Photo-fermentative Bio-hydrogen Production from Agricultural Residue Enzymatic Hydrolyzate and the Enzyme Reuse

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Reducing sugars contained in agricultural residue hydrolyzates can potentially be utilized in microbial fermentations to produce biofuels and biogas. Different types of agricultural residues were employed for photo-fermentative bio-hydrogen production, and the cumulative hydrogen production data fit well to the Modified Gompertz Model. Corncob was determined to have the highest reducing sugar yield and cumulative hydrogen production (12.64 mg mL^{-1} enzymatic hydrolysate, 228.94 mmol L^{-1}) and maximum hydrogen production rate (5.9677 mmol L^{-1} h^{-1}). Enzyme reuse was investigated by single factor experiment design to reduce the cost of bio-hydrogen production. Taking reducing sugar yield and activity recovery efficiency as reference, substrate re-adsorption method at different temperature and time, then enzyme immobilization method at different load and pH were investigated in the process of enzymatic hydrolysis. The efficiency of enzyme utilization was enhanced via substrate re-adsorption and enzyme immobilization methods, which resulted in a 4-fold increase in recycling efficiency. The optimal enzyme reuse condition by substrate re-adsorption was a re-adsorption time of 90 min at a temperature of 15 °C, while the optimal condition by enzyme immobilization method was a pH of 4.8 and immobilized enzyme load of 400 mg.

Keywords: Agricultural residues; Enzymatic hydrolysis; Enzyme reuse; Reducing sugar yield; Bio-hydrogen production; Photosynthetic bacteria group

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INTRODUCTION

The supply of fossil fuels is expected to hit rock-bottom in the coming decades (U.S. Energy Information Administration 2011). Unrestrained usage of fossil fuels has led to growing concerns over global warming and extreme climate events. Hence, alternative sources of energy need to be studied. Among these alternatives, hydrogen (H_2) is regarded as a promising energy resource because of its high energy yield (122 kJ/g) (Chen 2001; Nandi and Sengupta 1998), which is almost three times greater than that of hydrocarbon fuels. In addition, H_2 can directly produce electricity through fuel cells (Mizuno *et al.* 2000).

Biogas production can be increased by utilizing abundant lignocellulose materials such as agricultural residues consisting of cellulose, hemicelluloses, and lignin (Zhang and Cai 2006). Microbes could ferment these carbohydrate to produce biofuels such as bioethanol, biogas, and hydrogen (Zhang *et al.* 2012).

China has an abundance of agricultural residues according to statistics (Li and Wang 2012), which is estimated at approximately 5.0×10^9 (dry matter) tons per year. However, bio-hydrogen production from lignocellulosic biomass has not been very successful because of the complex structure of lignocellulose. Researchers have adopted various physical and chemical approaches to preprocess lignocellulose (Huang and Ding 2008; Liu and Shi 2005). Wu *et al.* found that straw biomass can be effectively micronized by the ball milling method, which breaks down more than 95% of the cell wall (Wu *et al.* 2011). The larger the specific surface area, the greater the accessibility of the cellulose (Li *et al.* 2006, 2007; Tian *et al.* 2006).

While there have been many experiments conducted on the production of hydrogen from agricultural residues. Microorganisms that have been used for hydrogen production in such studies can employ two pathways: fermentative and photosynthetic (Meher and Das 2008). Anaerobic, acidogenic bacteria and photosynthetic bacteria are two strains typically used for biological H_2 production. Anaerobic bacteria ferment the substrate to H_2 and organic acids, but cannot further utilize the organic acids, so energy conversion is typically only about 33% (Zhang *et al.* 2013). Alternatively, photosynthetic bacteria can use small-chain organic acids as electron donors for H_2 production with light energy and have high theoretical conversion (Shi and Yu 2006, Keskin and Hallenbeck 2012).

Studies have shown efficient production of H_2 from wheat straw hydrolyzate by dark fermentation (Kongjan and Angelidaki 2010). It was found that sorghum stover can be utilized in the photo-fermentative hydrogen producing process as substrate; a high rate of materials utilization was obtained, with the produced hydrogen concentration reaching 52% (Yue *et al.* 2010a,b). However, these processes still requires further improvement for commercial exploitation.

After considering previous studies that were performed with simple crushing of biomass, then in combination with other pretreatment methods, such as acid hydrolysis and enzymatic hydrolysis (Cara *et al.* 2007), we found that agricultural residues have different molecular structure, treatment process, reducing sugar yield, and hydrogen production potential. Therefore, the raw material type and enzymatic hydrolysis process need to be considered. Since the utilization of cellulase for saccharification of cellulosic residues increases the cost of bio-hydrogen production (Azocar and Ciudad 2011; Nguyen and Saddler 1991), research needs to be conducted to improve enzymatic hydrolysis efficiency and study enzyme reuse.

Kamyar (1999) listed a brief overview of enzymatic hydrolysis of natural or purified cellulosic materials. Lu *et al*. (2002) also showed a substantial amount of work about the enzyme reuse. The ultrafiltration recycling method, the substrate re-adsorption method, and the enzyme immobilization method are the major methods of enzyme reuse (Khoshnevisan *et al.* 2011; Echavarria *et al.* 2012).

In this study, a single factor experiment was employed to optimize photo-fermentative bio-hydrogen production process. The work presented here evaluated the reducing sugar yield and cumulative hydrogen production of several agricultural residues. After selection of the type of agricultural residues, diverse parameters used for cellulase reuse via substrate replacement and enzyme immobilization methods were investigated, such as the pH value, temperature, and enzyme load.

EXPERIMENTAL

Materials

Cellulosic materials for enzymatic hydrolysis and photo-fermentation

Corn stover, corncobs, sorghum stover, soybean stalks, cotton stalks, and rice straw were collected in the autumn of 2011 from a farm in Kaifeng, Henan province, China. The raw materials were air-dried, and pretreated by ball milling machine (Tai chi Ring Nano Products Co., Ltd., Qinhuangdao), respectively. The pretreatment method was described previously (Zhang *et al.* 2012). The agricultural residue powders (ARPs) after ball milling were stored in sealed plastic bags at room temperature at a relative humidity of 43% until use. The composition of the agricultural residues was analyzed according to the Van Soest Method (Van and Robertson 1991), with the results presented in Table 1.

Microorganism and medium for photo-fermentation

The strains used for photo-fermentation hydrogen production in this study were originally screened and isolated from silt sewage, pig manure, and cow dung. 40 mL of silt sewage and 200 g of mixed pig manure and cow dung were added into a 1000 mL reagent bottle with a ground stopper, then filled with the enrichment medium that contained the following components: NH_4Cl (1 g L^{-1}), $NaHCO_3$ (2 g L^{-1}), yeast extract (1 g L^{-1}), K_2HPO_4 (0.2 g L^{-1}), CH_3COONa (3 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.2 g L^{-1}), NaCl (2 g L^{-1}), and Micronutrient solution(1 mL) that was prepared by $FeCl_3 \cdot 6H_2O$ (5mg L^{-1}), $CuSO_4 \cdot 5H_2O$ (0.05 mg L^{-1}), H_3BO_4 (l mg L^{-1}), $MnCl_2 \cdot 4H_2O$ (0.05mg L^{-1}), $ZnSO_4 \cdot 7H_2O$ (1 mg L^{-1}), $Co(NO_3)2 \cdot 6H_2O$ (0.5mg L^{-1}). The pH value was adjusted to 7.0. The selected and augmented period was conducted for 4 cycles that lasted for almost 30 d.

The phenotypic identification of strains in mixed microorganism was carried out by 16S rDNA gene sequence analysis, then the sequences of the strains were analyzed by Basic Local Alignment Search Tool (BALST) comparative analysis method according to National Center For Biotechnology Information (NCBI). Results showed that the mixed strains were *Rhodospirillum rubrum, Rhodobacter capsulatus,* and *Rhodopseudomonas palustris* (Han *et al.* 2013).

The growth medium for the mixed strains culture had the following composition (in g L^{-1}): NH_4Cl (1), $NaHCO_3$ (2), yeast extract (1), K_2HPO_4 (0.2 g), CH_3COONa (4), $MgSO_4 \cdot 7H_2O$ (0.2), and NaCl (2).

The substrate solution utilizing for photo-fermentation consisted of reducing sugar contained in the ARPs enzymatic hydrolyzates as the carbon source. The C, N, P and micro-nutrient of the fermentation medium were supplied in the following dosages (in g L^{-1}): NH_4Cl (0.4), K_2HPO_4 (0.5), $MgCl_2$ (0.2), yeast extract (0.1), NaCl (2), and sodium glutamate (3.56).

Methods

Enzymatic hydrolysis procedure of ARPs

The enzymatic hydrolysis should be carried out via hydrolysis for the conversion of the ARPs into bio-hydrogen. The ARPs were pretreated as follow: ARPs weighing 2.5 g were loaded into the 250 mL flasks containing a 100 mL buffer solution (0.05 M citric acid/sodium citrate, pH 4.8), respectively. The enzyme employed during the experiments was the Solarbio cellulase (Japan), its enzyme activity is 30 units per mg, cellulase additive amount of each flask was 100 mg dry powder (Zhang *et al.* 2013). The

flasks were placed in a thermostatic oscillating incubator (Quantum Technology Co., Ltd. Changchun, China) with a rotation speed of 150 rpm to provide better contact among substrates under a constant temperature of 50 °C, the enzymatic hydrolysis lasted for 36 h. At each time interval, reducing sugar yield was measured. All the experiments were carried out independently two times.

Experiment procedures of enzyme reuse

The stability and reusability of free cellulase has been of great concern (Chimanage *et al.* 1986; Kumakura and Kaetsu 1988). Two common methods were adopted in this work. The first one was the substrate re-adsorption method, and the other one was the enzyme immobilization method. The simplest method for enzyme recovery is re-adsorption of the free enzymes in the supernatant onto fresh substrate (Kamyar 2005). A known amount of fresh corn cob powder was added to re-adsorb the free cellulase remaining in the liquid, which was allowed to sit for 60 min, centrifuged, and filtered. The second round of the hydrolysis then continued for another 48 h, and the same experimental procedures were performed. The experiment was continued for 4 times.

The enzyme immobilization method was adopted in this work to contrast effects with the re-adsorption by fresh substrate. Sodium alginate was the carrier to immobilize cellulase. The optimum embedding conditions of sodium alginate have been determined (Ani and Wahidin 2006), and the enzymatic hydrolysis conditions were the same as the procedures mentioned above. In order to obtain higher reusability of the cellulase, experiments on optimization of cellulase re-adsorption and immobilization for reuse were conducted. The activity recovery efficiency was calculated from Eq. 1,

$$\text{The activity recovery efficiency}(\%) = \frac{Q_n}{Q_{n-1}} \times 100 \quad (1)$$

where Q_n and $Q_{(n-1)}$ are the reducing sugar yield by enzymatic hydrolysis, where n represents the number of repetitions of enzymatic hydrolysis (as 1, 2, 3, 4).

Enzyme reuse experiments by substrate replacement and enzyme immobilization methods were carried out for 4 times to contrast enzyme activity recovery efficiency.

After that, the single factor experiment was taken to evaluate the effects of re-adsorption time (30, 60, 90, 120, and 150 min) and re-adsorption temperature (5, 15, 25, and 35 °C) on the substrate re-adsorption process. In the immobilization experiment, the pH value (3.6, 4.2, 4.8, and 5.4) and the immobilized enzyme load (200, 300, 400, and 500 mg) were investigated.

All experiments were conducted twice.

Experimental procedures for photo-fermentation hydrogen production

The batch experiments were performed in 250 mL flasks as batch reactors filled with 100 mL mixture comprising the substrate solution, 20% (v/v) mixed photosynthetic bacteria in exponential growth period. The initial pH values of the mixture were adjusted to neutral by 50% (mass fraction) KOH solution. The reactors were filled with argon (Ar) gas to remove oxygen to create the anaerobic environment, and then capped with rubber stopper. The photo-fermentation bioreactors were placed at a constant temperature incubator under temperature (30±1 °C), the light (3000 Lux) was provided by filament lamps.

At each 12-h time interval, the reducing sugar yield and cumulative hydrogen production were measured, and the experiments lasted for 4 days. All the experiments were conducted twice.

Analytical methods

The biogas volume was measured by water displacement using graduated cylinders filled with water and partially submerged in a tub of water. The volume and concentration of hydrogen were determined by gas chromatography and the hydrogen standard curve of the regression equation as follows: $y=0.0012x+0.9388$, $r^2=0.9991$, in which *x* is peak area of hydrogen and *y* is the relative hydrogen concentration.

The reducing sugar concentration of samples (5 mL) that were taken from the fermentation solution were determined by the di-nitro salicylic (DNS) colorimetric method (Miller 1959) by a HP8453 ultraviolet spectrophotometer (Agilent, USA), and reducing sugar yield was calculated according to the glucose standard curve, using the regression equation as follows: $y=0.3580x-0.0164$, $r^2=0.9990$, in which *x* is glucose concentration (mg per 0.5 mL solution) and *y* is optical density under 540 nm (OD_{540}).

Values for pH were measured by a pH meter (PHS-3C, Shanghai, China).

RESULTS AND DISCUSSION

Milling Pretreated Agricultural Residue Composition

The cellulose and hemicellulose contents of the pretreated agricultural residues were measured and shown below (Table 1). Corncob had the highest content of cellulose of 44 ± 0.5%, which was nearly twice that of the soybean stalk and cotton stalk.

Table 1. Composition of the Pretreated Agricultural Residues

Type	Moisture content, %	Cellulose, %	Hemicellulose, %
Corn stover	4.0 ± 0.3	33 ± 0.8	28 ± 0.4
Corncob	3.6 ± 0.4	44 ± 0.5	26 ± 0.7
Sorghum stover	4.2 ± 0.2	35 ± 0.6	26 ± 0.6
Soybean stalk	2.9 ± 0.3	25 ± 0.5	14 ± 0.6
Cotton stalk	3.1 ± 0.2	24 ± 0.6	25 ± 0.5
Rice straw	3.0 ± 0.4	37 ± 0.6	24 ± 0.4

Efficiency of Enzymatic Hydrolysis and Hydrogen Production Capacity of Pretreated Agricultural Residues

Different types of pretreated agricultural residues were utilized (Fig. 1). Although the lag phase of hydrogen production in this study with ARH lasted about 18 h, the cumulative H_2 production was still considerable. The maximum cumulative H_2 yield of 228.94 mmol per liter culture and reducing sugar of 12.64 mg per milliliter enzymatic hydrolysate were obtained with corncob utilization, which was almost 200% higher than other agricultural residues. The results verified that cellulosic plant materials represent a potential source of fermentable sugars (Nathan *et al.* 2005). The order of the enhancement for the reducing sugar yield (Fig. 1) and cumulative hydrogen production versus time (h) (Fig. 2) were as follows: corncob (12.64 mg mL^{-1} enzymatic hydrolysate, 228.94 mmol L^{-1}) > sorghum stover (8.44 mg mL^{-1} enzymatic hydrolysate, 149.93 mmol L^{-1}) > corn stover (8.28 mg mL^{-1} enzymatic hydrolysate, 145.67 mmol L^{-1}) > rice straw

(7.90 mg mL^{-1} enzymatic hydrolysate, 140.45 mmol L^{-1}) > soybean stalk (7.76 mg mL^{-1} enzymatic hydrolysate, 131.12 mmol L^{-1}) > cotton stalk (6.96 mg mL^{-1} enzymatic hydrolysate, 118.46 mmol L^{-1}). The reducing sugar yield and cumulative hydrogen production had the same range. When the reducing sugar yield was calculated based on per gram ARP, the results were 505.6 mg g^{-1} corncob, 337.6 mg g^{-1} sorghum stover, 313.2 mg g^{-1} corn stover, 316.0 mg g^{-1} rice straw, and 278.4 mg g^{-1} cotton stalk. Combined with the composition of the pretreated agricultural residues, it is clear that the total fraction of cellulose and hemicellulose may be the primary cause responsible for the higher efficiency of enzymatic hydrolysis by cellulase, the higher proportion of cellulose and hemicellulose, and the higher reducing sugar yield. What is more, the corncob possesses a loose structure and has nutrients that are beneficial for photosynthetic bacterial growth, such as elemental P 0.63 wt.% and elemental K 0.45 wt.%. The reducing sugar that was produced from the pretreated agricultural residues by cellulase was available in bio-hydrogen production using photosynthetic bacteria group, the cumulative hydrogen production per liter culture versus time (h) was illustrated in Fig. 2 for different types of ARP, respectively. To determine the effect of different ARP on the bio-hydrogen production potential, a modified Gompertz model (Zwietering *et al.* 1990), which is frequently-used to estimate hydrogen productivity (Sevinc *et al.* 2012; Keskin and Hallenbeck 2012) was employed.

The cumulative H_2 production data from each batch experiment fit to modified Gompertz Model using CurveExpert Professional 2.0.3 for Windows. The equation for the Modified Gompertz Model is shown in Eq. 2,

$$H = H_{max} exp\left\{-exp\left[\frac{R_{max}e}{H_{max}}(\lambda - t) + 1\right]\right\} \quad (2)$$

where H is the hydrogen accumulated per liter culture (mmol L^{-1}) at time t (h), H_{max} is the maximum cumulative hydrogen per liter culture (mmol L^{-1}), R_{max} is the maximum hydrogen production rate (mmol L^{-1} h^{-1}), λ is the lag time (h), and e is 2.718281828. The model parameters at different types of ARP are listed in Table 2. The correlation coefficients, r, were all close to 1, which meant that the Modified Gompertz Model was fit for the data, and well defined the photo-fermentative bio-hydrogen production with mixed microflora at using different types of ARP.

Table 2. Modified Gompertz Model Parameters at Different Types of ARP

Type	r	H_{max}, mmol L^{-1}	R_{max}, mmol L^{-1} h^{-1}	λ, h
Corncob	0.9988	230.0991	5.9677	17.67
Corn stover	0.9983	145.749	3.9463	17.59
Sorghum stover	0.9982	150.3886	4.0719	17.17
Rice straw	0.9984	140.2588	3.7564	18.25
Soybean stalk	0.9982	130.6472	3.3115	18.12
Cotton stalk	0.9970	119.285	2.7383	18.32

The maximum cumulative hydrogen production H_{max} and hydrogen production rate R_{max} were obtained when using corncob hydrolyzate as substrate. The highest value was 230.0991 mmol L^{-1}, which was at least 50% higher than other ARPs under the same condition. Although when using corncob hydrolyzate as substrate for photo-fermentation,

the lag time, λ, was not the shortest one, but there had no big differences. For obvious reasons, the corncob was utilized in further experiments.

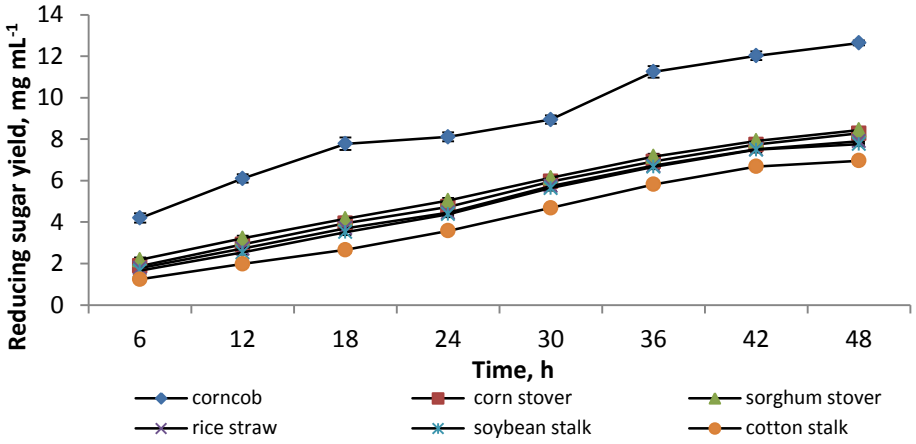

Fig. 1. Reducing sugar yield per mL enzymatic hydrolyzate of pretreated agricultural residues

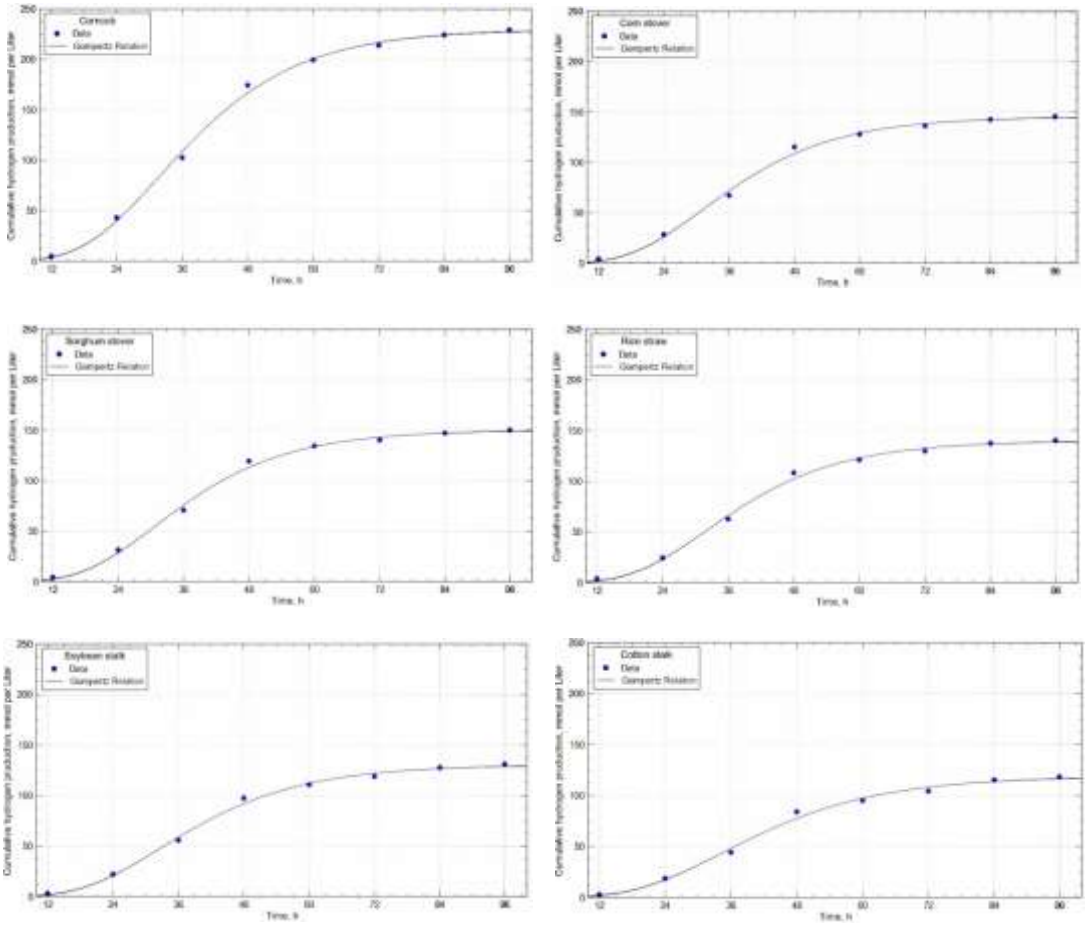

Fig. 2. Cumulative H_2 yield per liter culture versus time of pretreated agricultural residues hydrolyzate

Enzyme Reuse by Substrate Re-adsorption and Enzyme Immobilization Methods

Enzymatic hydrolysis was obtained under optimum conditions, and then enzyme reuse experiments were conducted by substrate re-adsorption and enzyme immobilization methods. The results are shown in Table 3.

Table 3. Results of Enzyme Reuse Experiments

Round	Reducing sugar yield, mg mL^{-1}		Activity recovery efficiency, %	
	Substrate re-adsorption method	Enzyme immobilization method	Substrate re-adsorption method	Enzyme immobilization method
1	12.43	12.26	85.2	91.4
2	10.56	11.37	84.9	92.7
3	8.77	10.14	83.1	89.2
4	7.01	8.91	80.0	87.9

The study showed that enzyme reuse *via* substrate re-adsorption and enzyme immobilization methods were feasible and efficient. After four rounds, the activity recovery efficiency by enzyme immobilization method (87.9%) was higher than by the substrate re-adsorption method (80.0%), while the reducing sugar yield of first round *via* the substrate re-adsorption method (12.43 mg mL^{-1}) was higher than by the enzyme immobilization method (12.26 mg mL^{-1}). Hence, to have the maximum reducing sugar yield and high activity recovery efficiency, enzyme deactivation and the loss in the process of centrifugal separation should be considered, along with the enzyme load and re-adsorption time and temperature. Further studies were carried out on the optimization of the enzyme reuse process.

After 36 h, the initial round of enzymatic hydrolysis was terminated and the initial substrates were filtered from the buffer solution. Then, 25 mg mL^{-1} fresh substrate was added to the enzyme hydrolysis solution for re-adsorption. Experiments proceeded under different temperatures (5, 15, 25, and 35 °C) and different time (30, 60, 90, 120, and 150 min). The substrate used for re-adsorption was then added to 100 mL fresh buffer solution to run the first round of recycling for another 36 h. At regular time interval (30 min), 5- mL samples were taken from the vessel to determine the reducing sugar yield and calculate the activity recovery efficiency (Fig. 3).

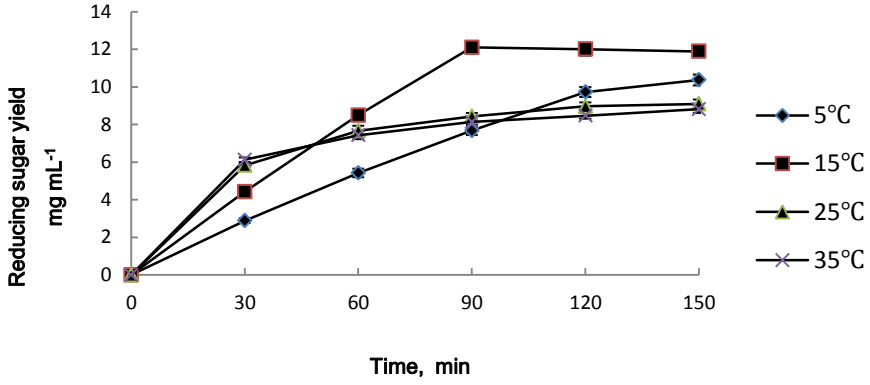

Fig. 3. Reducing sugar yield per mL enzymatic hydrolysate of substrate adsorption at different temperatures and different time (adsorption time intervals of 30 min)

By comparing the reducing sugar yield, the effect of adsorption time and temperature on the reducing sugar yield and activity recovery efficiency was examined (Fig. 3). The effect of different adsorption time was observed from the x-axis of Fig. 3. All the curves contained a point at which the growth rate of reducing sugar yield slowed down, or even presented a slight decline. The reason may be that the enzymatic hydrolysis reaction occurred when the re-adsorption proceeded, and substrate was consumed more with increasing time. It was judged that 15 °C was the optimal adsorption temperature providing the highest reducing sugar yield at 90 min (12.10 mg mL^{-1} enzymatic hydrolysate, activity recovery efficiency was 82.9%). At 5 and 15 °C, the substrate continued the adsorption process, so the reducing sugar yield increased with time; however, after 90 min of enzymatic hydrolysis, the reducing sugar yield decreased. The same situation did not occur at 5 °C because of the lower temperature, which inhibited enzymatic hydrolysis. So, even at higher temperatures (25, 35 °C), because the enzymatic hydrolysis occurred during substrate adsorption, the reducing sugar yield was lower than that at lower temperature. With the activity of free enzyme, the optimal condition for the substrate re-adsorption method was adsorption time of 90 min at 15 °C, which is similar to the results of other studies (Li *et al.* 2010).

The immobilizing of cellulase has advantages because it causes a decrease of the free enzyme loss. Single factor experiments were performed to observe the effect of the pH values and amount of cellulase on the reducing sugar yield and activity recovery efficiency (Fig. 4). The immobilized enzyme method showed an optimal pH of 4.8 when the reducing sugar yield was 11.92 mg mL^{-1} enzymatic hydrolysate, and activity recovery efficiency was 88.9%. On the other hand, pH values lower or higher than 4.8 were all detrimental to the enzymatic hydrolysis process.

Four different parameters were employed to find the suitable enzyme powder load and comparable reducing sugar yield. A slight increase of reducing sugar yield was observed with increasing immobilized enzyme load (from 11.47 mg mL^{-1} enzymatic hydrolysate to 12.31 mg mL^{-1} enzymatic hydrolysate), but no obvious growth was observed when the immobilized enzyme load reached to 400 mg. The reducing sugar yield of 400 mg immobilized enzyme load (12.24 mg mL^{-1} enzymatic hydrolysate) was almost the same as the 500 mg immobilized enzyme load (12.31 mg mL^{-1} enzymatic hydrolysate). This is most likely attributed to the fact that the active site plays a very crucial role of enzyme activity. When the enzyme load is low, the cellulase can effectively contact with cellulose activity sites and implement effective digestion. With more than 400 mg immobilized enzyme load, the addition of more enzyme did not enhance efficiency due to the inhibition of active site and inactivation because cellulase is adsorbed by non-degradable substrate.

A slight increase of reducing sugar yield occurred when more than 400 mg of immobilized enzyme load was added because the enzymatic hydrolysis reaction reached a certain critical point; the production of materials such as reducing sugar inhibits the activity of cellulase. Through the single factor experiments, the optimal condition was pH 4.8 and immobilized enzyme load of 400 mg (activity recovery efficiency was 82.9%).

The results showed that enzyme load and pH value are an important factors that should be considered for bio-hydrogen production by photo-fermentation. Through single factor design experiments, the impact of different factors on reducing sugar yield was obtained.

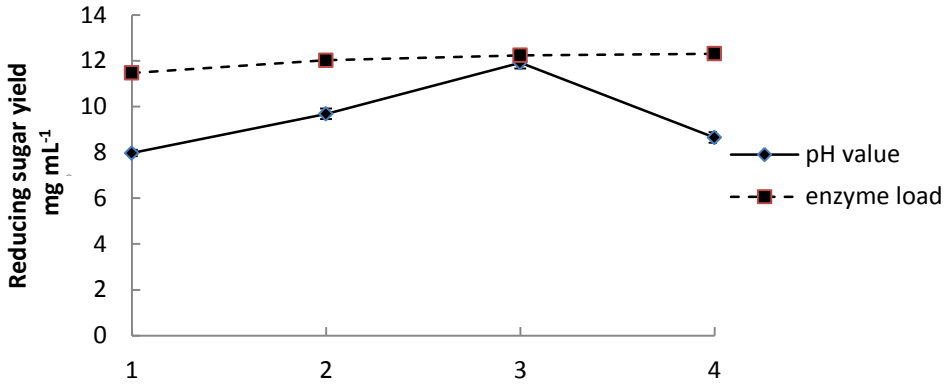

Fig. 4. Reducing sugar yield per mL enzymatic hydrolysate of immobilized enzyme at different enzyme loads and pH values (1, 2, 3, 4 represent four levels for each factor, such as pH value of 3.6, 4.2, 4.8, 5.4 and enzyme load of 20, 300, 400, 500 mg, respectively)

CONCLUSIONS

1. Different types of agricultural residues were utilized for photo-fermentative bio-hydrogen production. A modified Gompertz model was employed to describe the cumulative hydrogen production data, and a good fit to the data was achieved. Corncob had the highest reducing sugar yield and cumulative hydrogen production (12.64 mg mL^{-1} enzymatic hydrolysate, 228.94 mmol L^{-1} culture) and maximum hydrogen production rate (5.9677 mmol L^{-1} h^{-1}), such that corncob was determined to be the optimum type agricultural residue for bio-hydrogen producing.
2. The substrate re-adsorption and enzyme immobilization methods were effective for enzyme reuse and achieved efficient recycling of 4 rounds. The optimal enzyme reuse condition by the substrate re-adsorption method was at re-adsorption temperature of 15 °C and re-adsorption time of 90 min, and the optimal condition for the enzyme immobilization method was a pH value of 4.8 and immobilized enzyme load of 400 mg.

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