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Xinglian Geng · Ryuichiro Kondo · Kokki Sakai
Zhongzheng Lee

Improvement of dispersing property of sodium sulfite–formaldehyde–anthraquinone pulping effluent by treatment with *Coriolus versicolor**

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Abstract A strain of the fungus *Coriolus versicolor* was inoculated periodically into potato dextrose agar (PDA) slants containing the effluent to enhance the natural ability to grow in the effluent. The acclimated strain grown in the 50% effluent-containing PDA slant and the original strain were employed to treat the effluent. The acclimated strain could grow in a higher concentration of the effluent than the original unacclimated one. Both the original and acclimated strains improved the dispersing ability of the effluent, especially the acclimated strain because of its higher laccase secretion. The dispersing ability of the SFP effluent was improved to a level comparable to a commercial lignosulfonate product because it was strongly polymerized by the fungus. During the fungal treatment, more than 50% of the sugars were removed from the effluent, thereby increasing the purity of the SFP lignin product.

Key words *Coriolus versicolor* · Na_2SO_3-HCHO-AQ pulping effluent · Polymerization · Lignosulfonate · Laccase

Introduction

There is a large quantity of degraded lignin, cellulose, and hemicellulose as wastes produced from the pulp and paper industry. Most of the organic wastes are recycled as energy by combustion, but it is difficult for the effluent from the nonwood pulping process to be recycled in this way owing to its high silica content, which comes from the raw material. The pulp and paper industry has been forced to implement relevant changes in their manufacturing processes. One technique is the development of a high-yield sulfite process and utilization of its pulping effluent to produce valuable by-products. The Na_2SO_3-HCHO-AQ pulping (SFP) process[1] is a new sulfite cooking process that has been put into commercial practice to produce wheat straw pulp in China, and the pulping effluent is now dried directly and used as lignosulfonate products.

It is well known that lignosulfonate produced from the spent liquor of sulfite pulping processes has been widely used in industries as concrete additives, dispersants for carbon black, wax and dyes, oil well drilling mud additives, and so on.[2,3] The dispersing ability of lignosulfonate is one of the most important properties. Many of the chemical properties of lignosulfonate (e.g., molecular weights, sulfonic acid group content, and phenolic hydroxy group content) have an effect on its dispersing ability.[4] Moreover, SFP lignin products are hygroscopic because of the existence of sugar in it. Therefore, it is necessary to improve the purity of the SFP lignosulfonate product.

Three phenol oxidases, laccase I, II, and III, have been fractionated from the extracellular enzymes of the white-rot fungus *Coriolus versicolor*; laccase I mainly polymerizes lignin, and laccase III depolymerizes it.[5,6] The fungus is well known for its successful use for decomposing lignin in pulp by a biobleaching process and treatment of conventional chlorine bleaching effluents.[7,8] However, there is a tendency for lignosulfonate to be polymerized while chlorolignin[9] and alkaline lignin were depolymerized[10] by *C. versicolor* because the sulfonic acid groups may in some way inhibit depolymerization.

The motivation for this research is to find an effective utilization of the SFP effluent. To improve the dispersing ability and improve the purity of SFP products as dispersants, the SFP effluent was analyzed and then treated with *C. versicolor* to polymerize the SFP lignosulfonate components (SFP-LS) and decrease the sugar content in the efflu-

X. Geng · R. Kondo (✉) · K. Sakai
Department of Forest Products, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan
Tel. +81-92-642-2989; Fax +81-92-642-3078
e-mail: kondo @agr.kyushu-u.ac.jp

Z. Lee
College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, Peoples Republic of China

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ent. Some preliminary studies on this effluent have shown that *C. versicolor* has low ability to grow in the effluent, so a strain of *C. versicolor* was acclimated to have a high capacity of growing in the effluent by inoculating it on effluent-contained media. The original and acclimated strains were used to treat the effluent under the same incubation conditions. In addition, laccase crude preparation was employed to treat the effluent to elucidate the effect of polymerization of lignosulfonate on the dispersing ability.

Materials and methods

Chemicals. A commercial lignosulfonate product (CM-LS) was kindly provided by Nippon Paper Industries, Japan. All reagents were at least of analytical grade and purchased from Wako Pure Chemical Industries, Japan.

Microorganisms. *Coriolus versicolor* (IFO 6482) was maintained on 3.9% (w/v) potato dextrose agar (PDA) slants grown at 25°C and were kept at 4°C. All cultivation steps were carried out under sterile conditions.

Effluent. The effluent used in this study was black liquor obtained from a pulping mill (Xuzhou, China) where sodium sulfite, formaldehyde, and anthraquinone (AQ) were used to cook wheat straw. The murky brown effluent was vacuum-dried and stored in a solid state. The solid was dissolved in water and filtrated with glass fiber paper GA 200 before being used. No changes in the characteristics investigated here were observed before and after vacuum drying.

Strain acclimation. To enhance the natural ability to grow in the effluent, the strain was subcultured on PDA medium enriched with 10% (w/w) SFP effluent at 25°C step by step. The grown strain on 10% effluent-containing PDA medium was then subcultured repetitively on PDA medium enriched with increasingly higher concentrations of effluent at 20%, 30%, 40%, and 50% (w/w).

Culture conditions. For stationary cultures, five 5 mm diameter mycelia agar plugs derived from PDA plates incubated at 30°C were inoculated into 200-ml Erlenmeyer flasks containing 20 ml effluent and then incubated at 30°C. For shaking cultures, each Erlenmeyer flask (500 ml) containing 250 ml glucose-copper-peptone (GCP) (30 g glucose, 10 g peptone, 1.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 2 mg thiamine-HCl, and 16 mg $CuSO_4 \cdot 5H_2O$ per liter) liquid medium (pH 5.0) was inoculated into an agar plate that was incubated at 30°C and homogenized for 10 s. The flasks were incubated on a rotary shaker at 30°C and 140 rpm for 6 days. On day 6 of incubation, 50 ml of the culture was taken, homogenized again, and then further precultured in a 500-ml Erlenmeyer flask with 200 ml GCP medium and incubated as described above. On day 7 of incubation, the precultured mycelium of *C. versicolor* was separated from the fungal medium by filtration and used to treat the effluent. For polymerization of the SFP-LS, 0.53 g dry weight of mycelium was aseptically added to 200 ml effluent of 5 g/l in a 500-ml Erlenmeyer flask and then incubated on a rotary shaker at 30°C and 140 rpm. The effluent used in vivo was adjusted to pH 6.5 before autoclaving. All media were sterilized at 121°C for 20 min. No nutrient was added to the effluent during the treatment.

Crude laccase preparation. Cultivation of *C. versicolor* was carried out in 500-ml Erlenmeyer flasks containing 200 ml liquid medium (pH 4.5). The medium contained (per liter) 20 g glucose, 2.5 g L-asparagine, 0.15 g L-phenylalanine, 27.5 mg adenine, 50 μg thiamine-HCl, 1 g KH_2PO_4, 0.1 g $Na_2HPO_4 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, 10 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnSO_4 \cdot 4H_2O$, 1 mg $ZnSO_4$, and 2 mg $CuSO_4 \cdot 5H_2O$. The flasks were inoculated with an average dry weight of mycelia 1.3 g/l of PDA cultures. At the final concentrations of 0.2 mM and 0.1 mM in the flask, 2,5-xylidine as elicitor was added to the mycelia after 3 and 5 days of incubation, respectively. After 7 days of incubation on a horizontal shaker at 30°C and 140 rpm, the culture fluid was separated from the fungal mycelium by filtration with Advantec membrane filters (pore size 0.45 μm), precipitated with $(NH_4)_2SO_4$, dialyzed, and concentrated by two steps of ultrafiltration. The crude preparation exhibited a laccase activity of 29 μkat/ml. It was kept at 4°C before use.

Enzyme assay. Laccase activity was assayed at 37°C in reaction mixtures (3 ml) containing 3 mM 2,6-dimethoxyphenol, 50 mM malonate buffer (pH 4.5), and appropriate amounts of culture supernatant. The increase in absorbance at 470 nm due to the oxidation of 2,6-dimethoxyphenol was measured to calculate laccase activity.[11]

Laccase treatment. The treatment of the SFP effluent with 67 nkat/ml of final laccase activity was carried out in 500-ml Erlenmeyer flasks containing 200 ml SFP effluent of 5 g/l, pH of which was adjusted to 4.5. A control was run with distilled water instead of the laccase preparation. Samples were collected at the beginning and after 12, 24, 36, and 48 h; the reaction was stopped by heating in boiling water for 10 min. The laccase activity was analyzed separately.

Evaluation of the dispersing ability of lignosulfonate. The effect of lignosulfonate on the viscosity of 50% $CaCO_3$ slurry was used to evaluate its dispersing ability. After lignosulfonate was added, 50% $CaCO_3$ slurry was dispersed with a T.K. Homo mixer for 20 min at 7500 rpm, and the viscosity of the slurry was then determined with a BL viscometer at 30°C. The lower the viscosity of the slurry, the better is the effluent's dispersing ability. The pH of the lignosulfonate solution was adjusted to 7.0–7.5 before it was mixed with $CaCO_3$ solid.

Other analytical methods. Total sugar and uronic acid were determined with the phenol-sulfuric acid method[12] and the carbozole method,[13] respectively. The lignosulfonic acid content was determined by weight after separation with

Table 1. Characteristics of the SFP effluent

pH	7.8
Total dissolved solid (g/l)	20.0
Total sugar (g/l)	3.0
Lignosulfonic acid (g/l)	8.3
Ash (g/l)	9.3
Uronic acid (g/l)	1.6
Reducing sugar (g/l)	1.0

acetone[14] and ion exchange. Ash content was analyzed with Tappi standard method T629m-53.

Gel permeation chromatography (GPC) was done with a column of 40cm length and 3.5mm diameter filled with Sephadex G-75 column and 0.25M NaCl as eluant. After collection of fractions, absorbance at 280nm was measured with a Beckman DU 640 spectrophotometer.

Results and discussion

Effluent characteristics

The effluent used in this study was the black liquor from the Na_2SO_3-HCHO-AQ pulping process of wheat straw. Consequently, it contains all the organic and inorganic components dissolved from the raw material and residual pulping chemicals. The main characteristics of the effluent after dissolution into 20g/l and filtration with glass fiber paper GA 200 are shown in Table 1. The total sugar content was 15% of dissolved solid (DS) in the effluent. Therefore it seems that it is not necessary to add any external carbon source to grow the fungus because oligosaccharides and uronic acid, which are formed during the cooking process from hemicellulose or cellulose, can be used as a carbon source for fungal growth. The lignosulfonic acid content was only 41.6% of DS because of the high ash content in the effluent. The high ash content was caused by the high silica content from the pulping raw material, wheat straw, and heavy metals from water used during the pulping process (data not shown).

Strain acclimation

Whereas the original strain could not grow on the PDA medium containing more than 30% (w/w) of the SFP effluent, the acclimated strain could grow on the PDA medium containing 50% (w/w) of the effluent. The strain grown on 50% (w/w) effluent-contained PDA slant was used as the acclimated strain in this study.

Table 2 shows growth of the original and acclimated strains in the SFP effluent at different concentrations under stationary conditions. The acclimated strain could grow in the 20g/l effluent, but the growth of the original strain was inhibited when the concentration of the effluent was over 10g/l. This means the acclimated strain of *C. versicolor* could grow in higher concentrations of the SFP effluent than the original one.

Table 2. Growth of the original and acclimated strains in the SFP effluent at various concentrations

Strain	Growth at effluent concentrations of 2–25g/l						
	2	5	8	10	15	20	25
Original	G(2)	G(2)	G(3)	G(7)	N(10)	N(10)	
Acclimated	G(1)	G(2)	G(2)	G(3)	G(5)	G(7)	N(10)

G(2), growth was observed after 2 days of incubation; N(10), no growth was observed even after 10 days of incubation.

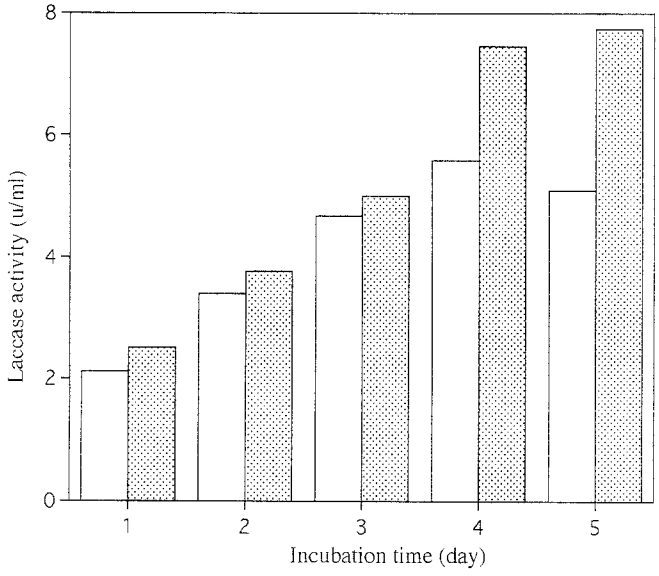

Fig. 1. Laccase activity in the effluent during treatment with the original (*open bars*) or acclimated (*gray bars*) strain under shaking condition

Polymerization of SFP-LS in the effluent with the fungus

Laccases usually polymerize lignin substrates rather than decompose them,[8] especially for lignosulfonate, although the mechanisms responsible for the competition between polymerization and depolymerization of lignin substrates are poorly understood. Both the original and the acclimated strains were employed to treat 5g/l of SFP effluent to polymerize lignosulfonate components in it.

The laccase activities in the effluent secreted by the original and acclimated strains are compared in Fig. 1. Along the incubation, the laccase activity of the acclimated strain was much higher than that of the original one. This shows the higher suitability of the acclimated strain for effluent treatment than the unacclimated one.

Figure 2 shows the molecular weight distributions of the untreated effluent as control and the treated effluent with the acclimated strain for 3 days and CM-LS analyzed by GPC. The molecular weight of SFP-LS in the treated effluent was increased because SFP-LS was polymerized by the fungus and the molecular weight of the main part was close to that of CM-LS.

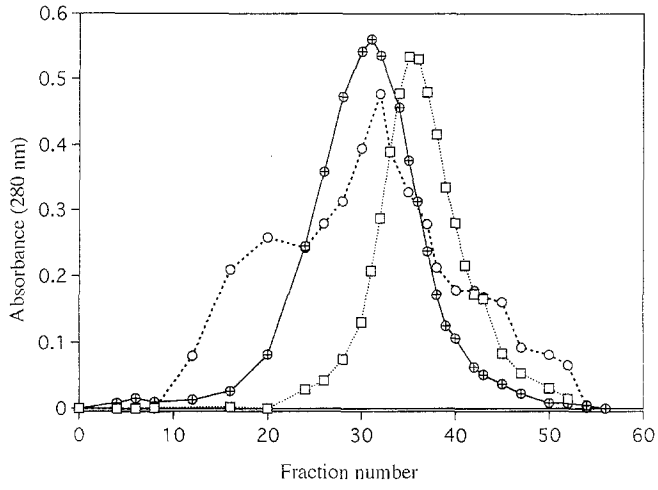

Fig. 2. Distribution of the molecular weight of lignosulfonates. Untreated SFP-LS (*squares*), treated SFP-LS (*crossed circles*), and CM-LS (*open circles*)

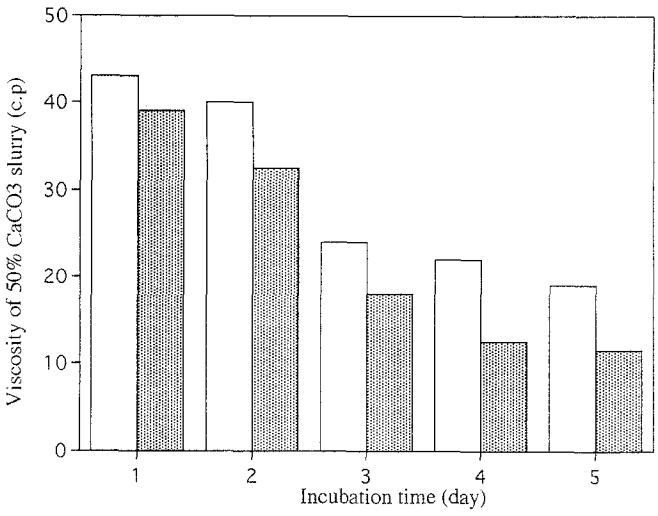

Fig. 4. Dispersing ability of the effluents treated with the original (*open bars*) or acclimated (*gray bars*) strain under shaking condition (0.5%, dissolved solid of effluents/$CaCO_3$ solid)

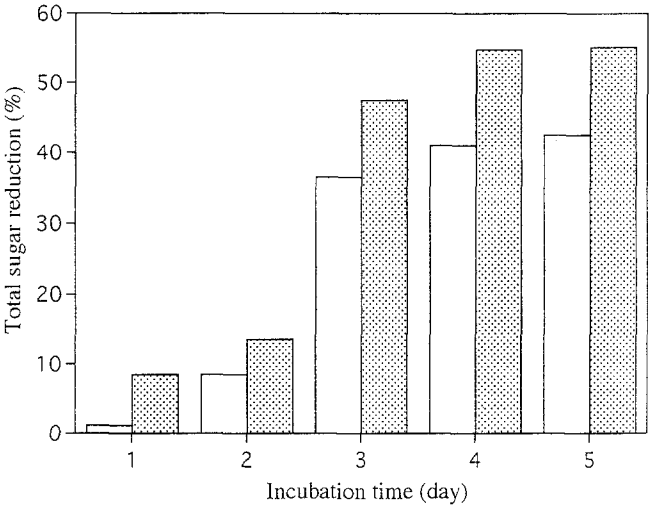

Fig. 3. Total sugar reduction in the effluent after treatment with the original (*open bars*) or acclimated (*gray bars*) strain under shaking condition

Reduction of sugar content in the effluent with the fungus

The total sugar reductions of the effluent by the original and the acclimated strains are shown in Fig. 3. Both acclimated and unacclimated strains could remove sugars quickly during 3 days of incubation. The total sugar reduction by the acclimated strain was more than that of the original one. This is consistent with the observation during the experiment that the growth of the acclimated strain was better than that of the original strain. This shows that sugar consumption was directly connected with fungal growth. Because the original and acclimated strains removed 42% and 54% of the sugars, respectively, the purity of SFP-LS in the effluent increased, resulting in a decrease of its hydrogroscopicity.

Improvement of the dispersing ability of SFP-LS with the fungus

The dispersing ability of lignosulfonate can be evaluated by the decrease in viscosity of a 50% $CaCO_3$-H_2O slurry. The dispersing abilities of the treated effluents with both the original and acclimated strains are compared in Fig. 4. When 0.5% (w/w) dissolved solid of the effluents was added to $CaCO_3$ solid, the dispersing ability of treated effluent with the acclimated strain was better than that with the unacclimated one. For both of them, the increase of dispersing abilities became slower after 4 days of incubation, although the laccase activity of the acclimated strain was still increasing. This means that an incubation time longer than 4 days and laccase activity higher than 117 nkat/ml are not necessary to improve the SFP-LS dispersing ability.

Figure 5 shows the effect of the lignosulfonate dose on the viscosity of the 50% $CaCO_3$ slurry. As can be seen, the addition of lignosulfonate has a great effect on its dispersing ability. The optimum dosage of the untreated effluent for the dispersing ability was 1.5% on $CaCO_3$ solid, whereas those of the effluents treated with the original and the acclimated strains for 3 days decreased to 1.0% and 0.5%, respectively. At the optimum dosages of the untreated and treated effluents, the dispersing ability of the treated effluents was better than that of the untreated effluent. Particularly, the dispersing ability of the treated effluent with the acclimated strain became as good as that of CM-LS.

Improvement of the dispersing ability of SFP-LS with laccase

Was the improvement of the dispersing ability of SFP-LS caused by polymerization of lignosulfonate or the contribu-

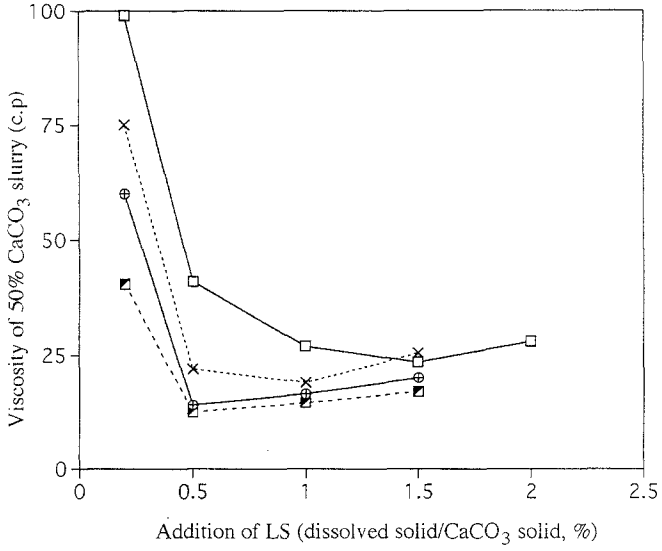

Fig. 5. Dispersing ability of the effluents. Untreated sodium sulfite–formaldehyde–anthraquinone lignosulfonate (SFP-LS) (*open squares*), treated SFP-LS with the original (*X*) or acclimated (*crossed circles*) strain, and Commercial lignosulfonate (CM-LS) (*half-filled squares*)

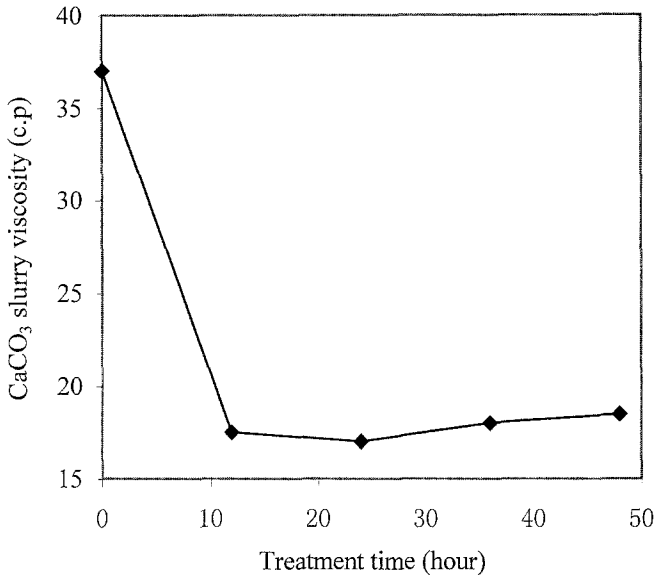

Fig. 6. Dispersing ability of the effluent during the treatment with crude laccase preparation. Effluent dosage as dissolved solid was 0.5% on $CaCO_3$ solid

tion of total sugar content reduction when it was treated with the fungus? The SFP effluent of 5 g/l was treated with crude laccase preparation at a 67 nkat/ml final laccase dose on a rotary shaker at 140 rpm and 30°C for 48 h. After collecting the samples at 12, 24, 36, and 48 h, the dispersing ability of the effluent was determined as shown in Fig. 6. The dispersing ability reached the optimum state after 24 h of treatment. The increased dispersing ability by laccase was comparable to the increase by the original strain. This is may due to the similar laccase dose secreted by the original strain on day 3 of incubation (Fig. 1).

The total sugar content remained at a stable level when SFP-LS was polymerized by laccase during the treatment with laccase (data not shown). This indicates that the increased dispersing ability of the SFP effluent was mainly caused by polymerization of SFP-LS.

Conclusions

The acclimated strain, grown on the 50% effluent-containing PDA medium, had higher suitability for the effluent treatment than the unacclimated strain. Both the original and the acclimated strains improved the dispersing ability of the effluent, especially the acclimated strain. The dispersing ability of the SFP effluent was improved by the acclimated strain to a level comparable to a commercial lignosulfonate product. During the fungal treatment, more than 50% of the sugars were removed from the effluent, thereby increasing the purity of the SFP lignin products. Laccase secreted by *C. versicolor* could polymerize SFP lignosulfonate, thereby increasing the dispersing ability of the SFP lignin products.

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References

1. He W, Tai D-S, Lee Z-Z (1993) Effect of formaldehyde during alkaine sulfite pulping of wheat straw. In: Proceedings of the Seventh International Symposium on Wood and Pulping Chemistry, Beijing, China, May 25–28, 1993, pp 523–531
2. Pearl IA (1982) Utilization of by-products of the pulp and paper industry. Tappi 65(5):68–73
3. Turner FS (1988) Process for making drilling fluid additives containing lignosulfonates. US patent 4755307
4. He W, Tai D-S, Lee Z-Z, Nakano J (1995) Sodium sulfite formaldehyde-quinone (SFQ) cooking of wheat straw and characteristics of SFQ lignins. In: Abstracts of the 40th anniversary conference of the JWRS, April 1995, Tokyo, p 393
5. Morohoshi N, Wariishi H, Muraiso C, Nagai T, Haraguchi T (1987) Degradation of lignin by the extracellular enzymes of *Coriolus versicolor*. IV. Properties of three laccase fractions fractionated from the extracellular enzymes. Mokuzai Gakkaishi 33(3):218–225
6. Morohoshi N, Fujita K, Wariishi H, Haraguchi T (1987) Degradation of lignin by the extracellular enzymes of *Coriolus versicolor*. V. Degradation of isolated lignin fractions by laccases I, II, and III. Mokuzai Gakkaishi 33:310–315
7. Archibald FS, Bourbonnais R, Jurasek L, Paice MG, Reid ID (1997) Kraft pulp bleaching and delignification by *Trametes versicolor*. J Biotechnol 53:215–236
8. Bergbauer M, Eggert C, Kraepelin G (1991) Degradation of chlorinated lignin compounds in a bleach plant effluent by the white-rot fungus *Coriolus versicolor*. Appl Microbiol Biotechnol 35:105–109
9. Bergbauer M, Eggert C, Kraepelin G (1990) Polymerization and depolymerization of waste water lignins by white-rot fungi. In: Kirk TK, Chang H-M (eds) Biotechnology in pulp and paper manufacture. Butterworth-Heinemann, Boston, pp 263–269

10. Manzanares P, Fajardo S, Cabanas A, Martin C (1996) Study of the ligninolytic activity in straw soda pulping effluents decolourized by *Trametes versicolor*. In: Srebotnik E, Messner K (eds) Biotechnology in pulp and paper industry. Facultas-Universitätsverlag, Vienna, pp 267–270
11. Hirai H, Kondo R, Sakai K (1994) Screening of lignin-degrading fungi and their ligninolytic enzyme activities during biological bleaching of kraft pulp. Mokuzai Gakkaishi 40:980–986
12. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
13. Bitter T, Muir HM (1962) A modified uronic acid carbazole reaction. Anal Biochem 4:330–334
14. Yokoyama S, Ikari Y (1980) Separation of organic components of NSSCP spent liquor by acetone and their characteristics. Jpn Tappi 34(7):48–56