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Pretreatment of Rice Straw by Proton Beam Irradiation for Efficient Enzyme Digestibility

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Abstract Biomass was pretreated with proton beam irradiation (PBI) in order to enhance enzyme digestibility. Rice straw and soaking in aqueous ammonia (SAA)-treated rice straw were treated with 1–25 kGy doses of PBI at a beam energy of 45 MeV. The optimal doses of PBI for efficient sugar recovery were 15 and 3 kGy for rice straw and SAA-treated rice straw, respectively. When PBI was applied to rice straw at 15 kGy, the glucose conversion reached 68% of the theoretical maximum at 72 h. When 3 kGy of PBI was applied to SAA-treated rice straw, approximately 90% of the theoretical glucose conversion was obtained at 12 h compared to a 89% conversion at 48 h. After 2 h of enzymatic saccharification, the initial reaction rates of raw rice straw pretreated with 15 kGy of PBI and SAA-treated rice straw pretreated with 3 kGy of PBI were 1.4×10^{-4} and 9.7×10^{-4} g L^{-1} s^{-1}, respectively. Further, the results of X-ray diffractometry support the effect of PBI on sugar recovery, whereas scanning electron microscopy images revealed a more rugged rice straw surface.

Keywords Proton beam irradiation · Pretreatment · Rice straw · Saccharification · Biomass

Introduction

Renewable biofuels such as bioethanol, biohydrogen, and biobutanol are expected to replace fossil fuel-based energies in the near future and are often produced from edible materials such as sugarcane in Brazil, corn in the United States of America, and wheat in

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France [1, 2]. Biofuel production using primary edible biomass has increased, resulting in destabilization of worldwide crop prices and the onset of food crises in underdeveloped countries [3, 4]. As the feedstock of a second-generation biofuel, inedible biomass is one of the energy resources that could alternate with fossil fuels as well as edible biomasses in the future. In particular, lignocellulosic biomass is a major energy resource due to its abundance and short carbon recycling period.

Cellulose, a component of lignocellulose that can be degraded into the fermentable sugar glucose, consists of bunches of polymeric backbone chains containing β (1→4) glucosidic bonds between each glucose monomer and relatively weak hydrogen bonds between each polymer bunch [5–7]. Thus, enzyme accessibility for cellulosic degradation and solubility is exceedingly low due to the crystalline structure and characteristics of the polymer. One of the main technical barriers for cellulosic ethanol production is the complex structure of lignocellulosic biomass which makes it difficult for enzymes to attack cellulose and liberate fermentable sugars. The rigid and complex structure of cellulose is a large barrier to its efficient enzymatic hydrolysis. To utilize biomass, appropriate pretreatment that improves enzyme accessibility to the substrate via chemical and mechanical modification of the biomass contents and surface is required. For the practical and commercial application of biomass as a biofuel feedstock, enhancement of enzyme digestibility is necessary before saccharification [8–10]. Various pretreatment methods such as reduction of particle size and irradiation exposure have been used to improve enzyme digestibility before saccharification [8].

Thermal pretreatment with or without dilute acid can be used to solubilize hemicelluloses to improve enzyme accessibility [6, 9, 11, 12]. However, these pretreatment processes cause some problems, including inhibition of fermentation. By-products such as furfural, hydroxyl methyl furfural, and volatile products generated by acid pretreatment affect ethanol productivity and conversion [13–16]. Further, the cost of heating cannot be negligible since it directly affects the process cost. Mechanical pretreatments such as crushing and milling do not produce chemical inhibitors, but they do contribute to the process cost similar to thermal and acid pretreatment [9, 11]. To overcome these problems, we considered a physical pretreatment process using irradiation energy, which produces no inhibitors and does not require large amounts of energy. Irradiation pretreatment for the utilization of cellulosic biomass was first reported in 1977 by Aoki et al. [17]. It was found that treatment with gamma-ray irradiation resulted in physical changes in the biomass. Since then, the number of researchers that have studied the relationship between irradiation pretreatment and biomass has increased [18–23]. Mark et al. [19] found a reduction in the crystallinity of biomass upon electron beam irradiation pretreatment, and Bak et al. [20] improved saccharification conversion using electron beam pretreatment. However, as electron beam irradiation is relatively low in energy (usually 1 ~ 5 MeV), a high dose would be needed for pretreatment [19, 20]. Proton beam irradiation (PBI), which provides 45 MeV of irradiation energy, has not yet been used for the pretreatment of biomass. Primarily, PBI is utilized in various areas such as nanotechnology, medical technology, information technology, and biotechnology due to its efficiency and many advantages. PBI could be used at low doses to efficiently degrade biomass. Further, if PBI is conducted following aqueous ammonia pretreatment, which is a well-known method for the efficient removal of lignin from biomass, the yield of fermentable sugars could be improved due to enhanced enzyme accessibility [23].

The objectives of this study were to investigate the pretreatment effect of PBI on rice straw and soaking in aqueous ammonia (SAA)-treated rice straw as well as to estimate the physical changes of the biomass surface after PBI pretreatment. Sugar recovery by enzyme saccharification was also performed to verify the effect of irradiation.

Materials and Methods

Rice Straws and Enzymes

Rice straw and SAA-treated rice straw were stored in the dark at 20 °C at about 70% relative humidity. SAA-treated rice straw was prepared for delignification as follows: Pretreatment with SAA was carried out at 60 °C, 250 rpm, 15% ammonia, 24 h reaction time, and a solid–liquid ratio of 1:12. After pretreatment, solids separated by filtration were washed with distilled water to remove the remaining ammonia until the pH was neutral, followed by drying at 45 °C until the weight did not change. Celluclast® 1.5L from *Trichoderma reesei* and Novozyme188® from *Aspergillus niger* were used for enzymatic saccharification of the biomass. The enzyme activities of Celluclast® 1.5L and Novozyme188® were 60 FPU and 10 CBU, respectively, and enzymes were purchased from Sigma Aldrich Korea.

Proton Beam Irradiation on Biomass

The proton beam (MC-50 Cyclotron) was irradiated at Korea Cancer Center Hospital (KCCH, Seoul, Korea). For PBI pretreatment, the biomass (5 g) was arranged into an array (150 mm in length) and compressed to a sample thickness of about 5 mm between two hard boards. The prepared samples were exposed to between 1- and 25-kGy doses of PBI at a beam energy of 45 MeV. During the irradiation treatment, the samples were turned over 180° to ensure irradiation equality of the biomass samples at a fixed time (Fig. 1a).

Saccharification and Sugar Analysis

Enzymatic hydrolysis of the biomass was performed at 50 °C using Celluclast® 1.5L and Novozyme188® diluted in 0.1 M citrate buffer (pH 4.8) and mixed with distilled water at a 1:1 ratio. The reaction mixture was sampled at regular time intervals during the 24-h reaction time, and samples were analyzed by HPLC (Shimadzu Instrument Set) using an Aminex® HPX-87 H column (Bio-Rad). The HPLC conditions were as follows: temperature of the column and refractive index detector, 55 °C; mobile phase, 0.005 N H_2SO_4; and flow rate, 0.8 mL/min.

Analysis of Biomass Composition

Solid analysis of the biomass was performed in order to estimate its composition and to determine the theoretical maximum according to National Renewable Energy Laboratory standard procedure [24]. For a two-step acid hydrolysis, the biomass was mixed with sulfuric acid (72%, *w*/*w*) for 4 h, followed by dilution with distilled water to an acid content of 4%. After then, the mixture was heated to 121 °C in an autoclave for 1 h. After heating and cooling, the mixture was neutralized with calcium carbonate and the supernatant was analyzed by the above HPLC method for an analysis of the biomass composition.

Analysis of Surface Change

To measure the crystallinity index (CrI), X-ray diffractometry (XRD; X'pert pro, PANanalytical, Netherlands) analysis was performed to take spectra by following the θ–2θ method [25, 26]. XRD was operated at 45 kV, 30 mA, and at room temperature. The anode material was copper

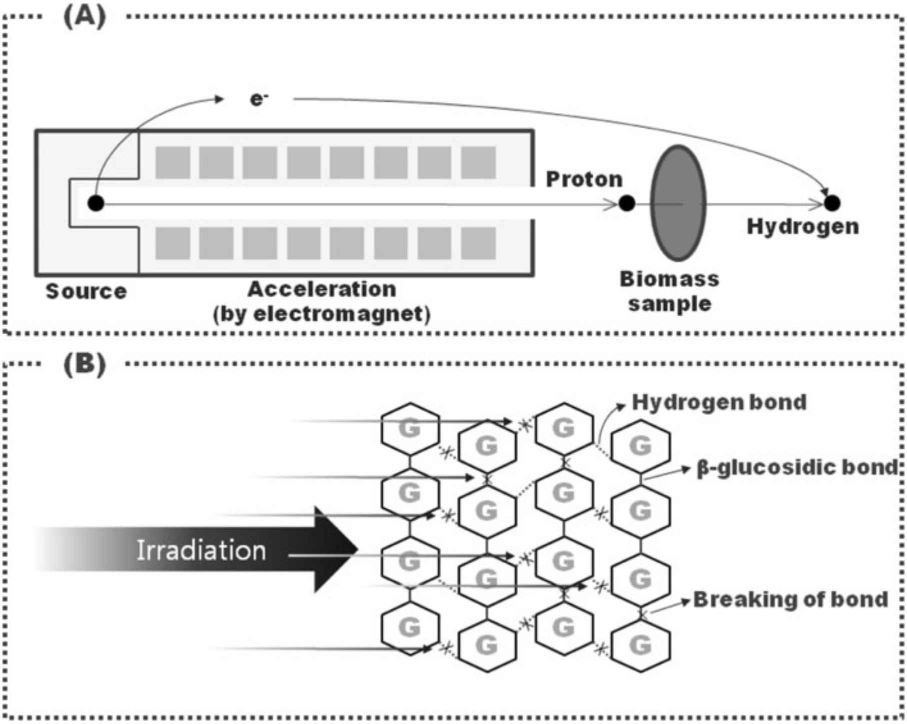

Fig. 1 Schematics of PBI pretreatment of biomass. **a** Schematic apparatus of PBI. **b** Expected effects of PBI

(Cu), and the K-α (irradiation) was 1.544 Å. Scan range was 10° to 90°, and step size was 0.167°. The intensity of amorphous (2θ=18°) and crystal region (2θ=22°) as reported by Segal et al. [25] was used to calculate the CrI. The CrI calculation was as follows:

$$\text{Crystallinity Index (CrI)} = \frac{I_{22^\circ} - I_{18^\circ}}{I_{22^\circ}} \times 100\ (\%)$$

where I_{θ° is intensity at the corresponding θ.

Scanning electron microscopy (SEM) analysis was performed to observe physical changes of the surface of the biomass before and after PBI pretreatment. Samples were dried at 70 °C for 24 h and coated with a thin electrical layer using heavy metal (Pt) for conduction. Images were taken at an accelerating voltage of 15 kV and 1.5 nm of resolving power with magnifications of 300-fold and 700-fold.

Results and Discussion

Effect of Proton Beam Irradiation on Rice Straw

Enzymatic hydrolysis of rice straw pretreated with various dosages of PBI was performed to evaluate enzyme digestibility (Fig. 2). Major linkages within cellulose such as β-glucosidic and hydrogen bonds can be broken by PBI treatment (Fig. 1b). The composition of rice straw ascertained by the solid analysis was as follows: 41.3% glucan, 28.4% xylan, 13.5% lignin, and 1.6% ash. The 41.3% (*w*/*w*) of glucan was the theoretical

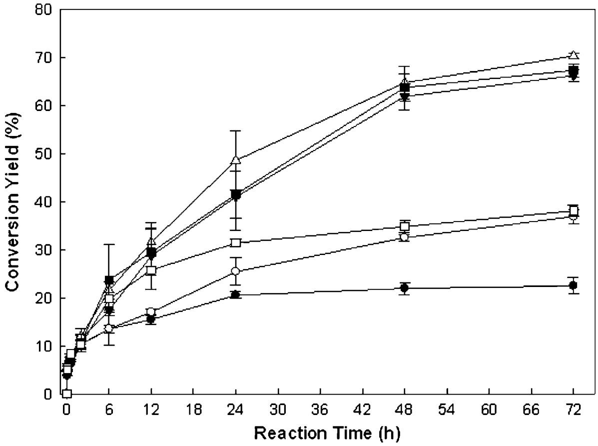

Fig. 2 Effect of PBI pretreatment on rice straw enzyme digestibility. Enzymatic hydrolysis was performed at pH 4.8, 50 °C, and 150 rpm using 60 FPU cellulase (Celluclast® 1.5L) and 15 CBU β-glucosidase (Novozyme® 188) per gram of glucan. *Filled circles*, non-treated rice straw; *open circles*, 5 kGy; *filled inverted triangles*, 10 kGy; *open triangles*, 15 kGy; *filled squares*, 20 kGy; *open squares*, 25 kGy

maximum of glucose conversion. The results of the solid analysis are the mean values of duplicate experiments. The maximum glucose conversion using rice straw pretreated with 15 kGy of PBI was 2.5-fold higher than that of non-treated rice straw (control). Further, the maximum glucose conversion at this dosage was approximately 70% of the theoretical maximum. Rice straw pretreated with 10 or 20 kGy of PBI showed a similar behavior as the 15 kGy-pretreated rice straw. The glucose conversion (approx. 36% of the theoretical maximum) of rice straw pretreated with 5 kGy of PBI was lower than that of the 10–20-kGy-pretreated rice straw. The 5-kGy dose was thought to be insufficient for breaking the hydrogen bonds. An interesting phenomenon was observed with the rice straw pretreated with 25 kGy of PBI; although it was exposed to the highest dose of PBI, glucose conversion was only about 38% of the theoretical maximum. This was thought to be due to the fact that any PBI dose over 20 kGy contained excess energy that broke not only the hydrogen bonds but also the fundamental structure of the glucose monomers. This result is in agreement with the phenomenon reported by Bak et al. [20] and Kumakura and Kaetsu [27]. Therefore, the appropriate dose for the pretreatment of biomass with irradiation may be used to decompose the hydrogen bonds between the bundles of polymeric backbone or the monomers that comprise the polymeric backbone of cellulose.

Table 1 shows the initial reaction rate of saccharification at 2 h. The initial reaction rate of the 15-kGy-pretreated rice straw was about 1.4×10^{-4} g L^{-1} s^{-1}, which was higher than those of others. In particular, the reaction rates of the 10-, 15-, and 20-kGy-pretreated rice straw were stable at 48 h and reached approx. 70% glucose conversion, whereas that of the

Table 1 Comparison of initial reaction rates between 15-kGy-pretreated rice straw and 3-kGy-pretreated SAA-treated rice straw at 2 h of saccharification

Initial reaction rate (× 10^{-4} g/L s)	Dose (kGy)								
	0	1	3	5	7	10	15	20	25
Rice straw	1.18	–	–	1.19	–	1.29	1.37	1.24	1.18
SAA-treated rice straw	7.65	8.05	9.71	8.76	8.42	7.90	6.12	6.10	5.68

– no irradiation

25-kGy-pretreated rice straw decreased and reached its maximum at 24 h. Further, the reaction rate of the 5-kGy-pretreated rice straw decreased in a manner similar to that of non-treated rice straw and slowly increased to 36% of the theoretical maximum at 72 h.

Effect of Proton Beam Irradiation on SAA-Treated Rice Straw

Although the results of PBI pretreatment on rice straw showed an enhancement of glucose recovery (Fig. 2), the reaction rate was relatively lower compared to the results of other well-known pretreatment methods due to microstructural inhibition. However, if the barrier disturbing enzyme access is removed from the rice straw by pretreatment followed by PBI, then the initial reaction rate can be improved. Lignin is a major component of lignocellulosic biomass and can be removed using alkali reagent such as NaOH, KOH, or ammonia. In this study, aqueous ammonia was utilized to remove lignin [28–30]. Soaking in aqueous ammonia was conducted at 60 °C, an ammonia concentration of 15%, and an agitation speed of 250 rpm for 24 h. After ammonia pretreatment, solid analysis was performed to determine the theoretical maximum of glucose recovery, and the composition was found to be 56.04% glucan, 24.2% xylan, and 3.6% lignin and others.

Figure 3 shows the results of glucose recovery of SAA-treated rice straw pretreated with various dosages of PBI. Compared to Fig. 2, these results indicate that the initial reaction rate of SAA-treated rice straw was dramatically increased and the time required to reach maximum conversion was reduced. Glucose recovery of the SAA-treated rice straw (control) reached about 89% of the theoretical glucose conversion at 48 h. Glucose conversion of 90% was obtained at 24 h and 12 h for 1- and 3-kGy-pretreated SAA-treated rice straw, respectively. When SAA-treated rice straw was pretreated with 15–25 kGy of PBI, the conversion rate was markedly decreased to levels below that of the control. These high doses also resulted in the decomposition of the fundamental structure of glucose due to excessive PBI energy.

The initial reaction rates of the SAA-treated rice straw pretreated with 1–10-kGy doses of PBI were higher than that of the control (SAA rice straw, 7.6×10^{-4} g L^{-1} s^{-1}), whereas the initial reaction rates of the straw pretreated with 15–25-kGy doses were slow (Table 1). The maximum initial reaction rate was found to be 9.7×10^{-4} g L^{-1} s^{-1} for rice straw pretreated with 3 kGy of PBI, and this figure was sevenfold higher than that of the 15-kGy-pretreated rice straw. Therefore, a PBI dose of 3 kGy was the optimum for pretreatment of SAA-treated rice straw.

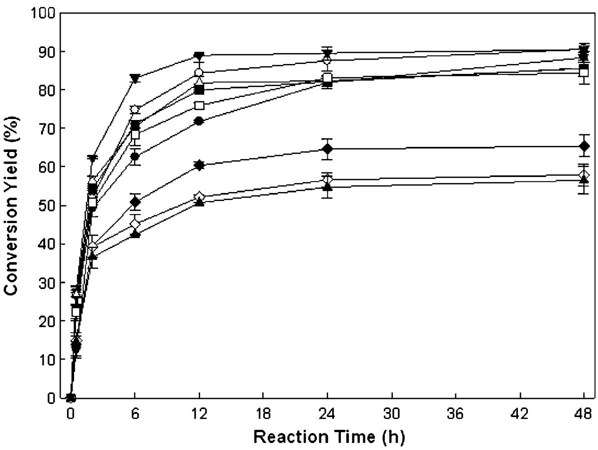

Fig. 3 Effect of PBI pretreatment on SAA-treated rice straw enzyme digestibility. Enzymatic hydrolysis was performed at pH 4.8, 50 °C, and 150 rpm using 60 FPU cellulase (Celluclast® 1.5L) and 15 CBU β-glucosidase (Novozyme® 188) per gram of glucan. *Filled circles*, non-treated; *open circles*, 1 kGy; *filled inverted triangles*, 3 kGy; *open triangles*, 5 kGy; *filled squares*, 7 kGy; *open squares*, 10 kGy; *filled diamonds*, 15 kGy; *open diamonds*, 20 kGy; *filled triangles*, 25 kGy

Though this process (PBI treatment after SAA pretreatment of rice straw) is actually two steps of primary existing pretreatment method and PBI and an increase in process cost is unavoidable, PBI could be made reasonable economically if a commercial PBI apparatus is developed. In case of gamma ray irradiation, commercial apparatus is well developed and it is broadly utilized at sterilization and others. Also, primary pretreatment processes such as aqueous ammonia pretreatment, dilute acid pretreatment, and others must be optimized. Further, as shown by the PBI pretreatment of SAA-treated rice straw, the required dose is relatively low. The irradiation dose (Gy) is directly concerned with time (3 kGy is approximately 3,000 s of exposure.). Therefore, when rice straw was pretreated by aqueous ammonia followed by PBI, the whole process time which is the integration of pretreatment and saccharification was shorter than when rice straw was pretreated with aqueous ammonia only. Thus, PBI can be utilized at biomass pretreatment and contribute to the pretreatment process of sugar platform using biomass.

Effect of PBI on Rice Straw Surface Changes

The relationship between crystallinity index and enzyme digestibility has been studied by a number of researchers [20, 25, 28, 31]. The results of the XRD analysis before and after PBI pretreatment of rice straw and SAA-treated rice straw are shown in Fig. 4. The CrI of the control rice straw and the 15-kGy-pretreated rice straw were 33.38% and 35.72%, respectively. This increase was not dramatic, but the glucose conversion results did show that PBI did have an effect. The primary crystallinity of lignocellulose is generally obstructed by amorphous structures; therefore, PBI pretreatment may have induced some physical changes. The increase in crystallinity could be indicating an exposure of the crystalline surface. However, as evidenced by the change in CrI, any change in the surface was minimal. After all, enzyme accessibility was slightly enhanced but still not sufficient to make the enzyme digestibility of the biomass swelling. Therefore, the glucose conversion of the 15-kGy-pretreated rice straw was higher compared to the non-treated rice straw, but the reaction rate was surprisingly low.

When PBI pretreatment was conducted on SAA-treated rice straw, the CrI before and after pretreatment with 3 kGy of PBI were determined to be 67.11% and 65.58%, respectively. Since SAA pretreatment solubilized a large amount of lignin from rice straw and made the biomass swell, the crystalline portion, which was veiled and supported by the

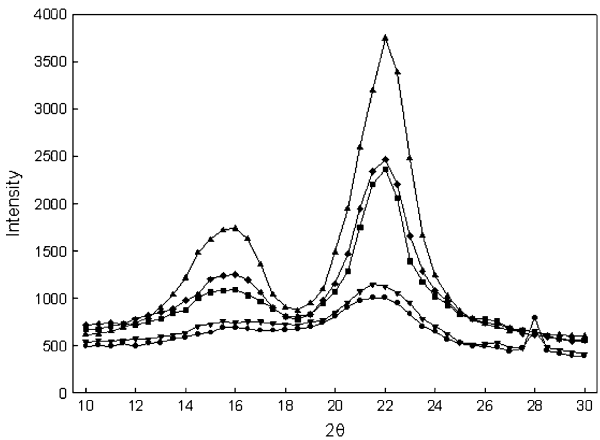

Fig. 4 XRD analysis of various biomasses. *Filled circles*, rice straw; *filled inverted triangles*, 15-kGy-pretreated rice straw; *filled diamonds*, SAA-treated rice straw; *filled squares*, 3-kGy-pretreated SAA-treated rice straw; *filled triangles*, α-cellulose

amorphous structure of lignin, could have been exposed. However, such a situation does not coincide with the increase in CrI. Though the result does not show much proportionality with PBI dosage, the CrI decreased as the PBI dosage decreased. Previous reports suggested that this means that the exposed crystalline surface can be modified by PBI, resulting in a rugged surface that was expressed as a reduced CrI [20, 21]. Due to microstructural modification induced by PBI pretreatment, the liquid permeation containing enzyme might be enhanced. However, as reported before, the conversion rate did not agree with the PBI dose or CrI since the decomposition of the fundamental structure of glucose occurred with excessive PBI doses [20, 27].

When rice straw before and after PBI pretreatment along with a sample of saccharified rice straw pretreated with PBI were analyzed by SEM, the observed physical surface changes were remarkable as the steps progressed. As a result, the PBI pretreatment process induced physical changes of the rice straw surface that are expected to promote enzyme accessibility to the biomass.

Conclusion

In this study, to enhance enzyme digestibility, PBI pretreatment process of rice straw was carried out with different proton beam doses (5–25 kGy) at a beam energy of 45 MeV. PBI pretreatment of SAA-treated rice straw was also conducted to address the problem of microstructural inhibition of rice straw in which lignin is twined around the cellulose. The optimal doses of PBI on rice straw and SAA-treated rice straw for efficient recovery of sugars were 15 and 3 kGy, respectively. The sugar recovery results were approximately 70% and 91% of the theoretical glucose conversion, respectively. In the case of rice straw pretreated with 15 kGy, the conversion was about 2.5-fold higher than that of the control (non-pretreated rice straw). The initial reaction rate (9.7×10^{-4} g L^{-1} s^{-1}) of 3-kGy-pretreated SAA-treated rice straw was sevenfold higher than that of 15-kGy-pretreated rice straw. Further, the structural surface changes of rice straw before and after pretreatment were also observed by SEM and XRD, which were used to check the crystallinity of the pretreated biomass.

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