1 **Effect of non-enzymatic proteins on enzymatic hydrolysis and simultaneous**
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4 **saccharification and fermentation of different lignocellulosic materials**
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Abstract:

Non-enzymatic proteins were added during hydrolysis of cellulose and simultaneous saccharification and fermentation (SSF) of different biomass materials. Bovine serum albumin (BSA), a model non-enzymatic protein, increased cellulose and xylose conversion efficiency and also enhanced the ethanol yield during SSF of rice straw subjected to varied pretreatments. Corn steep liquor, yeast extract, and peptone also exerted a similar effect as BSA and enhanced the enzymatic hydrolysis of rice straw. Compared to the glucose yields obtained after enzymatic hydrolysis of rice straw in the absence of additives, the glucose yields after 72 h of hydrolysis increased by 12.7%, 13.5%, and 13.7% after addition of the corn steep liquor, yeast extract, and peptone, respectively. This study indicated the use of BSA as an alternative to intensive pretreatment of lignocellulosic materials for enhancing enzymatic digestibility. The utilization of non-enzymatic protein additives is promising for application in glucose and ethanol production from lignocellulosic materials.

Key words: non-enzymatic protein, enzymatic hydrolysis, simultaneous saccharification and fermentation, pretreatment, rice straw

1. Introduction

Lignocellulosic biomass materials constitute to a substantial renewable substrate for bioethanol production (Limayem and Ricke, 2012). However, the conversion of cellulose to glucose, which constitutes the initial process of biorefining, has remained a bottleneck (Lynd et al., 2008). Cellulase mediated hydrolysis of cellulose is characterized by a decline in the rate of hydrolysis over the course of the reaction. Pretreatment is often used to break down the polymeric structures of lignocellulosic biomass and enhance the accessibility of enzymes to solid substrate during enzymatic hydrolysis step. It therefore becomes important to develop methods which reduce the cellulase dosage and without decreasing the conversion rate. On the other hand, lignocellulosic biomass is relatively recalcitrant to enzymatic hydrolysis and thus an appropriate pretreatment for its bioconversion is a crucial prerequisite in the process (Alvira et al., 2010; Chen et al., 2011; Bjerre et al., 1996).

Pretreatment of lignocellulose includes physical, chemical, and thermal methods often used in combinations. Although pretreatments are generally effective procedures, they also often cause problems in waste disposal. This leads to difficulties downstream, as well as significant environmental risks. Therefore, pretreatment has been regarded as one of the most expensive processing steps in conversion of lignocellulosic biomass-to-fermentable sugars (Mosier et al., 2005; Sui et al., 2015). Approximately 18–20% of the total projected cost for biological production of cellulosic ethanol is attributed to pretreatment procedures and accounts for a fraction higher than that

1 allocated to any other step during this production (Wooley et al., 1999; Yang and Wyman, 2006).

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4 Thus, the development of methods, which would increase efficiency and decrease bioconversion
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7 costs of lignocellulosic biomass are crucial.
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10 The use of additives such as nonionic surfactants (Tween 20 and Tween 80), non-catalytic proteins
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12 (Bovine Serum Albumin, BSA) and polymers (polyethylene glycol, PEG) have been reported to
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14 drastically enhance the enzymatic conversion of cellulose into soluble fermentable sugars and lower
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17 the cellulase concentration required for obtaining an optimum sugar yield (Eriksson et al., 2002;
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20 Kristensen et al., 2007; Kumar and Wyman, 2009). By adding Tween 20 (0.024–0.24 mM), Seo
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23 (2011) demonstrated an increased cellulose conversion rate at 72 h from 9 to 21% and 1% to 8.5%
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26 for samples with high and low lignin contents, respectively. Our recent study showed an
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29 improvement in cellulose stability and an increased filter paper conversion rate when BSA was used
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33 (Wang et al., 2013 and 2015). Based on this result, in the current study we have focused on
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38 investigating the effects of non-enzymatic proteins such as BSA, and other protein rich additives
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41 such as corn steep liquor, yeast extract, and peptone in bioconversion of biomass. BSA was used as
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44 model protein because it is a single component having explicitly structure. We aimed at optimizing
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47 pretreatment combinations, enzymatic hydrolysis, and the process of fermentation to realize the
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51 efficient utilization of biomass
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54 The goal of this work was to investigate the effects of non-enzyme proteins on enzymatic
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58 hydrolysis and simultaneous saccharification and fermentation (SSF) of lignocellulosic materials,
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1 especially using ordinary enzymes without outstanding hydrolysis capabilities. We focused on rice
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3 straw as a lignocellulosic material. Non-enzymatic proteins were used to reduce the severity of
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6 pretreatment of raw materials to the enzymatic hydrolysis and SSF. Because enzyme costs and the
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9 pretreatment of materials constitute a significant portion of the total process cost, the findings of the
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12 current study may have a significant economical impact on the final ethanol cost. Therefore,
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15 supplement non-enzymatic protein in lignocellulosic materials hydrolysis is a promising way for cost
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18 reduction and environment friendly in bioconversion of lignocellulosic materials. Lignocellulose is a
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21 very complex matrix and it is difficult to evaluate the influence of non-enzymatic proteins on
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24 enzymatic hydrolysis. The use of a simple filter paper model can be an effective approach to
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27 understand the influence of non-enzymatic protein on bioconversion of biomass materials such as
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30 cellulose, xylan (hemicellulose), and lignin. As well as rice straw, such model lignocellulosic
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33 materials were examined in this work.
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42 **2. Materials and Methods**

43 **2.1 Materials**

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48 **2.1.1 Model lignocellulosic biomass component:** Cellulose, filter paper (Whatman no.1, used as 1
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51 × 1 cm size square pieces); and hemicellulose, xylan (obtained from birchwood, Sigma, Co., Ltd.,
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55 USA).

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58 **2.1.2 Natural lignocellulosic material:** Rice straw (collected in 2010, Shinano-machi, Nagano,
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Japan, composition; cellulose 35.0%, hemicellulose 27.1%, and lignin 5.2%).

2.1.3 Model protein: BSA (Cohn Fraction V, pH 7.0, Wako Pure Chemical Industries Ltd., Japan).

2.1.4 Non-enzymatic protein additives: a) Corn steep liquor (CSL) (protein content: 220.6 mg/g),

Solvlys 095 E (Roquette Freres); b) Peptone (protein content: 323.8 mg/g), enzymatic digest of

protein (Bacto), REF: 211677; c) yeast extract (protein content: 238.9 mg/g), extract of autolysed

yeast cells (Bacto), REF: 212750.

2.1.5 Cellulase and yeast: a) Acremonium Cellulase (AC, derived from *A. cellulolyticus*; Meiji

Seika, Co., Ltd., Japan; No.: ACCF-4940.). Avicelase activity was 1040 u/g (pH, 5.7); b) Accellerase

1500 (Genencor, Co., Ltd., USA), Endoglucanase activity was 2200–2800 CMC u/g and

Beta-Glucosidase activity was 525–775 pNPG u/g (pH, 4.6–5.0).

Commercial dry yeast was used in the SSF test (Dry yeast ethanol RedTM, *S. cerevisiae*, Fermentis, Co., Ltd., France).

2.2 Methods

2.2.1 Non-enzymatic protein pretreatment: Biomass substrates (filter paper, 2 g dry weight/100

mL; xylan, 1 g dry weight/100 mL; pretreated rice straw, 2 g dry weight /100 mL) were added into a

shaking flask containing 50 mM citrate buffer (pH 4.8) and were then autoclaved.

Non-enzymatic protein was added into the flasks at a room temperature to adjust the initial protein

concentration to 1.0 mg/mL. These flasks were then placed in a shaking incubator at 150 rpm and

50 °C for 12 h. The substrate at the solid liquid ratio 2 g dry weigh/ 100 mL substrate could be fully stirred by the shaking incubator.

2.2.2 Enzymatic hydrolysis: Enzyme experiments were carried out using 50 mM citrate buffer (pH 4.8). Enzymatic hydrolysis of substrates was conducted at 7.5 and 15 FPU/g substrate cellulase, with and without a 12 h BSA pretreatment. Incubation conditions were identical as followed during BSA pretreatment (i.e. 150 rpm shaking at 50 °C). Aliquots of 0.5 mL were collected at 0, 3, 6, 24, 48, and 72 h and immediately chilled on ice followed by centrifugation at 10,000 rpm for 5 min. Supernatants were filtered using a 0.22 µm filter paper. Glucose concentration and free cellulase activity was estimated in the clarified supernatants as described below. Unless specified otherwise, all experimental results are an average of two experiments.

2.2.3 Simultaneous saccharification and fermentation (SSF): All SSF experiments were performed in 200 mL flasks (working volume, 100 mL) placed in a shaking incubator at 130 rpm at 35 °C. Basal medium was autoclaved 50 mM citrate buffer (pH 4.8). The SSF reaction mixture contained 2g substrate and enzyme dosage was adjusted to 15 FPU/g substrate. The living yeast cell content in the medium was about 1.5×10^7 cells/mL at the initial condition. SSF experimental protocols, performed using standard biomass analytical methods, were obtained from the National Renewable Energy Laboratory (NREL) (NREL/TP-510-42630).

2.2.4 Pretreatment strategies: Rice straw was cut into 2 cm pieces and pretreated using different

concentrations of NaOH (1, 0.5, 0.2, 0.05, 0.01 mol/L and 0.0 mM blank using only DI water) at 50 °C for 4 h at 160 rpm followed by overnight cooling to 25°C. This was followed by washing with DI water until the pH was neutralized and dried at 60°C until a constant dry weight was obtained. The pretreated rice straws were milled to pass through a 2 mm screen.

2.3 Analytical Methods

2.3.1 Cellulase activity: The enzyme activity of samples were estimated using Whatman No. 1 filter paper as a substrate. NREL FPU (NREL/TP-510-42628) was used for enzyme activity analysis.

Supernatants from the samples subjected to concentration and filter treatment were used to measure free cellulose activity.

Sugar and ethanol concentrations in solution were measured by high performance liquid chromatography (HPLC) system (SHIMADZU LC-10AD, with refractive index detector RID-10A). A SUGAR SH1011 (SHODEX) column was used. Material components were analyzed according to the NREL analytical method (NREL/TP-510-42617, 42620, 42622 and 42630).

2.3.2 Protein concentration determination: The Protein Assay Lowry Kit (Nacalai Tesque, product no. 29470) was used for quantitative protein analysis.

3. Results and Discussion

3.1 Characterization of commercial enzymes in combination with BSA

1 A wide repertoire of enzymes such as cellulases, xylanases, and hemicelluloses is required to
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4 degrade all the components of lignocellulose (Costa et al., 2015). These commercial enzymes are not
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7 optimized for degrading different varieties of biomass and therefore a combination of pretreatments
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10 as well as addition of non-enzymatic protein additives is needed to achieve optimum degradation. In
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13 order to test the effectiveness of non-enzyme protein additives, Acremonium Cellulase, Accellerase
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16 1500 and different lignocellulosic starting materials were analyzed for enzymatic hydrolysis.
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19 The yields of glucose and xylose, obtained after utilization of two commercial enzymes in
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22 conjunction with BSA, in the enzymatic hydrolysis of model lignocellulosic materials (filter paper
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25 and xylan) and natural lignocellulosic material (pretreated rice straw) are shown in Fig. 1 and Fig. 2,
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31 The addition of BSA showed a pronounced positive effect towards the enhancement of enzymatic
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34 hydrolysis of different lignocellulosic materials. As expected, the different enzymes showed varying
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37 levels of enzymatic hydrolysis of the model and natural lignocellulosic materials (Fig. 1 and Fig. 2).
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41 Enzyme A displayed a higher cellulose hydrolysis capacity compared to enzyme B, particularly in
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44 the case of rice straw. The disparity of hydrolysis capacity was reduced upon addition of BSA and
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47 the performance of enzyme B in the enzymatic hydrolysis of rice straw was 13.1% and 19.7%
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50 enhanced in the presence of 15 and 7.5 FPU/g substrate, respectively (Fig. 2). Further, the addition
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53 of BSA also lowered the enzyme loading to achieve the same **production yield**. The cellulose
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56 conversion rate after addition of BSA in the enzymatic hydrolysis of rice straw with enzyme B at 7.5
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FPU/g substrate was almost identical as that with enzyme B at 15 FPU/g substrate (Fig. 2).

A commercial scale production of glucose and ethanol from lignocellulosic biomass is hindered mainly by the prohibitive cost of the currently available cellulase preparations, which are the enzymes used for saccharification (Kumar et al., 2009). Reduction in the cost of cellulases can be achieved only by concerted efforts addressing several aspects such as enzyme production and utilization. In this study, the addition of BSA improved the enzyme performance and also increased glucose concentration of both the model biomass materials namely, filter paper and natural lignocellulosic materials (rice straw). Previous results have also pointed out that the supplementation of BSA might save cellulase loading by approximately 50% without decreasing the hydrolysis yield (Wang et al., 2013). For Asian countries such as Vietnam and China, rice is one of the most popular staple foods (Sereewatthanawut et al., 2008). However, there are several barriers such as high operational costs, particularly associated with high quality commercial cellulase, which limit the utilization of locally available agricultural biomass residues. In this context, it is very promising to use non-enzymatic proteins to improve the cellulose performance by local produce, which could contribute towards the evolution of biomass utilization projects in these developing countries.

3.2 Characterization of untreated and pretreated rice straw

The high cellulose content of rice straw makes it a very promising substrate for ethanol production (Galbe and Zacchi, 2007; Mochidzuki et al., 2015). In order to obtain different samples having composition, rice straw was pretreated under different severity concentrations of NaOH. A series of

tests with the pretreated samples were performed to understand the interplay between the proportion of three major biomass components (cellulose, hemicellulose, and lignin) and the performance of non-enzymatic proteins during enzymatic hydrolysis. The components of this pretreated rice straw are given in Table 1. As seen in Table 1, the cellulose component was significantly increased with increasing NaOH concentration, while the hemicellulose and lignin content was decreased. Removal of lignin or its disruption is essential for the efficient bioconversion of lignocellulosic materials to sugars (Van Dyk and Pletschke, 2012). The total weight loss of rice straw was obvious after pretreatment with NaOH and was 21.4, 29.6, and 34.1% after pretreatment with 0.2 M, 0.5 M, and 1 M NaOH solution, respectively.

In order to investigate the feasibility of addition of non-enzymatic protein to reduce severity of pretreatment, thus obtained rice straw samples under different NaOH concentrations was used in the tests described in the following sections.

3.3 Effect of BSA on enzymatic hydrolysis and SSF of rice straw using different pretreatments

The concentrations of glucose obtained after enzymatic hydrolysis of rice straw using different pretreatments are shown in Fig. 3a. An intensive pretreatment was observed to increase the cellulose concentration. However, the effect of BSA during enzymatic hydrolysis declined with an increase in NaOH concentration during the pretreatment. The glucose concentrations obtained from rice straw upon addition of BSA and after pretreatment with 0.05 M NaOH was 2.0 g/L, which was identical to that obtained after a stronger pretreatment using 0.2 M NaOH without adding BSA. Similarly,

glucose concentrations from rice straw pretreated with 0.5 M NaOH in the presence of BSA was 2.3 g/L, which was highly similar to that obtained after a 1 M NaOH pretreatment in the absence of BSA.

Further, we performed SSF of the pretreated rice straw samples to investigate the effect of BSA on ethanol production after pretreatment with different concentrations of NaOH. In every case, the ethanol concentration was observed to increase with the addition of BSA (Fig. 3b). After 144 h, ethanol concentrations obtained from rice straw increased by 25.4% (pretreatment with 0.01 M NaOH), 14.8% (pretreatment with 0.05 M NaOH), 6.1% (pretreatment with 0.2 M NaOH), 4.0% (pretreatment with 0.5 M NaOH), and by 3.8% (pretreatment with 1 M NaOH), respectively. During SSF, BSA was most effective when rice straw was pretreated with 0.01 M NaOH. Highest ethanol production; 2.4 g/L; was observed in SSF at 1 M NaOH pretreatment (Fig.3b). Thus, as mentioned above, BSA showed a pronounced effect on enzymatic hydrolysis of rice straw particularly after mild conditions of pretreatment.

Lignocellulose is a highly complex structure with myriad characteristics which influence and also limit the hydrolysis of carbohydrate polymers into fermentable sugars (Alvira et al., 2010). Although lignin is generally believed to be one of the most limiting factors in lignocellulose enzymatic hydrolysis (Eriksson et al., 2002), other factors may be equally important. In our experiment, pretreatment with varying concentrations of NaOH showed a more differentiated effect on the hemicellulose content compared to that of lignin. The hemicellulose content ranged from 14.0% (1

1 M NaOH) to 33.6% (DI water), whereas the lignin content ranged from 3.5% (1 M NaOH) to 6.5% (0.01 M NaOH) (Table. 1). Hemicellulose is known to form a protective physical barrier against enzymatic attack (Öhgren et al., 2007) and BSA adsorption on xylan was no less than that on lignin and much more than that on cellulose (Wang et al., 2015). Thus, the changes in hemicellulosic components would be more obvious when considered together with the effect of BSA on enzymatic hydrolysis of biomass materials.

The NaOH pretreatment could break down the structure of lignocellulosic biomass. According to the severity of pretreatment, the degree of the structure change was different. By a severe pretreatment, the distance (D) and fracture (F) could be increased, lignin and hemicellulose could be partly solubilized and increase accessibility of the enzymes to the inner surfaces of the material (Fig. 4). Thus, the enzymatic hydrolysis of lignocellulosic biomass is enhanced. On the other hand, by mild pretreatment, the structure change of material is limited. In this situation, addition of non-enzymatic protein could contribute to reduce un-productive adsorption of cellulase and also enhance saccharification. This could also explain that the effect of BSA was more significant for the samples pretreated with low concentration of NaOH.

This study also showed that the addition of BSA provided an alternative to intensive pretreatment of starting material for enhancing enzymatic digestibility. It has been suggested that the mechanisms of BSA function and pretreatment methods towards enzymatic hydrolysis enhancement are different (Table 2). The effect of surfactants is thought to arise due to the hydrophobic interaction between the

1 surfactant and lignin contained in the lignocelluloses, thereby either releasing unspecifically bound
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4 enzyme or preventing unproductive enzyme adsorption (Eriksson et al., 2002; Kristensen et al.,
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7 2007). Seo et al. (2011) reported that Tween 20 treatment contributed to increase non-freezing bound
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10 water considerably to increase accessible cellulose surface and increased cellulose conversion.
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13 Previous studies also showed BSA not only increased the amount of free enzyme, but also increased
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16 the amount of adsorbed enzymes during the enzymatic hydrolysis of rice straw (Wang et al., 2015).
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19 In this study, results revealed that in the enzymatic hydrolysis and SSF of mild pretreated rice straw,
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22 non-enzymatic proteins was play a much more prominent role to increase the accessibility of
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25 enzymes onto cellulose than in the enzymatic hydrolysis and SSF of severe pretreated rice straw.
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28 Therefore, the use of non-enzymatic proteins for reducing the severity of the pretreatment to enhance
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31 the bioconversion efficiency of biomass in enzymatic hydrolysis and SSF deserves further attention
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34 and merits potential as suitable alternative to intense pretreatment procedures.
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37 38 39 **3.4 Effect of non-enzymatic protein additives on SSF** 40

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42 Owing to their high protein contents; CSL, yeast extract, and peptone were used as non-enzymatic
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45 protein additives and their effect on enzyme activity was evaluated by measuring the concentration
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48 of glucose during enzymatic hydrolysis. Similar to the model protein BSA, all the tested
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51 non-enzymatic proteins also enhanced enzymatic hydrolysis of rice straw (Fig. 5). Compared to the
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54 concentrations obtained in the absence of additives, the glucose concentration after 72 h of
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57 hydrolysis of pretreated rice straw increased by 12.7%, 13.5%, and 13.7% after addition of CSL,
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1 yeast extract, and peptone, respectively. The positive effect exerted by the non-enzymatic proteins
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4 was clearly better than that exerted by model protein BSA.
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7 A previous study showed that the addition of BSA and CSL before the addition of cellulase
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10 enhanced enzyme activity in solution and increased cellulose and xylose conversion rates by
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12 affecting the adsorption and desorption of enzymes (Wang et al., 2015). Fig. 5 shows that CSL, yeast
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14 extract, and peptone display a function similar to BSA in enhancing the enzymatic hydrolysis of
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16 lignocellulosic materials. Additionally, the favorable nutrient composition of CSL, yeast extract, and
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18 peptone could also render them useful in microbial cultures for ethanol production. Mochidzuki
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20 (2015) showed that the agricultural byproduct rice bran, used as an inexpensive nutrient supplement
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22 in SSF for ethanol production from rice straw, increased the efficiency of SSF and also reduces the
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24 cost of the nutrients used in cellulosic ethanol fermentation. This indicates that high protein content
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26 agricultural/industrial byproducts such as CSL and rice bran could be employed as inexpensive
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28 non-enzymatic protein additives in biomass utilization projects.
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42 3.5 Mass balance of enzymatic hydrolysis and SSF of rice straw

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44 The values of glucose yield after 72 h of enzymatic hydrolysis and 144h of SSF of rice straw was
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46 used to outlines a mass balance scheme starting from 100 g of DI water pretreated rice straw and
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48 illustrates the impact of pretreatment and addition of non-enzymatic proteins on its bioconversion
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52 (Fig.6). It can be seen that compared to DI water pretreated rice straw, 20.6%, 15.4%, 14.2 % of
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58 cellulose was lost after pretreatment with 1 M, 0.5 M, and 0.2 M NaOH, respectively. Furthermore,
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1 in spite of an enhanced enzymatic hydrolysis by pretreatment with high concentration of NaOH, the
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4 final glucose concentration from rice straw remained similar to that obtained after enzymatic
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7 hydrolysis upon pretreatment with 0.01 M or 0.05 M NaOH. A pretreatment is aimed at breaking
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10 down the lignin structure and disrupting the crystalline structure of cellulose for enhancing enzyme
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13 accessibility during hydrolysis (Mosier et al., 2005). However, a tremendous loss in the amount of
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16 fermentable sugars after pretreatment procedures has been reported (Alvira et al., 2010). 32.3 g
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19 glucose was obtained upon addition of BSA and after 72 h of enzymatic hydrolysis of rice straw
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22 pretreated with 0.05 M NaOH pretreatment. In comparison, a slight decrease in the glucose yield
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25 amounting to 29.8 g was obtained without the addition of BSA after pretreatment with 1 M NaOH.
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28 A similar result was also observed in case of the SSF process where 8.7 g ethanol was obtained after
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31 144 h of SSF after addition of BSA and pretreatment with 0.05 M NaOH, which was slightly more
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34 than that obtained using 1 M NaOH. Thus, the utilization of non-enzymatic protein additives is
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37 promising for application in glucose and ethanol production from lignocellulosic materials.
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45 4. Conclusions

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47 Non-enzymatic proteins increased the enzymatic conversion of model lignocellulosic materials
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49 (filter paper and xylan) and natural lignocellulosic material (pretreated rice straw). The effect of
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52 BSA on the enzymatic hydrolysis of lignocellulosic materials varied according to the initial biomass
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58 used. Compared to utilizing intense pretreatment procedures, the addition of non-enzymatic proteins
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1 to lignocellulosic materials can lead to higher biomass conversion efficiency during the process of
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4 enzymatic hydrolysis and SSF, and reduce the wastes caused by pretreatment procedures. Utilization
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7 of inexpensive non-enzymatic proteins is promising for future applications in glucose and ethanol
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10 production from lignocellulosic materials.
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Figure legends

Figure 1: Effect of commercial enzymes used individually or in combination with BSA on the enzymatic hydrolysis of model biomass materials at 50 °C. (A, Acremonium Cellulase; B, Accellerase 1500; enzyme dosage, 15 FPU/g substrate)

Figure 2: Effect of different dosages of commercial enzymes used individually or in combination with BSA on the enzymatic hydrolysis of pretreated rice straw at 50 °C. (A, Acremonium Cellulase; B, Accellerase 1500. Substrate concentration, pretreated rice straw 2% w/v)

Figure 3 Effect of BSA on enzymatic hydrolysis and SSF of rice straw subjected to varying pretreatment procedures. (a. Enzymatic hydrolysis; b. SSF)

Figure 4 Predicted model of alkali pretreatment and non-enzymatic protein enhance enzymatic hydrolysis of lignocellulosic material. (D: distance; F: fracture)

Figure 5: Effect of non-enzymatic proteins on enzymatic hydrolysis of rice straw pretreated with 1 M NaOH

Figure 6: Mass balance after pretreatment, saccharification and ethanol production using rice straw as substrate

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3 **Tables**

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6 **Table 1: Component analysis of biomass materials**

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Pretreatment	Cellulose %	Hemicellulose %	Lignin %	Total weight lost %
DI Water	41.1	33.6	5.6	-
0.01 M NaOH	41.7	30.4	6.5	3.6
0.05 M NaOH	43.0	30.0	6.1	13.0
0.2 M NaOH	44.9	20.8	5.5	21.4
0.5 M NaOH	49.4	18.2	5.3	29.6
1.0 M NaOH	49.4	14.0	3.5	34.1

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Table 2: Explanation of pretreatment and additives effect on cellulose enzymatic hydrolysis

Mechanism	Alkali pretreatment	Surfactant	Non-enzymatic protein
Structure change	Reduce the crystallinity of cellulose (Mirahmadi et al., 2010)	Tween disrupt the lignocellulose matrix (Kaar and Holtzapple 1998; Kurakake, Ooshima and Harano, 1994)	BSA facilitate decrystallinization and size reduction of biomass particles, resulting in a highly digestive substrate (Brethauer et al., 2011)
	Removal or disruption of lignin (Van-Dyk and Pletschke, 2012; Talebnia et al., 2010)	Tween 20 treatment increase non-freezing bound water considerably to increase the contact of cellulose surface with	
	Reduce hemicelluloses (Mirahmadi et al., 2010; Wang et al., 2013)	water (Seo et al., 2011)	
		Tween 20 treatment increase cellulose reducing-ends (Seo et al., 2011)	
Adsorption control	Expose more accessible surface area of cellulose to cellulase (Singh et al., 2011; Lu et al., 2002)	Surfactants affecting enzyme-substrate interaction, e.g., reduces un-productive adsorption to lignin (Kaar and Holtzapple 1998;	BSA prevents the non-productive adsorption of enzyme onto lignin of corn stover (Yang and Wyman, 2006; Brethauer et al., 2011)
	Reduce un-productive adsorption on lignin (Van-Dyk and Pletschke, 2012; Wang et al., 2015)	Eriksson et al., 2002; Yang and Wyman, 2006; Kristensen et al., 2007)	BSA increase the free enzyme during enzymatic hydrolysis of biomass material (Brethauer et al., 2011; Wang et al., 2015)
Others		Tween protects the enzymes from thermal deactivation (Kaar and Holtzapple 1998)	Hydrophobic protein could alleviate the feedback inhibition of cellobiose (Han and Chen, 2010)
			BSA protects the enzymes from thermal deactivation (Wang et al., 2013)
			BSA relieved the cumulative sugar inhibition of hydrolysis (Wang et al., 2015)

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6