Promotional Effects of Water-soluble Extractives on Bamboo Cellulose Enzymolysis

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The release of fermentable monosaccharides from cellulose is a key step for the economical and efficient production of ethanol from lignocellulosic biomass. However, some residual substances in pretreated biomass negatively affect enzymolysis by reducing the activity of the enzyme due to the nonproductive and competitive binding of enzymes. To improve enzyme efficiency, heterologous proteins have been introduced as an additive for cellulase during the hydrolysis process. In this study, the enzymatic hydrolysis of cellulose from pretreated bamboo was enhanced by adding an aqueous extract of the bamboo to the hydrolysis system. The cellulose to glucose conversion yield (CGCY) increased to different extents when different substrates were used with different enzyme loadings. The promotional effect of bamboo extractives on enzymatic hydrolysis of different bamboo varieties was observed. In conclusion, the deactivation of the cellulolytic enzyme by negative binding to residual lignin in substrate was reduced due to the competitive effects of proteins in the extract. In addition, other effects, such as easy accessibility of the substrate (amorphogenesis), were also possible reasons. Overall, the promotional effect of bamboo aqueous extractives played an important role in the enzymatic hydrolysis of pretreated bamboo.

Keywords: Bamboo; Cellulose; Cellulase; Aqueous extract; Pretreatment; Protein

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INTRODUCTION

Lignocellulosic biomass is the most abundant resource on the earth. It can be transformed into various chemicals or biofuels, which can effectively reduce the overwhelming depletion of petrochemical resources (Himmel *et al*. 2007). The efficient and economical conversion of lignocellulose into soluble sugars is a major challenge for lignocellulosic biorefinery (Zacchi and Axelsson 1989; Brodeur *et al*. 2011; Huang and Fu 2013). Biomass is mainly composed of three natural molecules: lignin, hemicellulose, and cellulose, which are tightly bonded by physical and chemical interactions. The complex structure of biomass materials blocks cellulase from accessing cellulose and makes it resistant to enzymatic hydrolysis. Various pretreatment methods are used on lignocellulosic biomass to enhance the accessibility of cellulose-degrading enzymes (Sousa *et al*. 2009; Qiu and Aita 2013; Guo *et al*. 2018).

The removal of lignin improves the enzymatic hydrolysis of lignocellulose (Soares and Gouveia 2013; Wang *et al*. 2013; Zeng *et al*. 2014). Currently, it is difficult to thoroughly remove lignin from biomass feedstocks. There is always some lignin that remains in pretreated substrates, which is usually called residual lignin (Yang and Pan 2015). The residual lignin in pretreated lignocellulose can negatively affect the hydrolysis

of cellulose by reducing the enzyme activity due to the irreversible binding of cellulase to the residual lignin (Ximenes *et al*. 2011). Several possible measures have been reported for blocking this nonproductive adsorption, including the addition of metal ions, exogenous proteins such as bovine serum albumin (BSA), or proteins extracted from biomass, as well as surfactants such as Tween 20, Tween 80, and polyethylene glycol (PEG) (Yang and Wyman 2006; Kumar and Wyman 2009; Han and Chen 2010; Tu and Saddler 2010; Brethauer *et al*. 2011; Zhang *et al*. 2011; Akimkulova *et al*. 2016). Proteins extracted from biomass were reported to minimize enzyme deactivation and loosen the highly compact zones of the cellulose (Han and Chen 2007; Arantes and Saddler 2010; Smit and Huijgen 2017). For example, proteins isolated from fresh corn stover and wheat straw enhance the hydrolysis rate of cellulose and the glucose yield (Han and Chen 2007; Smit and Huijgen 2017). Some other proteins extracted from biomass can synergize cellulase during cellulose hydrolysis (Lu *et al*. 2006).

Bamboo is becoming a promising feedstock for biofuel and chemical production due to its fast growing speed and high cellulose content. In this study, unpurified bamboo extract was employed as a direct additive into the enzymatic hydrolysis system of pretreated bamboo (Fig. 1). The effects of bamboo extract on enzymolysis were examined. This study mainly used moso bamboo (*Phyllostachys heterocycla*) as the research object and other bamboo varieties were also considered.

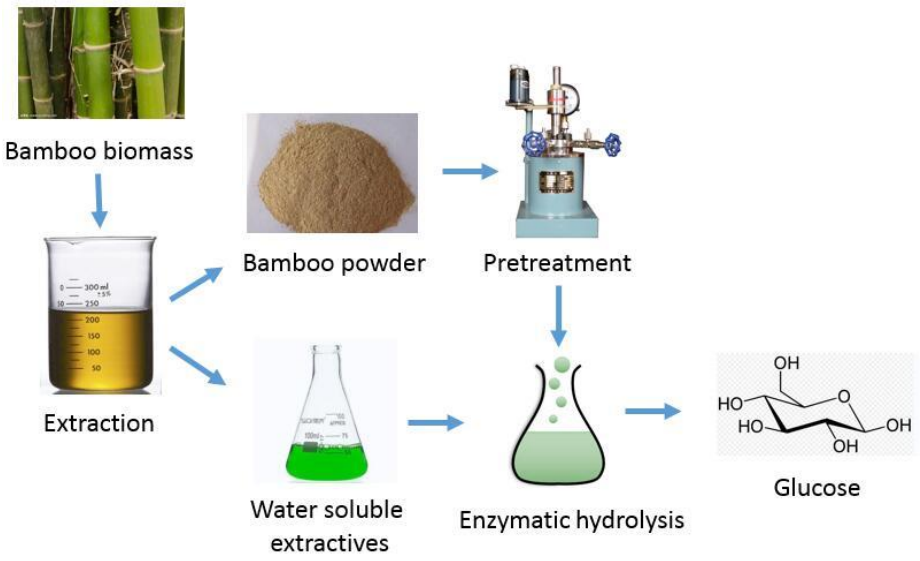

Fig. 1. Sketch map of bamboo hydrolysis with water soluble extractives

EXPERIMENTAL

Materials

Moso bamboo (*Phyllostachys heterocycla*, PH bamboo), *Phyllostachys pubescens* (PP bamboo), and *Neosinocalamus affinis* (NCA bamboo) were harvested from the bamboo germplasm resources nursery of the Southwest University of Science and Technology in Mianyang, Sichuan Province, China. After hot-air drying, the bamboo culms (2 years old) were milled with a hammer mill (screen opening size: 2.0 mm). The

moisture content of the bamboo culms was measured in an oven at 103 ± 2 ℃ for 24 h.

Extraction Method of Bamboo

The water-soluble extractives removal was slightly modified from the reported procedure (Smit and Huijgen 2017). Bamboo (1.0 kg) (moisture content 11%) and 2 kg of hot demineralized water (approximately 50 ℃) were mixed in a glass beaker. The sample was then heated in an oven at 50 ℃ for 120 min. The extracting solution was first purified with gauze and then with a filter (equipped with 0.1 mm filter element). Next, the solution was concentrated with a cross flow ultra-filtration system (Sartorius, 1 kD). The concentrated solution was preserved with 0.02% NaN_3 (sodium azide) and stored at 4 ℃.

Pretreatment

The pretreatment conditions are shown in Table 1. Organosolv (OS) pretreatment was performed with the bamboo extraction residue in an autoclave reactor (10 L) using conditions based on a study by Li *et al*. (2012). A mixture of extracted bamboo, 75% aqueous ethanol, and 2% sulfuric acid (solid-to-liquid ratio: 1 kg dry weight extracted bamboo/10 L mixed liquid) was heated to 180 °C and maintained for 60 min. After cooling, the slurry was separated by filtration through a Whatman GF/D filter paper, and the solid substrates were first washed with 75% aqueous ethanol and then washed with hot water (approximately 60 °C). The subsample was dried at 50 °C to determine its solid recovery and its composition. The solid substrate then was stored at 4 °C for subsequent study.

Pretreatment with dilute acid (DA) and alkaline (AL) solution was performed on the extracted bamboo (Leenakul and Tippayawong 2010; Li *et al*. 2016). During this step, the extracted bamboo was pretreated in an autoclave at a pressure of 1.1 bar, a temperature of 140 °C with a residence time of 90 min, and a sulfuric acid concentration of 1.2% (temperature of 121 °C with a residence time of 60 min and a NaOH concentration of 1%). Solid and liquid fractions were separated by filtration, solid residues were washed with water and stored in a refrigerator (4 ℃). The subsamples were dried at 50 ℃ to determine the solid recovery and composition.

Table 1. Applied Pretreatment Methods

Substrate code	Pretreatment	*T* (°C)	*t* (min)	Solvent	Catalyst
OS*	Organosolv	180	60	75% Aqueous Ethanol	2% sulfuric acid
DA*	Dilute acid	140	90	Water	1.2% sulfuric acid
AL*	Alkaline	121	60	Water	1% NaOH
* Organosolv (OS), dilute acid (DA), and alkaline (AL)					

Composition of Bamboo and Extract Analysis

The composition of the bamboo before and after its pretreatment was analyzed using the National Energy Laboratory (NREL) analytical procedure (Sluiter *et al*. 2008). The protein content of the unpretreated bamboo was determined by using the Kjeldahl method (N×6.25) and the protein concentrations of the bamboo extract were determined by using the BCA protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The sugar composition of the extract were determined according to the method presented by (Ostovareh *et al*. 2015).

Hydrolysis

Enzymatic hydrolysis was performed in 150 mL flasks at 50 °C on a rotary shaker for 48 h with the agitation speed at 140 rpm. Based on the cellulose content in the substrate, bamboo samples equivalent to 1.0 g glucan (cellulose) (the amount of bamboo sample used for every hydrolysis experiment was calculated according to the cellulose content showed in Table 2) was loaded with 50 mL of a sodium citrate buffer (0.05 M, pH 4.8). Cellulase and β-glucosidase were used for the enzymatic hydrolysis experiments. The samples were mixed with 5 to 15 FPU cellulase and 10 to 30 FPU β-glucosidase, respectively, per gram of glucan in the substrate and were taken for colorimetric determination of the glucose concentration after 3 h, 6 h, 12 h, 24 h, and 48 h. Different from the control group, the bamboo extract was added to the reaction system, replacing the demineralized water, and the amount of the extract was set at the maximum according to the experiment.

RESULTS AND DISCUSSION

Biomass Pretreatment

The composition of the different bamboos before and after pretreatment are shown in Table 2. The protein content of the moso bamboo was 4.1% as determined by the Kjeldahl method (N×6.25). From the total amount of proteins present in the moso bamboo, 30.7% was dissolved resulting in a protein concentration of 3.6 g/L in the bamboo extract (Table 3.). The untreated moso bamboo contained 37.8% cellulose, 15.9% xylan, and 25.6% lignin. Most of the lignin and hemicellulose of the moso bamboo were solubilized by using the organosolv pretreatment method. The solid residue after the separation was rich in cellulose and had an enhanced accessibility to hydrolysis enzymes.

Table 2. Composition of Moso Bamboo Before and After Pretreatment

Pretreatment	Compositions of bamboo (%, dry weight)				Solid recovery[a] (%)	Lignin removal[b] (%)
	Cellulose	Xylan	Klason lignin	Ash		
Moso bamboo	37.8 ± 1.9	15.9 ± 1.2	25.6 ± 0.8	1.5 ± 0.3	100	0
PP bamboo	32.4 ± 2.3	18.3 ± 0.8	32.3 ± 2.1	1.2 ± 0.3	100	0
NCA bamboo	41.3 ± 2.2	22.7 ± 1.5	21.8 ± 1.6	1.3 ± 0.3	100	0
OS[f]	55.6 ± 1.9	6.2 ± 0.4	16.3 ± 1.3	1.4 ± 0.5	69.2	33.4
DA[f]	49.1 ± 1.3	8.8 ± 0.7	37.1 ± 2.9	1.3 ± 0.1	63.8	9.8
AL[f]	53.1 ± 1.7	20.9 ± 1.1	14.1 ± 1.6	1.4 ± 0.4	66.2	56.1

[a] Solid recovery (%) = WP/WI × 100 (WI is the initial weight of bamboo solid, WP is the weight of bamboo solids after pretreatment)

[b] Lignin removal rate (%) = (WIL− WPL)/WIL × 100 where WIL is the initial weight of lignin (g per 100 g raw bamboo material), and WPL is the weight of lignin in different pretreated bamboo solids (g per 100 g raw bamboo material)

[f] Calculated with moso bamboo

Approximately 63.8% to 69.2% of solids were recovered from the moso bamboo after different pretreatments. As Table 2 shows, the pretreatment with dilute acid effectively removed the xylan. With DA, the xylan content was 8.8% and the cellulose content increased to 49.1%. The removal of xylan in turn increased the Klason lignin content of the moso bamboo from 25.6% to 37.1%. Lignin does not dissolve in the acid solution, but its structures and characteristics can be altered by depolymerization, condensation, and relocation (Trajano *et al*. 2013). In addition, dilute acid can solubilize hemicellulose in different biomass materials such as rice straw, bamboo, olive trees, *etc.*, (Leenakul and Tippayawong 2010; Yan *et al*. 2017; Martínez-Patiño *et al*. 2018).

Table 3. Protein and Soluble Sugar Content of Moso Bamboo Extract (g/L)

Protein	Glucose	Fructose	Sucrose	Arabinose	Xylose
3.6	0.04	0.03	0.01	0.03	0.008

The xylan and cellulose contents of moso bamboo increased from 15.9% to 20.9% and from 37.8% to 53.1% after alkaline pretreatments, respectively. Most of the lignin that was solubilized in the alkaline solution resulted in a delignification of 56.1% and an increase in the xylan and cellulose contents.

Hydrolysis

Organosolv pretreated moso bamboo substrates

The enzymatic hydrolysis results for the organosolv-pretreated moso bamboo substrates are shown in Fig. 2(a). Using an enzyme loading of 15 FPU/g cellulase and 30 IU/g β-glucosidase, most of the cellulose was converted to glucose within 48 h, which suggested that the enzyme activity was sufficient. At lower enzyme loadings, the enzyme activity was not enough to ensure that all the bamboo cellulose converted to glucose within 48 h.

The bamboo extract increased the hydrolysis rate during this study. The cellulose to glucose conversion yield (CGCY) was increased by 2.3% after 48 h with enzyme loadings of 15 FPU/g cellulase and 30 IU/g β-glucosidase when the extract was added. Similarly, when the enzyme loadings were 10 FPU/g cellulase + 20 IU/g β-glucosidase, 5 FPU/g cellulase + 10 IU/g β-glucosidase, extract addition resulted in an 11.1% and 25.6% relative increase in CGCY, respectively. As discussed earlier, the nonproductive adsorption of cellulase onto residual lignin in the pretreated bamboo substrates led to a reduction of the enzyme activity. The proteins or other substances in the bamboo extract could ease the deactivation of enzymes by the competition mechanism.

In addition, the promotional effect of the bamboo extract was found when microcrystalline cellulose was used as the substrate for enzymatic hydrolysis (Fig. 2(b)). Using an enzyme dose of 15 FPU/g + 30 IU/g, the CGCY increased from 70.1% to 77.3% when the extract was added. The CGCY increased from 46.6% to 58.2% and 32.3% to 48.5% when enzyme doses were 10 FPU/g + 20 IU/g and 5 FPU/g + 10 IU/g, respectively. In summary, the decrease in the cellulase inactivation and increase in the substrate accessibility by amorphogenesis can be important, as reported by Arantes and Saddler (2010).

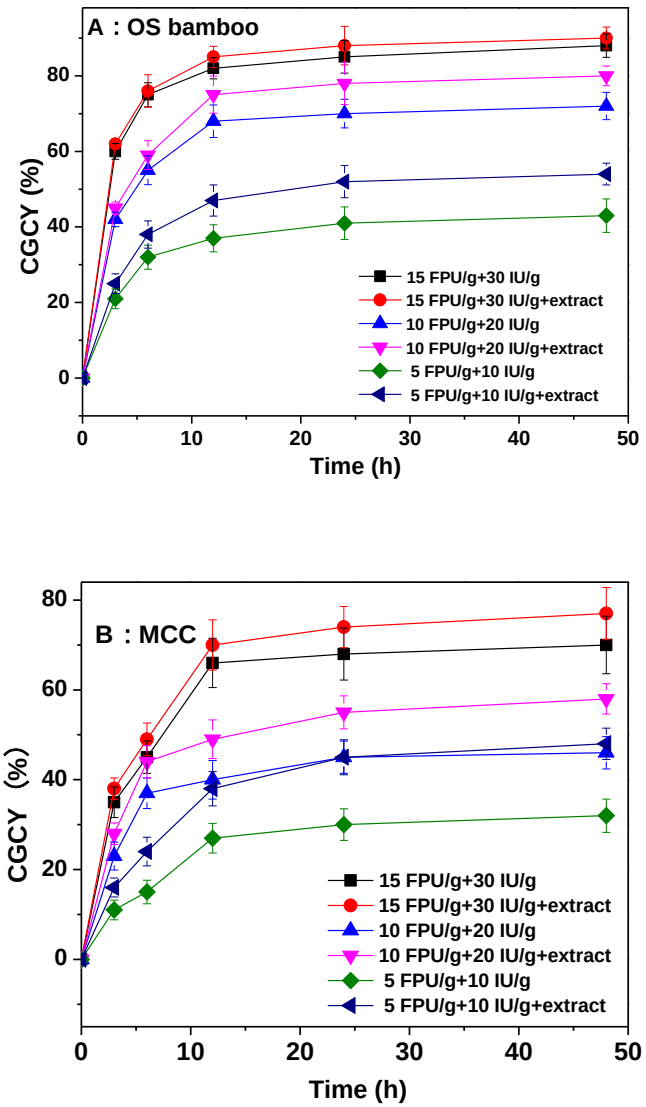

Fig. 2. Effect of bamboo extract on enzymatic hydrolysis of A (organosolv pretreatment moso bamboo) and B (microcrystalline cellulose (MCC)) at different enzyme loadings

To confirm that no cellulase was present in the bamboo extract, a group of control experiments were performed. As shown in Table 4, no product was detected from the reaction system when no enzyme was added (entry 1). Using an enzyme loading of 5 FPU/g + 10 IU/g, the added extract increased the CGCY of the moso bamboo pretreated with organosolv from 43.3% to 54.7% (entry 2 and 4). These experiments showed that the bamboo extract had no cellulolytic activity (entry 3).

Another experiment was performed to determine the main reason for the positive effect of the bamboo extract. The addition of the bamboo extract, which was filtered with an ultrafiltration membrane (1 kD), resulted in a negligible increase of CGCY at an enzyme loading of 5 FPU/g + 10 IU/g (entry 5). Therefore, the main substance in the extract that improved the enzymatic hydrolysis was a biological macromolecule, possibly a protein.

Table 4. Control Experiment

Entry	OS bamboo	Enzyme[a]	Extract	CGCY (%)
1	yes	no	no	0
2	yes	yes	no	43.3
3	yes	no	yes	0.1
4	yes	yes	yes	54.7
5	yes	yes	yes[b]	44.1
[a] Enzyme loading was 5 FPU/g + 10 IU/g				
[b] Extract was filtered through a 1 kD ultrafiltration membrane				

To compare the promotional effect of the bamboo extract with the foreign protein bovine serum albumin (BSA), another control experiment was performed. As previously mentioned, the protein concentration of the bamboo extract was 3.6 g/L. The bamboo extract was then concentrated with an ultrafiltration membrane (1 kD) and added to the reaction system. The additional dose was expressed with mg of protein added per 1 g of the substrate. For comparison, the foreign protein BSA was added to the system.

As shown in Fig. 3, the addition of the bamboo extract increased the glucose yield. The relative yield increase reached 26.0% when the protein contents were 120 mg/g substrate. With the addition of the same amount of BSA, the maximum conversion increase was only 15.9% and 16.0%, respectively. When comparing the results of the two groups, the bamboo extract exhibited a better performance than the BSA on the enzymatic hydrolysis of the organosolv-pretreated moso bamboo. As described by Smit and Huijgen (2017), the differences in the protein compositions between the extract and the BSA can be a major reason for the different tightness of adsorption to lignin or the different extents of the amorphogenetic effect on the substrate.

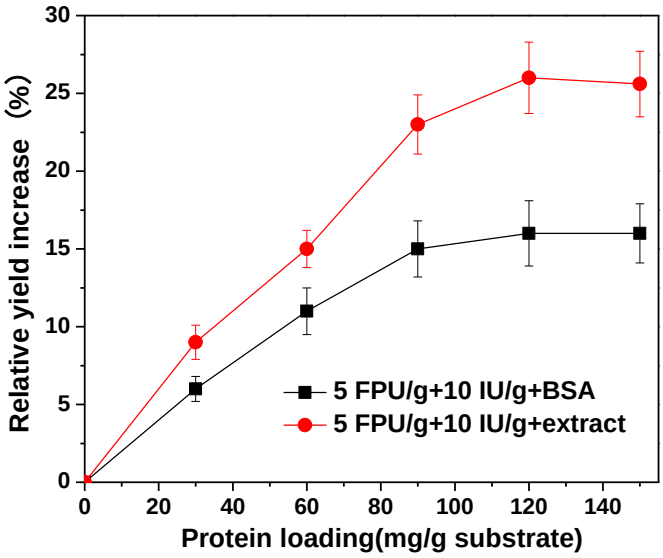

Fig. 3. Effect of bamboo extract and BSA addition on enzymatic hydrolysis after 48 h with enzyme loading of 5 FPU/g + 10 IU/g

Bamboo substrates from other pretreatment methods

As shown in Fig. 4, the bamboo extract additionally exhibited a promotional effect for enzymatic hydrolysis of moso bamboo with other pretreatment methods (dilute acid and alkaline). When using an enzyme loading of 5 FPU/g + 10 IU/g, the CGCY of DA, AL, and OS pretreated bamboo was 21.6%, 35.2%, and 43.3%, respectively. With the addition of the bamboo extract, the CGCY of the enzymatic hydrolysis was 24.1% (DA, increased 11.6%), 39.2% (AL, increased 11.4%), and 54.7% (OS, increased 26.3%), respectively. As the results showed, OS pretreated bamboo has the highest CGCY among the three substrates. For DA pretreated bamboo, the lowest CGCY attribute to its higher lignin content (Table 2) because cellulase tends to bind on the lignin-rich surfaces. Both AL and OS pretreated bamboo have low lignin and high cellulose content. According to (Gunawan *et al*. 2017), high content of xylan (Table 2) in AL pretreated bamboo was an important factor for relative low cellulase activity. The promotional effect of the extract not only directly correlated to the amount of lignin present in the substrate, but also depend on characteristics of residual lignin and affinity for cellulase adsorption (Meng *et al*. 2016).

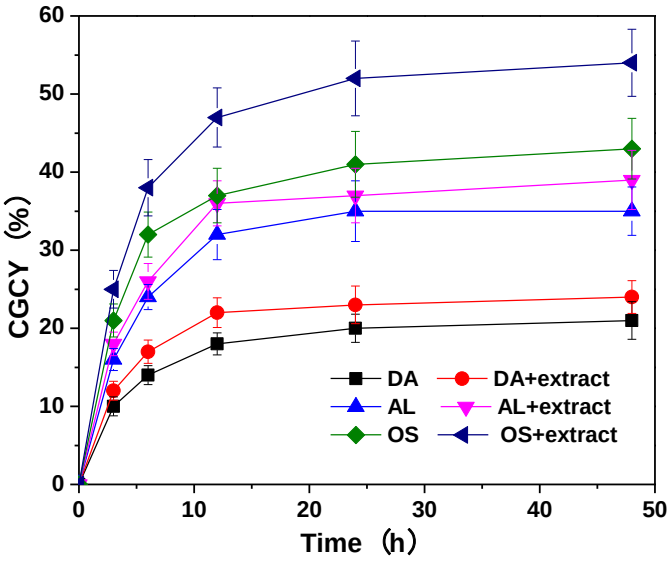

Fig. 4. Effect of additional bamboo extract on the enzymatic hydrolysis of bamboos with different pretreatment methods after 48 h with enzyme loading of 5 FPU/g + 10 IU/g

Substrates from other bamboo varieties

To confirm whether proteins in the bamboo extract played the same role in the promotional effect on enzymatic hydrolysis, two other common bamboo varieties from the Sichuan Province were used (*Phyllostachys pubescens* and *Neosinocalamus affinis*; different in cellulose and lignin contents: see Table 2) as substrates. As shown in Fig. 5, the addition of extracts from different bamboo varieties to the enzymatic hydrolysis system with organosolv-pretreated substrate increased the CGCY. The PH bamboo increased from 43.3% to 51.1%, the PP bamboo increased from 37.2% to 45.7%, and the NCA bamboo increased from 46.2% to 56.8%. The promotional effect on bamboo cellulose enzymolysis can be observed not be limited to PH bamboo, but also was evident for other bamboo varieties.

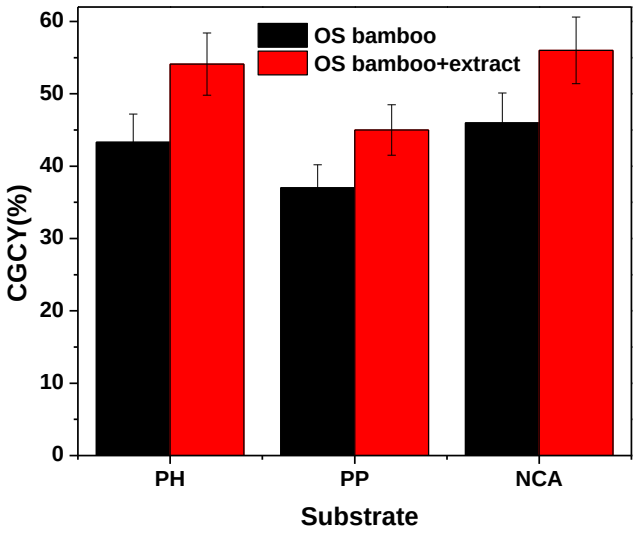

Fig. 5. Effect of the extract on the enzymatic hydrolysis of different organosolv-pretreated bamboo with an enzyme loading of 5 FPU/g + 10 IU/g after 48 h. The PH, PP, and NCA are different bamboo varieties (see Table 2)

CONCLUSIONS

The structure of lignocellulose is very complex, and many proteins exist in the cell wall of bamboo and other plants. The present research found that water-soluble components from crude bamboo extracts (without purification) had a clear promotional effect on the enzymatic hydrolysis of different bamboo substrates with different pretreatment methods (dilute acid, alkaline, and organosolv). The promotional effect originated from bamboo extractives larger than 1 kD, most likely proteins. The proteins in the extract could ease the deactivation of enzymes by the competition mechanism. In addition, these inaccessible regions of cellulose are disrupted or loosened by proteins in the extract, thereby increasing the cellulose surface area and making it more accessible to the cellulase enzyme complex. In summary, the promotional effects of bamboo aqueous extractives played an important role in the enzymatic hydrolysis of pretreated bamboo. Thus, the required enzyme dose was reduced, resulting in lower production costs for fuels and chemicals from bamboo.

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