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ESR Estimation of Lignin Structure of Japanese Cedar Pulverized Using a Vibration Mill with Ring Media

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Lignocellulosic biomass has a recalcitrant structure, with hemicellulose and lignin bound tightly to cellulose. Furthermore, lignin might be an inhibitor factor in enzyme reaction. In our earlier study, a vibration mill with ring media was developed to obtain Japanese cedar fine powder, which reached high saccharification efficiency in enzyme reaction. Nevertheless, the influence of lignin was not examined. This study used a lignin quantification test and ESR analysis to assess the influence of lignin in Japanese cedar powder pulverized using a vibration mill with ring media. Lignin quantification test results show that the Klason lignin content of Japanese cedar remained unchanged irrespective of the pulverization time. The ESR analysis results of the radical amount of Japanese cedar revealed that the radical concentration of Japanese cedar was maximized at the pulverization time of 20 min. Results suggest that improvement of the saccharification efficiency of pulverized powder cleaved the lignin binding in the particle, easing access of the enzyme to cellulose.

リグノセルロース系バイオマスは、ヘミセルロースおよびリグニンがセルロースに強固に結びついた複雑な網目構造を有しており、酵素糖化においてリグニンが阻害要因であると言われている。秋田県立大学では、リング媒体利用粉砕機を開発し、杉を微粉砕することで高い酵素糖化率となる杉粉末を得ているが、リグニンの影響についてはこれまで検討されていない。本研究では、杉をリング媒体利用粉砕機で粉砕した際における杉粉末中のリグニン構造変化、またその変化が酵素糖化率に与える影響について検討した。リグニン定量試験の結果、杉粉末のクラソンリグニン含有量は粉砕時間が増加しても変化しなかった。一方で、ESR 試験より得られたスペクトルから杉粉末中のラジカル濃度を算出した結果、杉粉末中のラジカル濃度は粉砕時間 20 分で最大値を取り、その後は緩やかに減少した。粉砕することでリグニンの主要結合であるフェニルエーテル結合を開裂し、酵素糖化率の向上に寄与しているのではないかと予想された。

Key Words

Lignocellulosic biomass, Pulverization, Enzymatic saccharification, Radical

1. Introduction

Lignocellulose conversion technology has become necessary for processing lignocellulosic biomass into fuel¹⁾. However, improved development of pretreatment is necessary to achieve energy conservation and high productivity because lignocellulosic biomass has a recalcitrant structure, with hemicellulose and lignin bound

tightly to cellulose^{2) 3)}. Furthermore, lignin might be an inhibitor of enzyme reactions³⁾. Therefore, a sufficient pretreatment should be applied to eliminate the inhibition of lignin, and to promote the contact of enzyme to holocellulose (cellulose and hemicellulose).

At Akita Prefectural University, a vibration mill with ring media was developed to produce Japanese cedar fine powder, achieving high saccharification efficiency in the enzyme reaction⁴⁾. The relation between the powder particle size and saccharification efficiency has been examined repeatedly because of the enzyme contact increased as a result of the particle size reduction by pulverization. Although the influence of lignin on saccharification has not been elucidated. Furthermore, Seino et al. reported

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from ESR (Electron Spin Resonance) analysis results that phenyl ether bond, a main bond of lignin, is cleaved during wood pulverization⁵⁾. Clarifying whether cleavage of lignin is related to enzyme reaction is extremely important. Our research team investigated structural changes of lignin generated during pulverization using a vibration mill with ring media to realize efficient pretreatment.

Lignin quantification testing and ESR analysis were conducted in this study to estimate the lignin structure change in Japanese cedar pulverized by a vibration mill with ring media. The influence of lignin on saccharification efficiency was examined.

2. Experimental methods

2.1 Pulverization examination

Fig. 1 presents a vibration mill with ring media. The vibration mill consisted of two mill chambers, ring media installed the mill chamber, a vibrating bed, two 3.7 kW motors, and support springs.

Coarse powder of Japanese cedar was prepared to dry basis moisture content of approximately 15% and particle diameter less than 2 mm. Simultaneous drying and a coarse grinding were carried out using KDS-2 (JP Steel Plantech Co.) to prepare the wood chips for pulverization using the vibration mill with ring media. Then 10 ring media and 800 g Japanese cedar coarse powder were put into the mill chamber. The pulverization conditions were 1600 rpm for 60 min pulverization time.

2.2 Estimation of pulverization effects

After pulverization examination, the Japanese cedar powder particles were measured. The distribution was found using a particle size analyzer (Microtrac MT3300II; Nikkiso Co. Ltd.). The resolution of this analyzer is as high as 2000 μm . A 0.1 g sample was dispersed in 20 ml water with a drop of surfactant using an ultrasonic bath. Then the sample was examined using the particle size analyzer. To evaluate the particle size change during pulverization of the cedar, the median and 80% pass particle size were

estimated from the particle size distribution.

The enzymatic saccharification test of the pulverized powder was applied to ascertain whether the enzymatic saccharification was improved by pulverization using a vibration mill with ring media. The test uses 2 w/v% Japanese cedar powder and 0.1 wt% enzyme (Cellic CTec2; Novozymes Japan Ltd.) for 2 ml acetic acid buffer (5.5 pH and 0.1 M). The mixture of Japanese cedar powder, enzyme, and acetic acid buffer was set into a shaking incubator (BR-23MR; Taitec Corp.) at 50 °C, with 150 rpm agitation speed, for 48 h reaction time. Then the saccharification efficiency was estimated using a spectrophotometer (U-3900H; Hitachi High-Technologies Corp.) with 420 nm wavelength using Schales method and a standard curve of glucose. For this enzymatic saccharification test, the enzymatic saccharification efficiency was defined as a ratio of the amounts of monosaccharides to those of holocellulose in terms of weight.

A lignin quantification test of pulverized powder was applied to estimate the change of lignin contents during pulverization. After using sulfuric acid method in the lignin quantification test, a refluxing for acid hydrolysis treatment of a sample was carried out using the refluxing equipment in Fig. 2. The lignin quantification test procedure was conducted as follows. 1) Absolutely dried Japanese cedar powder 1 g and 72 wt% sulfuric acid 15 ml were mixed at room temperature. They were left to stand for 4 h to react sufficiently. 2) The mixture of Japanese cedar powder and sulfuric acid was diluted to 3 wt% sulfuric acid concentration with distilled water. 3) The diluted mixture of Japanese cedar powder and sulfuric acid was refluxed for 4 h to hydrolyze it sufficiently. 4) A refluxed mixture of Japanese cedar powder and sulfuric acid were filtrated to separate a solid and liquid. Then the separated solid was dried. The

Fig. 1 Vibration mill with ring media

Fig. 2 Refluxing equipment for acid hydrolysis treatment of the pulverized powder sample

dried solid was weighed to estimate the Klason lignin (acid insoluble lignin) content. In this lignin quantification test, the Klason lignin content was defined using a ratio of the amounts of the Klason lignin to absolutely dried Japanese cedar powder by weight. The acid-soluble lignin content in Japanese cedar is only about 0.2 wt%, which is regarded as negligible.

To investigate the amount of bond cleavage (radical) of lignin in powder, ESR analysis was done with an ESR spectrometer (EMXplus; Bruker BioSpin). Before ESR analysis, Japanese cedar powder was dried to avoid effects of water in the sample. Then the absolutely dried sample was measured to ascertain the spectral intensity using the ESR spectrometer with output of 0.1 mW. The measured spectral intensity was calculated with double integration using software (Xenon; Bruker BioSpin) to estimate the radical concentration of Japanese cedar.

3. Results and Discussion

Fig. 3 shows particle size distributions of Japanese cedar powder before and after pulverization. From the measured distributions, the median and 80% pass particle size were calculated. Results show that the median pass particle size at pulverization times of 0, 20, 40, and 60 min were, respectively, 408.3, 47.63, 46.85, and 43.67 μm . The 80% pass particle sizes at pulverization times of 0, 20, 40 and 60 min were, respectively, 619.0, 79.01, 70.4, and 67.49 μm . Therefore, the powder particle size was almost unchanged after pulverization for 20 min.

Fig. 4 depicts a relation between the pulverization time and the enzymatic saccharification efficiency. The enzymatic saccharification efficiency increased concomitantly with increasing pulverization time. Results show that the pulverized powder had improved enzymatic saccharification along with increasing pulverization time. However, the powder particle size was unchanged after pulverization time of 20 min, which suggests that the improved enzymatic saccharification is attributable to reasons other rather than the powder particle size. Endo reported that enzymatic saccharification in wet-grinding improves because of the increased enzyme reaction area created by advanced fibrillation of cellulose⁶⁾. However, during dry grinding using a vibration mill with ring media in this study, lignocellulose destruction probably occurred rather than cellulose fibrillation because this method uses high impact force. Actually, an earlier report describes that enzymatic saccharification was not changed by application of ligninolytic enzyme to Japanese cedar powder pulverized by a vibration mill using ring media⁷⁾. Results suggest that enzymatic saccharification was unchanged because this

pulverization method decomposed the lignin sufficiently.

Fig. 5 portrays a relation between the pulverization time and the Klason lignin content. In this result, the Klason lignin content of Japanese cedar was maintained over 30% during this pulverization time. The Klason lignin content was invariant irrespective of the pulverization time. The enzymatic saccharification efficiency of Japanese

Fig. 3 Particle size distributions of Japanese cedar powder before and after pulverization

Fig. 4 Pulverization time and enzymatic saccharification efficiency

Fig. 5 Pulverization time and Klason lignin content

cedar was improved even if the amount of lignin was unchanged. However, the structural change of lignocellulosic biomass might have occurred even if the lignin amount was unchanged. The following are ESR analysis results of investigations of the lignin decomposition to estimate the structure change.

Fig. 6 presents the measured ESR spectral intensity before and after pulverization. A peak of the ESR spectral intensity from pulverized powder was higher than that before pulverization. Fig. 7 shows the radical concentration changes with pulverization time. Regarding the radical concentration of Japanese cedar from the calculated ESR spectral intensity, the radical concentration increased rapidly at the initial stage of pulverization and reached a maximum value at the pulverization time of 20 min. These results confirmed that pulverized powder generated radicals. Considering that the lignin dissociation energy is lower than that of saccharides and that radicals can be stably detected, results suggest that phenyl ether bonds (α -O-4 and β -O-4 bonds), which are the main bonds of lignin, were cleaved, generating phenoxy radicals⁵⁾ during vibration milling using ring media pulverization of Japanese cedar. Results suggest that improvement of the

enzymatic saccharification efficiency of pulverized powder occurred because lignin binding in particles was cleaved. The enzyme accessed the cellulose more easily because the exposed amount of cellulose increased as a result of lignin cleavage. However, the radical concentration decreased slowly after 20 min pulverization time. Recondensation is known to have occurred because of secondary reactions in the lignin decomposition⁸⁾. Therefore, from the decreased radical concentration after pulverization time of 20 min, we inferred that the progress of secondary reaction was exceeded the generated radicals because radical formation is suppressed as molecular weights decreased. However, Kåldström *et al.*⁹⁾ reported that lignin fragments formed by the cleavage of the α -O-4 and β -O-4 bonds might react with vicinal sugars from hemicellulose because of their mutual proximity. By contrast, the formation of a covalent linkage of lignin fragments to glucan fragments is expected to occur only rarely because the cellulosic fibers constitute the core of the lignocellulose composite⁹⁾. Results suggest that decomposed lignin continues to be liberated from cellulose, even if recondensation reaction occurs. Additionally, results suggest that enzymatic saccharification is promoted by increasing the exposed cellulose. For these reasons, it can be expected that one factor improving the enzymatic saccharification in this pulverization method is an increase in the exposure of cellulose because of lignin decomposition.

4. Conclusion

This study investigated the influence of lignin for enzymatic saccharification of Japanese cedar pulverized by the vibration mill with ring media. The pulverized powder was evaluated using the enzymatic saccharification test, the lignin quantification test, and ESR analysis. The main results obtained from this study can be summarized as presented below.

- (1) The pulverized powder particle size was unchanged beyond pulverization for 20 min.
- (2) The enzymatic saccharification efficiency of Japanese cedar increased concomitantly with increased pulverization time.
- (3) The Japanese cedar lignin content was invariant despite increasing pulverization time.
- (4) The peak of the ESR spectral intensity from pulverized powder was higher than that before pulverization.
- (5) The radical concentration of Japanese cedar was maximized at the pulverization time of 20 min. It decreased slowly after 20 min pulverization time.
- (6) Improvement of the enzymatic saccharification efficiency of pulverized powder was expected because of the phenoxy radical was generated and the enzyme accessed

Fig. 6 Measured ESR spectral intensity before and after pulverization

Fig. 7 Pulverization time and radical concentration

the cellulose more easily.

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