1 **Assessment of the structural factors controlling the enzymatic saccharification of rice**
2 **straw cellulose**

3

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8

9 **Abstract**

10 In this study, the structural factors controlling the enzymatic saccharification of rice straw
11 cellulose were examined by preparing structure-modified rice straw samples such as dewaxed,
12 alkali-treated, oxidized, and swollen rice straw. It was found that the initial enzymatic
13 saccharification rate of the various structure-modified samples was largely controlled by the
14 initial cellulose surface area of the cellulose unit. Although the presence of lignin limited the
15 cellulose surface area, there was no strong relationship between the lignin content and the
16 initial reaction. On the other hand, the long-term enzymatic saccharification of rice straw
17 cellulose highly depended on the lignin removal rate (lignin content). It was also found that
18 silica is not a crucial factor in controlling the enzymatic saccharification.

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20 *Keywords: Rice straw, Pretreatment, Cellulase, Saccharification*

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1. Introduction

The securement of sustainable feedstock is one of the most important factors affecting the feasibility of biorefinery. For this reason, lignocelluloses have attracted great attention as sustainable feedstock for biorefinery. Lignocelluloses can be classified into the following four groups [1]: 1) forest products/residues (e.g., eucalyptus, pine, and poplar), 2) agricultural residues (e.g., corn stover, wheat straw, sugarcane bagasse, and rice straw), 3) agricultural processing by-products (e.g., corn fiber, rice hull, and barley hull), and 4) dedicated energy crops (e.g., hybrid poplar, switchgrass, and willow). Since the factors affecting availability of the above lignocelluloses, including the land use for their cultivation, are geologically and seasonally diverse, a strategy for biorefinery from these sources cannot be generalized. This is the reason why many countries have been evaluating the potential of indigenous biomass for biorefinery.

For many Asian countries including Japan, among the agricultural residues, rice straw accounts for the major portion of the available biomass [2]. According to statistics supplied by the Japanese government, the annual generation of rice straw in 2005 reached about 9 Mt; however, most of this product is plowed down, with a little effective utilization (i.e., animal feed and composting) that accounts for only about 20 % of the total production. Considering this situation, the utilization of rice straw as feedstock of biorefinery is quite meaningful in those Asian countries in which the land use for energy crop cultivation is highly limited in

1 terms of establishing an agricultural society for carbon circulation. From this viewpoint, the
2 feasibility of sugar-based biorefinery (e.g., ethanol production) using rice straw has been
3 widely discussed [2, 3]. However, basic studies on the pretreatment and enzymatic
4 saccharification of rice straw, taking into account its intrinsic structural features, have not
5 been fully carried out, even though it is very important to optimize the sugar-based
6 biorefinery using rice straw.

7 In contrast to wooden stem, the stem of the rice straw monocots lacks a cambium, but it
8 has numerous vascular bundles in different layers consisting of a waxy cuticle, silicated
9 epidermis, cortex (collenchyma), a thick lignified layer (sclerenchyma), and ground tissue
10 (parenchyma), as shown in Fig. 1 [4].

11 Figure 1 here

12 Compared to woody biomass and other monocots, one of the intrinsic features of rice
13 straw is its silica-enriched body, which is known to be the main physical barrier to material
14 transportation along with the lignin that reduces the susceptibility to enzymatic degradation
15 [5]. Jin and Chen [4] reported that the proportion of lignin and the silica content in different
16 parts of rice straw (e.g., the leaf blade, node, and internode) showed a negative correlation
17 with the hydrolysis performance of cellulose.

18 Nonetheless, the enzymatic saccharification cannot be fully explained based on the
19 proportion of lignin to silica, because rice straw has a three-dimensional structure consisting

1 of various layers. Moreover the negative effects of lignin and silica on the digestibility of rice
2 straw as an animal feed have been elucidated extensively for the last several decades [6], but
3 the structural factors governing the enzymatic saccharification of rice straw cellulose are still
4 not clear. Therefore, in this report, the structural factors controlling the enzymatic
5 saccharification of cellulose in rice straw have been studied taking into consideration the
6 intrinsic structure of rice straw. Based on the results, an effective pretreatment strategy for
7 rice straw was also discussed.

8

9 **2. Material and Methods**

10 **2.1. Preparation of structure-modified rice straw samples**

11 Straw of Japanese rice (*Akitakomachi*) harvested at maturity on October 2011 from a
12 single paddy field in Shinano (36°48' N, 138°12' E, Nagano prefecture, Japan) was kindly
13 supplied by the rural rice farmer. Air-dried rice straw was coarsely cut to less than 30 mm by
14 cutting machine and stored in a dry space until further use. Rice straw was crushed to (3 ± 2)
15 mm $\times$ (10 ± 5) mm pieces by using a manual roll crusher and air-dried to reduce the water
16 content (below 10 %, weight fraction). The crushed raw rice straw is termed RAW. The
17 authors intended to modify the rice straw structure in a stepwise approach to estimate more
18 clearly how each modification step (e.g. removal of wax and silica-enriched layers)
19 contributes to changes in the accessible cellulose surface area, since various components of

1 rice straw affect exposure of the cellulose surface together. First, ethanol Soxhlet extraction
2 was carried out for 24 h to remove the wax from the cuticles of rice straw. The dewaxed
3 sample is hereafter termed DW. DW was treated to control silica-enriched layer (i.e.
4 epidermis) by immersion in a 940 mol m⁻³ Na₂CO₃ (soda ash) solution under the following
5 conditions: liquid-to-solid ratio (L/S) of 20 (dm³ kg⁻¹), temperature of 62 °C, and reaction
6 times of 20, 40, 90, and 180 min. For the modification of densely lignified layer, DW was
7 treated by immersion in the oxidizing agent (25 % CH₃COOH + 15 % of 30 % H₂O₂ + 2.5 %
8 H₂SO₄ + 57.5 % distilled water, on a volume basis) described elsewhere [7] under the
9 following conditions: liquid-to-solid ratio (L/S) of 30 (dm³ kg⁻¹), temperature of 80 °C, and
10 reaction times of 20, 40, 90, and 180 min. Alkali-treated and oxidized samples are referred to
11 as AL and OX, respectively. Swollen rice straw (referred to as SW hereafter) was also
12 prepared by wet ball milling of the alkali-treated sample for 180 min (AL180) at 0.4 m s⁻¹ for
13 3 h in a 0.5-L milling pot (PM001, As one, Japan).

14

15 **2.2. Structural assessment of the model samples**

16 The compositional analysis was performed according to the method suggested by the
17 National Renewable Energy Laboratory (NREL, Denver, CO, US) [8, 9, 10]. For scanning
18 electron microscopy (SEM), the samples were air-dried and spread uniformly on the sample

1 mounter and then were coated with Au before the observation using a field emission SEM
2 (FE-SEM) instrument (S-4500, Hitachi).

3 As an indirect structural analysis, cellulase (Celluclast® 1.5L; CAS #: 9012-54-8; Sigma
4 Aldrich Co.; protein concentration: $51.43 \pm 1 \text{ g dm}^{-3}$) adsorption (equilibrium and rate) and
5 astra blue (Santa Cruz biotechnology, Inc.) adsorption (equilibrium) were carried out. Since
6 the catalytic activity of cellulase can be maintained across a wide range of temperatures, the
7 amount of sample used as an adsorbent can be altered by the hydrolysis occurring during the
8 cellulase adsorption. Therefore, cellulase adsorption experiments were performed at 0–4 °C
9 to maintain the catalytic activity of cellulase at a negligible level [7]. The protein
10 concentration was measured using the Bradford protein assay kit according to the instructions
11 provided by Thermo Scientific. Besides, since the adsorption of astra blue is very sensitive to
12 the adsorption conditions (e.g., pH and concentration of the adsorbate in the solution), the
13 adsorption conditions of astra blue were controlled to obtain monolayer adsorption using 25
14 mol m^{-3} $\text{Na}_2\text{B}_4\text{O}_7/\text{HCl}$ buffer solution as described in Peterlin et al. [11]. The experimental
15 conditions for cellulase and astra blue adsorption are listed in Table 1.

16 Table 1 here

17 The Langmuir adsorption equilibrium and rate data were interpreted using the following
18 equations:

19 Adsorption isotherm $q = \frac{q_s K C_e}{1 + K C_e} \quad (1)$

1 ▷ detector: RI (refractive index).

2 The conditions of the enzymatic saccharification of the samples are also listed in [Table 1](#).

3

4 **3. Results and discussion**

5 **3.1. Composition, surface morphology, and accessibility of the model samples**

6 [Fig. 2a](#) and [Table 2](#) show the compositional changes in rice straw following the different
7 treatments. During the 3-h alkali treatment, 87.08 % of ash and 41.88 % of lignin were
8 removed but the hemicellulose and cellulose levels remained were almost unchanged. During
9 the 3-h oxidative treatment, 93.64 % of lignin and 76.86 % of hemicellulose were removed;
10 however, the ash (mainly silica) and cellulose contents were slightly decreased. Therefore, it
11 can be considered that the alkali treatment and oxidative treatment mainly affected the silica-
12 lignin matrix and lignin-hemicellulose matrix, respectively.

13 Figure 2 and Table 2 here

14 The SEM images of some representative samples are shown in [Fig. 2b](#). The waxy silicated
15 epidermis of RAW can be observed in Panel [2b-1](#). Although the ethanol extractives (wax)
16 accounting for about 15 % of RAW were removed, the surface morphology of DW was not
17 very different from that of RAW. In contrast to the silica skeleton ([2b-3](#)) of RAW, the
18 structure of silica seemed to be changed during the alkali treatment ([2b-4](#)). Some silica
19 remained on the surface, appearing as a white ball. After the 3-h oxidative treatment, rice

1 straw was bleached (whitened) and its cellulose surface was exposed (2b-5). However, the
2 silica body was still retained on the surface. The inner surface of the fibrils of the swollen rice
3 straw (2b-6) was exposed.

4 Based on the above results and previous references, it can be inferred how the structure of
5 rice straw was modified by each treatment. For DW, the RAW sample structure remained yet
6 the waxy cuticle layer was removed. During alkali treatment, the silica-lignin matrix was
7 removed preferentially. This is because the silica in the epidermis is not stand-alone but is
8 complexed with, mainly, lignin [12]. During the oxidative treatment, lignin and hemicellulose
9 were removed from across the various layers, yet the silica remained. Although silica-lignin
10 and lignin-hemicellulose were removed during the alkali and oxidative treatments
11 respectively, the frame of the cell wall was retained. However, the cell wall collapsed during
12 the wet ball milling (SW), exposing the inner fibril surface.

13 One of the structural properties of the model samples, the “accessibility to cellulase,” can
14 be also deduced from the cellulase adsorption rate [13, 14]. In particular, it is described as
15 initial accessibility, because the accessibility is altered by the cellulase action known as
16 amorphogenesis [15]. The cellulase adsorption parameters are summarized in Table 3.

17 As shown in Fig. 3b and Table 3, the cellulase adsorption rate cannot be increased merely
18 by the removal of the silicated and lignified layers (i.e., exposure of the outer surface of rice
19 straw fibrils). On the other hand, the exposure of the inner surfaces of rice straw fibrils (SW)

1 significantly increased the initial accessibility to cellulase. Oxidative treatment largely
2 increased the monolayer saturation amounts of the adsorbed cellulase for the model samples
3 (q_s); however, q_s of the alkali-treated samples (AL40 and AL180) was not very different from
4 that of RAW and DW.

5 Table 3 and Figure 3 here

6 Since cellulase is supposed to adsorb also on non-cellulosic components (e.g., lignin, silica,
7 and hemicellulose) non-specifically [16], we cannot say that the amount of cellulase adsorbed
8 accurately reflects the surface reactivity (i.e., cellulose surface area) of the substrate to
9 cellulase. Therefore, the cellulose surface area of the model samples was examined by astra
10 blue adsorption and the details are explained in the following section.

11

12 **3.2. The structural factor of rice straw determining the initial cellulase reaction rate**

13 **3.2.1. The modification of the previous cellulose conversion model**

14 Although the structural properties of the model samples were provided as mentioned
15 above, it is still very difficult to quantitatively predict the factors governing the enzymatic
16 saccharification rate by using those fragmentary pieces of information. Moreover, in contrast
17 to the cellulosic substrates, the non-productive and irreversible adsorption of cellulase onto
18 non-cellulosic components should be monitored to examine the cellulase reaction with
19 lignocelluloses [17]. Unfortunately, it is extremely difficult to monitor those phenomena

1 quantitatively. In this study, the structural factor of rice straw controlling the cellulase
2 reaction was examined by using the cellulose conversion model with modifications [7].

3 In the original work, a simple cellulose conversion model considers the cellulase-derived
4 (cellulase overcrowding and product inhibition) and substrate-derived (cellulose surface area)
5 factors controlling the reaction rate of cellulosic substrates [7]. In the previous model, the
6 substrates were assumed to be cylindrical cellulose fibrils, and the concept of “initial radius
7 of cellulose fibrils (R_0)” was adopted to describe the initial surface area of the cylindrical
8 cellulose fibrils (as a reaction rate-controlling factor) as shown below.

9
$$A_c = \frac{2}{\rho_c R_0} , (4)$$

10 where A_c and ρ_c are the cellulose surface area per unit weight of the cylindrical cellulose
11 fibrils ($\text{m}^2 \text{kg}^{-1}$) and the density of cellulose (kg m^{-3}), respectively.

12 The basic form of the previous model, which did not consider the reaction retardation, was
13 described as:

14
$$\frac{d\alpha}{dt} = \frac{k_1}{R_0^2} \left(\frac{Q}{S_0} \right) , (5)$$

15 where α , t , k_1 , R_0 , Q and S_0 are the cellulose conversion ratio (-), time (s), reaction rate
16 constant ($\text{m}^2 \text{s}^{-1}$), initial radius of cellulose fibrils (m), total amount of cellulase adsorbed onto
17 cellulose fibrils (kg), and total initial weight of cellulose fibrils (kg), respectively. This basic
18 form corresponds to the specific activity of cellulase proposed by Igarashi et al. [18].

1 The final form considering the exponential decrease of the reaction rate due to cellulase
 2 overcrowding and inhibition of products is expressed as

$$3 \quad \frac{d\alpha}{dt} = \frac{k_1}{R_0^2} \left(\frac{Q}{S_0} \right) \left[\exp \left(-k_2 \frac{1}{q_m} \frac{\alpha}{1-\alpha} \right) + k_3 \right], (6)$$

4 where q_m is the monolayer saturation amount of the adsorbed cellulase onto the unit weight of
 5 cellulose fibrils, and k_2 and k_3 are dimensionless reaction rate constants. The derivation and
 6 development of Eq. (5) and (6) are omitted because they are described in detail in Seo et al.
 7 [7].

8 Here, the authors considered that the specific surface area (SSA) of cellulose ($\text{m}^2 \text{kg}^{-1}$)
 9 shown in Eq. (7) is equivalent to A_c [Eq. (4)], since it is almost impossible to measure the
 10 surface area of cellulose fibrils correctly.

$$11 \quad SSA = \frac{N_A a_m q_m}{M}, (7)$$

12 where N_A , a_m and M are the Avogadro's number (mol^{-1}), the occupied surface area of one
 13 molecule of the adsorbate (m^2), and the molecular weight of the adsorbate (kg mol^{-1}),
 14 respectively. Therefore, q_m can be expressed as a function of the initial radius of cellulose
 15 fibrils as reported below.

$$16 \quad \frac{1}{q_m} = R_0 \left(\frac{\rho_c}{2} \right) \left(\frac{N_A a_m}{M} \right) (8)$$

17 Therefore, Eq. (6) can be rewritten as:

$$18 \quad \frac{d\alpha}{dt} = \frac{k_1}{R_0^2} \left(\frac{Q}{S_0} \right) \left[\exp \left(-k_2' R_0 \frac{\alpha}{1-\alpha} \right) + k_3 \right], (9)$$

where k_2' is the revised reaction rate constant (m^{-1}). It can be noted that the increase of the cellulose surface area (i.e., decrease in R_0) contributes not only to increasing the reaction rate but also to relieving the reaction retardation.

Since the previous model was constructed and validated with cellulosic substrates, the cellulase adsorption isotherm was used to examine the cellulose surface area. However, cellulase adsorption cannot be used to examine the cellulose surface area of rice straw samples as mentioned in the beginning of section 3.2. Therefore, the authors adopted the astra blue adsorption technique. Astra blue is a well-known dye for the selective staining of cellulose due to its strong affinity for cellulose molecules [19, 20].

Furthermore, since the total amount of cellulase adsorbed onto the cellulose fibrils (Q) cannot be specified, the total amount of cellulase loaded (C_L , kg) was used instead of Q in the revised cellulose conversion model. The revised equation is shown as Eq. (10).

$$\frac{d\alpha}{dt} = \frac{k_1}{R_0^2} \left(\frac{C_L}{S_0} \right) \left[\exp \left(-k_2' R_0 \frac{\alpha}{1-\alpha} \right) + k_3 \right] \quad (10)$$

3.2.2. Model calculation and validation

Fig. 4a shows the astra blue adsorption isotherms of the model samples. The adsorption parameters are listed in Table 3.

Figure 4 here

1 For the bleached rice straw (OX180), the monolayer saturation amount of adsorbed astra
2 blue onto the unit sample (13.3 g kg^{-1}) was very similar to that of the bleached pulps (13.8 –
3 14.6 g kg^{-1}) reported by Peterlin et al. [20]. Therefore, the SSA (i.e., cellulose surface area) of
4 the unit samples was calculated using Eq. (7) by using q_s instead of q_m . a_m and M of the astra
5 blue were 5 nm^2 and $1.06875 \text{ kg mol}^{-1}$, respectively. The specific cellulose surface area
6 according to the removal rate of non-cellulosic materials is shown in Fig. 4b.

7 As shown in Fig. 4b-1, the cellulose surface area of the unit sample did not increase much
8 by removing the waxy layer. On the contrary, the cellulose surface area of the cellulose unit
9 (Fig. 4b-3) decreased due to the increase in the DW cellulosic fraction (Fig. 4b-2). The
10 cellulose surface area increased largely at around 40–60 % of removal rate. However, the
11 cellulose surface area of the unit sample was not increased over $45 \text{ m}^2 \text{ g}^{-1}$ sample, although
12 nearly 80 % of the non-cellulosic materials were removed. On the other hand, a larger value
13 was observed for SW compared to that of the other samples. As discussed in Section 3.1, this
14 is because the inner cellulose surface cannot be exposed merely by removing the outer layers.

15 Fig. 5a shows the initial cellulose conversion ratio (1 hr) of the model samples under
16 various cellulose-loading conditions. The experimental data were interpreted by using Eq.
17 (10).

18 Figure 5 here

1 The parameters of the control (raw rice straw), estimated by fitting the measured data to
2 Eq. (10), are shown in Fig. 5a. The initial cellulose conversion ratio for the experimental
3 structure-modified samples was simulated by changing the initial radius (“relative R_0 ” to
4 control’s). All the calculated data are shown using the solid line. The calculations based on
5 the model were performed using the Scilab software. As shown in Fig. 5a, the simulated data
6 corresponded well to the experimental data for a wide range of cellulase loadings.

7 It is interesting to note that DW showed a lower initial cellulose conversion ratio than raw
8 rice straw, probably because DW has lower cellulose surface area of unit weight of cellulose
9 than RAW (Fig. 4b-3). In other words, the relatively larger initial radius (R_0) of DW
10 compared to that of raw rice straw resulted in an initial poor cellulose conversion ratio.

11 The suggested model was validated by comparing the relative R_0 of the structure-modified
12 samples to R_0 of the control (RAW) by examining the astra blue adsorption and calculations
13 based on the model. As shown in Fig. 5b, the relative R_0 of all the samples but DW obtained
14 by model calculation was very close to the relative R_0 measured by astra blue adsorption.
15 Therefore, it can be concluded that measured suggested model quantitatively describes the
16 effect of the cellulose surface area on the initial enzymatic saccharification rate of rice straw
17 cellulose.

18 Similarly to a previous report showing that the initial enzymatic saccharification rate of
19 cellulosic substrates is largely controlled by the initial cellulose surface area of the cellulose

unit [7], the initial enzymatic saccharification rate of rice straw cellulose is also mainly affected by the initial cellulose surface area of the cellulose unit, although rice straw is a much more heterogeneous substrate, based on the cellulase reaction.

Furthermore, the results shown in Fig. 4 clearly elucidated that the initial enzymatic saccharification of rice straw cellulose was controlled not by the lignin or silica content itself but by the initial cellulose surface area of the cellulose unit strongly related to the removal of non-cellulosic materials, although previous work roughly estimated that the initial enzymatic hydrolysis of rice straw would seem to be determined by the structure features, such as number of accessible sites to cellulase [21].

3.3. The rice straw structural parameter determining the long-term cellulose conversion

Although the sensitivity of cellulose conversion of the cellulosic substrate to R_0 decreased with the reaction time, the variation of the long-term reaction also could be described as a function of R_0 [7]. However, for the model rice straw samples, the variation of the long-term enzymatic saccharification rate could not be successfully described by Eq. (10) using estimated parameters for the initial reaction.

For lignocelluloses including rice straw, the uncertainties with respect to the cellulase reaction might increase with the reaction time, because the probability that a biospecific reaction between cellulose and cellulase could occur would decrease with the progression of

1 cellulose conversion. In other words, irreversibly-/non-productively-bound cellulase to the
2 non-cellulosic materials might increase with the reaction time [17]. Therefore, a reliable
3 description of the available cellulase quantity considering its irreversible/non-productive
4 adsorption is necessary to enhance the accuracy of Eq. (10) for the long-term cellulose
5 conversion [17]. However, although this is an old, unresolved issue in this field, it is not an
6 objective of this study to address this.

7 Fig. 6a shows the variation in the cellulose conversion ratio during the 72-h period for the
8 model samples.

9 Figure 6 here

10 Not only at the beginning of the reaction, but also during the reaction, the cellulose
11 conversion ratio of DW was lower than that of raw rice straw. Therefore, it can be inferred
12 that any treatment that does not contribute to increase the initial cellulose surface area of the
13 cellulose unit cannot be a pretreatment during the process of enzymatic saccharification of
14 rice straw cellulose. Although SW showed higher initial reaction rate than other samples due
15 to the higher initial cellulose surface area of the cellulose unit (Fig. 4 and 5), the variation of
16 the cellulose conversion ratio over time was quite similar to that of AL180, which shows a
17 same composition. The reason underlying this finding is unclear and hard to explain by
18 referring to the results obtained in this study. Nonetheless, it can be expected that a physical
19 treatment (e.g., ball milling) may expose not only the inner cellulose surface, but also more of

1 the lignin surface covering the cellulose microfibrils. This can also be inferred from the
2 results shown in Fig. 4a (inner panel). Considering that creating a finer particle by crushing
3 could not increase the cellulose-containing surface area of raw rice straw, it is thought that
4 the physically increased surface area obtained by crushing may be a lignin-containing surface.

5 The impact of structural factors (i.e. silica, lignin and hemicellulose) on the long-term
6 cellulase reaction was estimated by multiple linear regression as follows:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3, (11)$$

7 where y and β_i ($i=0-3$) are the cellulose conversion ratio at 72 h as a response variable and
8 the regression coefficients, respectively. x_1 , x_2 and x_3 are independent variables given by:

$$x_1 = \left(\frac{Si}{C_L}\right), x_2 = \left(\frac{L}{C_L}\right) \text{ and } x_3 = \left(\frac{H}{C_L}\right),$$

10 where Si , L , H and C_L are the amounts of silica, lignin, hemicellulose and cellulase loaded
11 for enzymatic saccharification. The statistical analysis was performed using the Origin
12 program. The relationships between the fitted cellulose conversion ratio at 72 h (y) by
13 multiple linear regression and independent variables (x_1 , x_2 and x_3) are shown in Fig.6b. A
14 summary of the multiple linear regression is also listed in Table 4. Considering the p -values
15 and standardized coefficients, silica does not seem to be a crucial structural factor for
16 determining the long-term cellulase reaction, whereas lignin was estimated to have a high
17 impact. Yang et al., also pointed out that the ratio of delignification during pretreatment is a
18 crucial factor affecting enzymatic saccharification of rice straw cellulose [22].

1 Actually, the silica layer works as a barrier to protect the vascular system by limiting the
2 materials' access during the growth phase of the rice crop (see Fig. 1). However, it is
3 expected that the silica layer (micrometer-scale) cannot be a crucial factor in controlling the
4 materials' access once the rice straw is crushed because the effect of the directional property
5 of the silica layer on the limitation of materials' access is weakened, in contrast to that of
6 lignin directly covering the cellulose microfibrils on a nano-scale order. If silica combines
7 with cellulose fibrils in rice straw as the silica-carbohydrates complex found at the early stage
8 of secondary wall formation for some reed grasses [23], silica may hinder the cellulase
9 reaction. However, it is reported that silica mainly binds with phenol- or lignin-moieties but
10 not with carbohydrate in rice plant [12].

11 Hemicellulose located between lignin and cellulose fibrils is one of the barriers that limits
12 the accessibility of cellulase to cellulose fibrils [24, 25], but some recent studies have claimed
13 that hemicellulose has a positive effect on enzymatic saccharification of lignocelluloses [4,
14 26] probably because of the negative effect of hemicellulose on the crystallinity of cellulose
15 fibrils [26]. Although further specific discussion about this is not available in this study, at
16 least hemicellulose does not seem to have a strong negative effect on the long-term
17 saccharification of rice straw cellulose since the standardized coefficient of x_3 was estimated
18 to be a positive number.

1 Consequently, the initial and long-term enzymatic saccharifications of rice straw cellulose
2 are controlled by the cellulose surface area of the cellulose unit and the lignin content,
3 respectively. As mentioned above, it is inferred that the highly sensitive behavior of cellulase
4 to cellulose surface area at the initial reaction stage could be possibly changed to be more
5 sensitive to lignin as the reaction time increased. Nevertheless, a topochemical approach is
6 needed to discuss which lignin (e.g. lignins in middle lamella, primary and secondary cell
7 wall for different vessels and parenchyma cell) is more crucial for enzymatic saccharification
8 of rice straw cellulose [25, 27].

9

10 **3.4. Effective pretreatment of rice straw**

11 Pretreatments of lignocelluloses enhance the accessibility of cellulose to cellulase, namely
12 the biospecific adsorption between cellulose and cellulase is increased by pretreatments.
13 Furthermore, pretreatments have the function of increasing the theoretical yield of the end-
14 products (e.g., glucose or ethanol) in the reaction system (e.g., saccharification or SSF;
15 simultaneous saccharification and fermentation). Considering that solid (substrate) loading
16 for the reaction is physically limited to certain levels to maintain proper mixing conditions, it
17 is considered that pretreatments contribute to obtaining a higher theoretical concentration of
18 the end-products in the reaction system by increasing the cellulose content of rice straw (i.e.,
19 removal of non-cellulosic materials).

1 Likewise, the above mentioned functions of pretreatments are directly affected by the
2 degree of removal of non-cellulosic materials, in particular, the first two are highly related to
3 the removal of lignin. Therefore, a superior pretreatment method of rice straw for the removal
4 of non-cellulosic materials (especially, lignin) and for the preservation of cellulose should be
5 adopted.

6 Actually, there are many pretreatment methods of lignocelluloses such as biological (e.g.,
7 white/brown-rot fungi), chemical (e.g., diluted acid, alkali, wet oxidation, organosolv, and
8 ionic liquid), physicochemical (e.g., ammonia fiber expansion, steam explosion, and CO₂
9 explosion) and physical or mechanochemical (e.g., wet/dry milling, wet disk,
10 ultrasonification, and microwave irradiation) [28]. Nonetheless, alkali-based pretreatment is
11 considered to be the most effective on rice straw, because the lignin content of rice straw is
12 lower than that of woody biomass and the “peeling effect” of alkali can contribute to remove
13 multiple layers of rice straw [29]. In addition, rice straw is rich in *p*-hydroxyphenyl, which
14 solubilizes straw lignin in alkali solutions [30]. As shown in this study, silica and lignin can
15 be simultaneously removed by using alkali. According to the pretreatment conditions (e.g.,
16 temperature, pressure, and alkali loading), hemicellulose can also be removed. Moreover,
17 alkali-based pretreatment is superior to other pretreatments in preserving cellulose. This also
18 agrees with the opinion of previous studies reporting that alkali-based pretreatments (e.g.,
19 ammonia, lime, and NaOH) are more effective on herbaceous biomass and agricultural

1 residues than on woody biomass [29, 31, 32]. However, many factors such as process
2 capacity, targeted end-product, and following treatments should be considered
3 comprehensively in order to choose the appropriate pretreatment method, because each
4 method has its advantages and disadvantages.

5

6 **4. Conclusions**

7 In this study, the structural factors controlling the enzymatic saccharification of rice straw
8 cellulose were examined by preparing structure-modified rice straw samples and the main
9 findings are as follows:

10 (i) The initial enzymatic saccharification of rice straw cellulose was not controlled by the
11 silica or lignin itself but by the initial cellulose surface area of the cellulose unit strongly
12 related to the removal of non-cellulosic materials (e.g. silica, lignin and hemicellulose).

13 (ii) The removal rate of lignin affected the long-term enzymatic saccharification of rice
14 straw cellulose significantly, but that of silica did not.

15 Although lignin removal is crucial to enhance the initial and long-term reaction by
16 exposure of the cellulose surface and reduction of the non-productively bound cellulase,
17 respectively, the removal of other non-cellulosic materials and the preservation of cellulose
18 during the pretreatment are also important, because high cellulose contents in feedstock are
19 preferable for obtaining abundant end-products.

1

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Table 1. The summary of the conditions of the adsorption and enzymatic saccharification experiments.

	Cellulase adsorption		Enzymatic saccharification	Astra blue adsorption
	Equilibrium	Rate		Equilibrium
Buffer (pH)	50 mol m ⁻³ Acetic buffer (pH 4.8)		25 mol m ⁻³ Na ₂ B ₄ O ₇ /HCl (pH 8)	
Temp. (°C)	0–4 °C		50 °C	Room
Time (h)	6	1, 2, 4, 8, and 12	1, 3, 6, 12, 24, 48, and 72	1.5
L/S (dm ³ kg ⁻¹)	100		60	120–150
Adsorbate loading	0.005–1.2	0.13	0.014–0.136 (g g ⁻¹ of cellulose)	0.004–0.2 (g g ⁻¹ of sample)
	(g g ⁻¹ of sample)			
Measurement method	Bradford protein assay		HPLC	UV spectrometer (609 nm)

Table 2. The composition of model rice straw samples and loss of major components during the treatments

	EtOH-extractives	Water-solubles	Ash (silica)	Lignin	Cellulose	Hemi-cellulose	Total mass (%)	Mass loss (%)				
								Si	L	H	Si+L	L+H
RAW	15.35	7.87	8.60	11.27	33.54	15.92	100	-	-	-	-	-
DW	-	9.3	10.15	13.32	39.62	18.8	84.65	-	-	-	-	-
AL20	-	-	5.97	12.55	46.12	22.11	68.44	52.47	23.85	4.91	36.23	12.77
AL40	-	-	3.89	12.65	47.12	22.01	65.77	70.24	26.23	9.02	45.27	16.16
AL90	-	-	3.14	12.18	50.51	27.01	63.19	76.94	31.74	na	51.29	8.91
AL180 (SW)	-	-	1.82	10.75	53.66	28.4	60.99	87.08	41.88	na	61.43	12.2
OX20	-	-	12.87	10.38	42.71	22.48	60.65	9.2	44.17	14.35	29.04	26.71
OX40	-	-	15.9	7.02	48.77	15.95	52.06	3.7	67.59	47.82	39.95	56.02
OX90	-	-	15.51	2.45	60.88	10.13	48.04	13.3	89.55	69.41	56.57	77.76
OX180	-	-	17.03	1.54	62.26	7.9	46.60	7.66	93.64	76.86	56.45	83.82

Si: silica, L: lignin, H: hemicellulose

Table 3. The adsorption parameters for model rice straw samples.

Samples	Cellulase adsorption			Astra blue adsorption	
	q_s	K	$k_{ad} (\times 10^{-4})$	$q_s (\times 10^{-3})$	K
RAW	0.104	0.308	1.416	6.109	10.128
DW	0.115	0.261	1.661	6.689	10.128
AL40	0.126	0.294	1.884	11.221	5.000
AL180	0.173	0.215	1.757	13.360	4.700
OX20	0.373	0.145	1.970	9.212	8.000
OX180	0.500	0.105	2.907	15.779	4.664
SW	0.495	0.184	502	21.912	7.963

Table 4. The summary of the multiple linear regression.

	coefficient (β_i)	SE	<i>t</i> -value	<i>p</i> -value	SC	Statistics
Intercept	1.0470	0.1281	8.1740	5.32E-06		N=15 Adj. R ² =0.8584
x_1	-0.0047	0.0121	-0.3873	0.7059	-0.04977	
x_2	-0.1375	0.0188	-7.3075	1.53E-05	-1.08209	
x_3	0.0411	0.0162	2.5310	0.0279	0.382134	

SE: standard error, SC: standardized coefficient

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Figure 1 (a) Schematic image of the structure of rice straw

Figure 2 (a) Compositional changes of rice straw according to the structure modification (b) SEM images of structure-modified rice straw samples (b-1: RAW; b-2: DW; b-3: Silica skeleton obtained by ashing the raw rice straw at 570 °C; b-4: AL180; b-5: OX180; b-6: SW).

Figure 3 Adsorption isotherms (a) and adsorption rates (b) of cellulase.

Figure 4 Adsorption isotherms of astra blue for model samples (a) and different particle sizes of raw rice straw (a: inner). Changes in cellulose surface area of the unit sample (b-1), cellulose unit (b-3), and cellulose portion (b-2) according to the removal rate of non-cellulosic materials [EtOH-extracts (Wax), ash/silica (Si), lignin (L) and hemicellulose (H)].

Figure 5 Simulation of the initial cellulose conversion ratio of model samples (a) and comparison between the relative R_0 of model samples obtained by astra blue adsorption and model calculation (b).

Figure 6 Variation of the cellulose conversion ratio of the model samples during 72 h (cellulase loading: 0.05 g g⁻¹ of cellulose) (a) and relationships between the fitted cellulose conversion ratio at 72 h by multiple linear regression and independent variables (b).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6