode could produce hydrogen continuously until 120 h while bacteria in static mode stopped hydrogen production after 20 h of fermentation time (Figure 1).

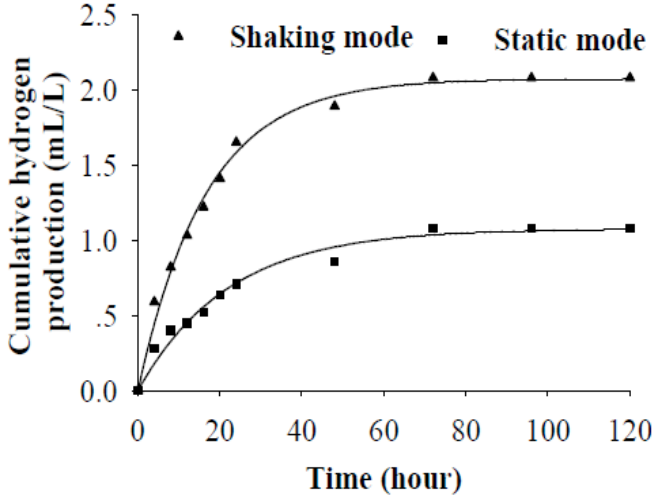

Figure 1. Effects of operation mode on hydrogen production (symbols: observed data, curves: predicted data with the Gompertz equation).

The potential of direct bio-hydrogen production was estimated by Gompertz equation. Table 1 summarizes the maximum cumulative hydrogen production, rate of hydrogen production and hydrogen yield. The results showed that shaking mode was more effective than static mode for hydrogen production. Maximum cumulative hydrogen production (2 mL/L), rate of hydrogen production (0.08 mL/L·h) and hydrogen yield (7.96×10^{-3} mmol H_2/g-dw) were higher than those in static mode.

It was due to that in shaking mode, the mixture of BA medium and water hyacinth was homogeneous which enhanced the ability of bacteria to approach to nutrient. While in static mode, water hyacinth settled on the bottom, causing difficulty for bacteria to approach to nutrient. Therefore, the shaking mode was the optimal operation mode for bio-hydrogen production and used for the further experiments.

Table 1. Potential of direct bio-hydrogen with operating in static and shaking modes

Operation mode	P (mL/L)	R_m(mL/L·h)	HY (mmol H_2/g-dw)
Static	1	0.04	3.08×10^{-5}
Shaking	2	0.08	7.96×10^{-3}

P = hydrogen production; R = hydrogen production rate, HY = hydrogen yield; dw = dry weight

Soluble metabolite products such as volatile fatty acids (acetic acid, propionic acid, butyric acid and formic acid) and ethanol from hydrogen production using two operation modes are shown in Table 2. For shaking mode, bacteria only produced acetic acid and butyric acid which were produced along with hydrogen when bacteria used glucose as substrate (Eq. (3) and (4))[8]. For static mode, the production of propionic acid by bacteria caused a reduction of hydrogen production because bacteria would also use hydrogen as a substrate to produce propionic acid according to Eq. (5)[9]. Thus, the shaking mode was more potential for hydrogen production than the static mode.

Table 2. Soluble metabolite products from hydrogen production using static and shaking modes

Operation mode	Soluble metabolite products (g/L)		
	HAc	HPr	HBu
Static	2.97±0.02	0.58±0.01	0
Shaking	2.14±0.01	N.D.	4.08±0.01

HAc = acetic acid; HPr = propionic acid;
HBu = butyric acid; N.D. = not detectable

$$C_6H_{12}O_6 + 4H_2O \rightarrow 4H_2 + 2C_2H_4O_2 + 2HCO^-_3 + 4H^+ \quad (3)$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2H_2 + C_4H_8O_2 + 2HCO^-_3 + 3H^+ \quad (4)$$

$$C_2H_4O_2 + H_2 + CO_2 \rightarrow CH_3CH_2COOH \quad (5)$$

C. diolis C32-KKU released cellulase to hydrolyze cellulose in water hyacinth to sugars. However, the produced glucose, xylose and arabinose in fermentation broth were not detectable by HPLC. It might be due to the immediate consumption of hydrolysable sugars by bacteria.

3.2 Effects of initial pH on direct hydrogen production

Investigation of the effect of initial pH of BA medium which used water hyacinth as carbon source on direct hydrogen production was carried out by fixing water hyacinth at 10 g-dw/L. The initial pH of BA medium was varied to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0. The control was without pH adjustment (pH 9.6). Most cumulative hydrogen production increased noticeably from 0 to 20 h of fermentation time (Figure 2). The highest cumulative hydrogen production was obtained at the initial pH of 5.5.

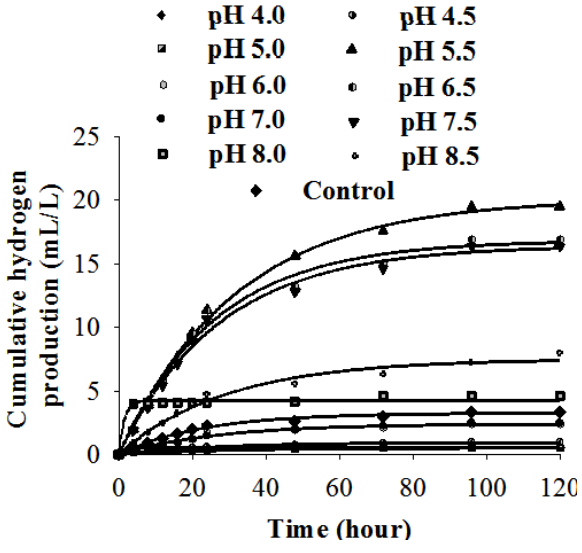

Figure 2. Effects of initial pH on hydrogen production (symbols: observed data, curves: predicted data with the Gompertz equation).

The potential of direct bio-hydrogen production was estimated by Gompertz equation. Table 3 summarizes the maximum cumulative hydrogen production, hydrogen production rate and hydrogen yield. The results showed that the highest cumulative hydrogen production (19 mL/L), hydrogen production rate (0.52 mL/L·h) and hydrogen yield (7.54×10^{-2} mmol H_2/g-dw) were achieved at the initial pH of 5.5 followed by the initial pH of 6.5, 7.5, 8.5, 8.0, 9.0, 7.0, 6.0, 4.5, control, 4.0 and 5.0, respectively. At mild acid condition (pH 5-6), the ability of bacteria for hydrogen production was high. It is possible that *Clostridia* bacteria are urged to excrete protons in the cytoplasm so that the cells grow well[10]. The process of hydrogen production of *Clostridia* bacteria occurs when the cell grows into early exponential phase. The results obtained from this study were similar to those of Masset et al. (2010)[11], who reported that the optimal pH for hydrogen production from starch and glucose by *C. butyricum* CWBI1009 were 5.2 and 5.6, respectively.

Table 3. Potential of direct bio-hydrogen at various initial pH values

Initial pH	P (mL/L)	R_m (mL/L·h)	HY (mmol H_2/g-dw)
4.0	0	0	0
4.5	1	0.03	3.63×10^{-3}
5.0	1	0.01	1.97×10^{-3}
5.5	19	0.52	7.54×10^{-2}
6.0	2	0.08	9.18×10^{-3}
6.5	16	0.53	6.42×10^{-2}
7.0	2	0.06	9.57×10^{-3}
7.5	16	0.50	6.24×10^{-2}
8.0	4	3.84	1.67×10^{-2}
8.5	7	0.22	2.71×10^{-2}
9.0	3	0.11	1.26×10^{-2}
control	1	0.02	3.78×10^{-3}

P = hydrogen production; R = hydrogen production rate, HY = hydrogen yield; dw = dry weight

The lowest cumulative hydrogen production, hydrogen production rate and hydrogen yield at initial pH was 4.0. Adjustment of the initial pH of the medium to 4.0 by the addition of 1 N HCl increased free protons in the substrate. These protons could pass through cell wall into cytoplasm and inhibited the glycolytic enzyme activity and disrupt/disrupted the structures of cell wall, DNA and protein. Therefore, the overall cell growth was inhibited resulting in low hydrogen production[10].

The production of soluble metabolite products such as volatile fatty acids (acetic acid, propionic acid, butyric acid and formic acid) and ethanol from hydrogen production at various initial pH of BA medium are shown in Table 4. The results showed that at the initial pH of 5.5 bacteria only produced acetic and butyric acid. At the initial pH of 4.0, no volatile acids and solvent were produced. In addition, at the initial pH of 4.5 bacteria only produced ethanol because bacteria changed metabolic pathway to produce alcoholic solvent and most of research which studied ethanol and butanol production always adjusted the initial pH to 4.3-4.5[13]. At other initial pH values, bacteria also produced propionic acids and formic acids, which resulted in low hydrogen production because bacteria used hydrogen as a substrate to produce propionic acid and formic acid as shown in Eq. (5)[9] and Eq. (6)[12], respectively.

$$CO_2 + H_2 \rightarrow HCO_2H \quad (6)$$

Accordingly, bacteria could produce hydrogen effectively at the initial pH of 5.5 and this initial pH was used to further optimize direct bio-hydrogen by variation of initial concentrations of water hyacinth.

3.3 Effects of initial concentrations of water hyacinth on direct hydrogen production

Water hyacinth was hydrolyzed to sugar and then used to produce hydrogen by cellulolytic bacteria. Effects of initial concentrations of water hyacinth (5, 10, 15,

20, 25, 30 g-dw/L) on direct hydrogen production were studied. The control was without water hyacinth addition. The initial pH of BA medium was fixed at 5.5. Cumulative hydrogen production slightly increased from 20 to 40 h of fermentation time except the initial water hyacinth of 5 g-dw/L and control. The highest cumulative hydrogen production was obtained at the initial water hyacinth of 10 g-dw/L (Figure 3).

Table 4. Soluble metabolite products from hydrogen production at different initial pH values

Initial pH	Soluble metabolite products (g/L)				
	HFr	HAc	HPr	HBu	EtOH
4.0	N.D.	N.D.	N.D.	N.D.	N.D.
4.5	N.D.	N.D.	N.D.	N.D.	6.51±0.01
5.0	N.D.	0.50±0.01	N.D.	1.38±0.01	N.D.
5.5	N.D.	3.71±0.01	N.D.	1.50±0.01	N.D.
6.0	N.D.	2.96±0.01	0.57±0.01	N.D.	N.D.
6.5	N.D.	1.87±0.01	0.09±0.01	6.99±0.01	N.D.
7.0	0.65±0.01	4.86±0.02	0.81±0.01	0.95±0.01	N.D.
7.5	0.26±0.01	2.86±0.01	0.51±0.01	7.88±0.01	N.D.
8.0	5.39±0.01	5.06±0.01	0.69±0.01	0.56±0.02	N.D.
8.5	1.60±0.01	3.85±0.01	N.D.	N.D.	N.D.
9.0	1.55±0.01	1.68±0.01	N.D.	N.D.	N.D.
Control	2.37±0.01	5.14±0.01	3.35±0.01	N.D.	N.D.

HFr = formic acid; HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; EtOH = ethanol; N.D. = not detectable

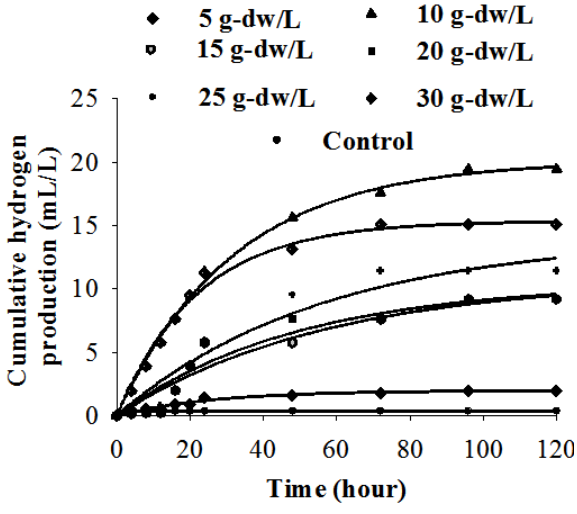

Figure 3. Effects of initial concentration of water hyacinth on hydrogen production (symbols: observed data, curves: predicted data with the Gompertz equation).

The potential of direct bio-hydrogen production was estimated by Gompertz equation. Table 5 summarizes the maximum cumulative hydrogen production, hydrogen production rate and hydrogen yield. The results showed that the maximum cumulative production increased with increasing concentrations of water hyacinth from 5 to 10 g-dw/L while increased concentrations of water hyacinth higher than 10 g-dw/L caused the maximum cumulative hydrogen production to decrease. It is possible that excess substrate concentration causes bacteria to produce a lot of volatile fatty acids such as acetic acid, butyric acid, propionic acid and formic acid. The pH values of fermentation broth decreased, resulting in hydrogen production inhibition[14]. Therefore, the highest maximum cumulative hydrogen production (19.22 mL/L), hydrogen production rate (0.43 mL/L·h) and hydrogen yield (0.08 mmol H_2/g-dw) were obtained at the initial water hyacinth of 10 g-dw/L. The results obtained from this study were similar to those of Alalayah et al. (2008)[15], who reported that the optimal initial concentration of substrate for hydrogen production from glucose by *C. saccharoperbutylacetonicum* N1-4 (ATCC 13564) was 10 g/L.

Table 5. Potential of direct bio-hydrogen at different initial concentrations of water hyacinth

Initial concentration (g-dw/L)	P (mL/L)	R_m (mL/L·h)	HY (mmol H_2/g-dw)
5	2	0.05	0.012
10	19	0.43	0.076
15	9	0.21	0.025
20	9	0.53	0.018
25	11	0.50	0.017
30	15	0.39	0.019
control	0	0	0

P = hydrogen production; R = hydrogen production rate, HY = hydrogen yield; dw = dry weight

The minimum cumulative hydrogen production, hydrogen production rate and hydrogen yield were obtained at the initial water hyacinth concentration of 5 g-dw/L due to the deficiency of carbon source. The results showed that carbon source was used up within 50 h. Normally, fermentation time should be 100 h[16]. Therefore, less cumulative hydrogen production was obtained. The results indicated that the concentration of carbon source affected hydrogen production because bacteria could not produce hydrogen without carbon source (Table 5). To achieve maximum effective of hydrogen production, the optimal initial concentration of carbon source should be investigated.

The production of soluble metabolite products such as volatile fatty acids (acetic acid, propionic acid, butyric acid and formic acid) and ethanol from hydrogen production at various initial concentrations of water

hyacinth are shown in Table 6. At the optimal concentration of water hyacinth (10 g-dw/L), bacteria only produced acetic acid. For other initial concentrations, bacteria also produced acetic acid and butyric acid. Without water hyacinth, bacteria produced propionic acid, which negatively affected the hydrogen production because bacteria used hydrogen as a substrate to produce propionic acid, resulting in less cumulative hydrogen production. Therefore, the optimal conditions for direct bio-hydrogen production were the shaking mode at 150 rpm, 37°C, the initial pH of 5.5 and the initial concentration of water hyacinth of 10 g-dw/L.

Table 6. Soluble metabolite products from hydrogen production at variation of initial concentration of water hyacinth

Initial concentration (g-dw/L)	Soluble metabolite products (g/L)		
	HAc	HPr	HBu
5	2.20±0.02	N.D.	1.15±0.01
10	4.00±0.01	N.D.	N.D.
15	2.23±0.01	N.D.	3.30±0.01
20	3.55±0.01	N.D.	2.17±0.01
25	7.55±0.01	N.D.	2.11±0.01
30	9.23±0.01	N.D.	2.30±0.01
Control	1.68±0.01	3.56±0.02	0.98±0.01

HAc = acetic acid; HPr = propionic acid; HBu = butyric acid; N.D. = not detectable

3.4 Activity of cellulase

During fermentation, *C. diolis* C32-KKU released cellulase to hydrolyze water hyacinth to produce hydrogen and volatile fatty acids. Therefore, the cellulose activity at the optimal conditions was observed. The highest activity of cellulase was obtained in 12-24 h of fermentation (Figure 4). It might be due to the good adaptability of bacteria in the environment, resulting in high cellulase production. After that, the activity of cellulase tended to decrease with increasing fermentation time because bacteria entered a death phase. This result was in line with the results of hydrogen production which showed that hydrogen production was slightly increased in 12 to 24 h of fermentation.

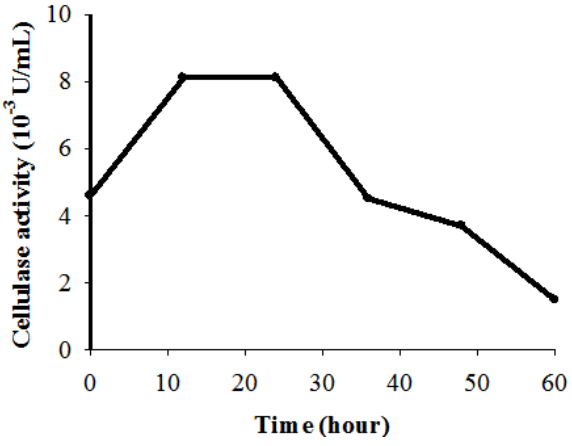

Figure 4. Activity of cellulase under the optimal conditions for direct hydrogen production

4. Conclusions

Clostridium diolis C32-KKU could directly produce hydrogen from water hyacinth because it could release cellulase to hydrolyze water hyacinth to sugar and then used hydrolyzed sugar for producing hydrogen. The optimal conditions for direct hydrogen production were at shaking speed of 150 rpm, the initial pH of 5.5 and the initial dry weight of water hyacinth of 10 g-dw/L. Under the optimal conditions, maximum hydrogen production of 19 mL/L, hydrogen production rate of 0.43 mL/L·h, hydrogen yield of 0.076 mmol H_2/g-dw of water hyacinth and maximum cellulase activity of 0.0081 unit/mL were obtained. The results showed that direct bio-hydrogen production from lignocellulosic materials could be feasible.

5. Acknowledgment

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