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Influence of rice straw polyphenols on cellulase production by *Trichoderma reesei*

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In this study, we found that during cellulase production by *Trichoderma reesei* large amounts of polyphenols were released from rice straw when the latter was used as the carbon source. We identified and quantified the phenolic compounds in rice straw and investigated the effects of the phenolic compounds on cellulase production by *T. reesei*. The phenolic compounds of rice straw mainly consisted of phenolic acids and tannins. Coumaric acid (CA) and ferulic acid (FA) were the predominant phenolic acids, which inhibited cellulase production by *T. reesei*. When the concentrations of CA and FA in the broth increased to 0.06 g/L, cellulase activity decreased by 23% compared with that in the control culture. Even though the rice straw had a lower tannin than phenolic acid content, the tannins had a greater inhibitory effect than the phenolic acids on cellulase production by *T. reesei*. Tannin concentrations greater than 0.3 g/L completely inhibited cellulase production. Thus, phenolic compounds, especially tannins are the major inhibitors of cellulase production by *T. reesei*. Therefore, we studied the effects of pretreatments on the release of phenolic compounds. Ball milling played an important role in the release of FA and CA, and hot water extraction was highly efficient in removing tannins. By combining ball milling with extraction by water, the 2-fold higher cellulase activity than in the control culture was obtained.

[**Key words:** Rice straw; Phenolic compounds; Tannin; Phenolic acid; Cellulase; *Trichoderma reesei*]

Biofuels are alternatives to petroleum-derived fuels, and their demand is increasing because of fossil fuel shortages and environmental issues (1,2). The production of biofuels derived from crops, such as sugarcane and corn, using limited land resources would potentially cause a biomass deficit. However, the use of lignocellulosic materials, such as forest and agricultural residues, as a potential alternative feedstock for the production of bioethanol would not affect the food supply. The bioconversion of lignocellulosic biomass in ethanol production requires cellulases that can hydrolyze complex polysaccharides in lignocellulosic biomass. Commercial cellulase is commonly produced by fungi, particularly *Trichoderma reesei*, which has been researched extensively due to its high cellulase production capability (3–12). However, the economic bioconversion of lignocellulosic biomass by cellulase is greatly hindered by the current high production cost of cellulose (3). Because the production of low-cost cellulase is a key step in the bioconversion of lignocellulosic biomass to ethanol, improvements in cellulase production have been investigated using cheaper substrates (4–7).

Rice straw is a major lignocellulosic residue produced worldwide, with an estimated availability of 685 million tons per year (13). The use of this waste for cellulase production could reduce the production cost and address the environmental rice straw disposal problem. Before its use as a substrate for cellulase production, rice straw is usually pretreated to make it easier to use by microorganisms (14,15). However, inhibitors, such as furfural and 5-hydroxymethylfurfural, are formed during the pretreatments (14,15), and much research has focused on how to alleviate inhibition problems (14–18).

Plant polyphenols are secondary metabolites. They can be divided into two categories (19). One is polyphenol monomers, including a variety of flavonoid compounds, chlorogenic acids, gallic acid and ellagic acid, and the other is tannin substances produced by the polymerization of monomers and oligomers or polymers, such as proanthocyanidins and gallotannins. In higher plants, these compounds are found in high concentrations and mainly consist of ferulic acid (FA), caffeic acid and coumaric acid (CA) (20). Crops and their straws contain a large amount of polyphenols. At present, research on the polyphenols in straws is mainly concentrated on hydroxy acid derivatives, such as FA and CA (21–23). Phenolic acids are known to exhibit antimicrobial activities against a variety of microorganisms, such as lactic acid bacteria (24) and yeast (25). Most of the phenolic acids, including FA and CA, are ether-linked to lignin and polysaccharides (26,27). The linked phenolic acids can be released with the aid of enzymes. Alrahmany et al. (28) reported that polysaccharide enzymes could increase the release of phenolic acids from oat bran. Compared with bran, straw has higher contents of cellulose and lignin. This feature makes it harder to release phenolic acids. However, cellulase and xylase are accumulated in the broth, and hydrolyze cellulose and hemicelluose into glucose and xylose, respectively, when straw is used to culture *T. reesei*. Breaking structures during *T. reesei* culturing makes it easier to release phenolic acids from rice straw. Thus, the

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release of phenolic acids during *T. reesei* culturing should be investigated to confirm that it influences cellulase production.

Unlike phenolic acids, tannins are rich in the outer layers of plants (29). Tannins are classified into hydrolysable and condensed. The condensed tannins form the most important group, accounting for over 95% of commercial tannin production (30). They are extracted, usually with hot water, mainly from the bark, stem or heartwood of the plants (31,32).

In this study, during *T. reesei* culturing, phenolic compounds, including phenolic acids and condensed tannins, were released from rice straw. However, the influence of phenolic compounds, particularly those derived from rice straw, on the behavior of *T. reesei* is not well understood. Thus, this study focused on whether the phenolic compounds in broths influenced cellulase production by *T. reesei* and on how to alleviate inhibitory effects. Since chemical pretreatments, such as acid and alkali pretreatments, form inhibitors that could influence the growth of microorganisms and efficiency of fermentation (33–35), ball milling, a kind of physical pretreatment, was chosen in this study. First, we investigated the effects of phenolic compounds on cellulase production with commercial cellulose. Second, we investigated the effects of the release of phenolic compounds from rice straw on cellulase production. Third, we proposed a process for the efficient production of cellulase. To the best of our knowledge, this is the first report on the effects of phenolic compounds from rice straw on cellulase production by *T. reesei*.

MATERIALS AND METHODS

Rice straw pretreatment Rice straw was obtained from fields on Chongming Island, Shanghai, China. The rice straw was thoroughly washed with tap water, and dried in an oven at 55 °C. After being ground to make a 1.0–0.5 mm particle size powder, the rice straw was milled at 400 rpm for 5–20 cycles at room temperature using a Desk-Top Planetary Ball Miller (SFM-1, Hefei, China). A cyclic mode of 10 min milling, followed by a 5 min pause, was used. The milling time indicated in this study refers to the actual milling time, excluding the pause time.

***T. reesei* cultivation** *T. reesei* (M305) was purchased from the China Center for Industrial Culture Collection (CICC). Seed cultures were prepared by shaking the inoculated media in 100-mL flasks in a rotary shaker at 200 rpm and 30 °C for 3 days. The medium for seed culture contained 1% peptone, 0.05% yeast extract, 1% corn steep liquor, 2.4% KH_2PO_4, 0.47% $C_4H_4O_6K_2$, 0.12% $MgSO_4 \cdot 7H_2O$, 0.5% $(NH_4)_2SO_4$, 2% Avicel PH-101 cellulose microcrystalline and 0.1% Tween 80 (36). The initial pH of the medium was 4.0. The components of cellulase production medium have been previously described (10), and they consisted of 10 g/L yeast extract, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4.7H_2O$, 8 g/L KH_2PO_4, 0.4 g/L $CaCl_2.2H_2O$, 0.0014 g/L $ZnSO_4.7H_2O$, 0.0016 g/L $MnSO_4.6H_2O$, 0.005 g/L $FeSO_4.7H_2O$ and 0.0002 g/L $CoCl_2$. Either 3% Avicel PH-101 cellulose microcrystalline or 7.5% rice straw was used as the carbon source. For cellulase production, 20 mL of the cellulase production medium in a 100 mL flask was inoculated with 1 mL seed culture broth. The culturing was carried out at 200 rpm at 30 °C for 8 days. The samples were taken at day 2, 4, 6, and 8, and were centrifuged at 7370 rcf for 10 min. The supernatants were used for the assay of cellulase activity. All of the fermentation studies were repeated 2–3 times, and average values are reported.

Enzyme activity measurement Cellulase activity, measured by a filter paper assay (FPase) and represented by filter paper units (FPUs). The activity of 1 mL of the diluted enzyme was determined at 50 °C for 60 min by the dinitrosalicylic acid method, according to the method recommended by the Commission of Biotechnology, IUPAC. One unit of FPase activity was defined as the amount of enzyme required to release a 1 μmol equivalent of reducing sugar per min. Absorbance measurements were taken at 540 nm to measure FPase activity.

Tannin measurement To extract tannins from rice straw, water and 70% methanol with 1% HCl, or 70% acetone with 1% HCl, was used as the solvent. The extraction conditions reported in literature were used (37,38), with some modifications. In this study, 10 g of the 15-cycle milled straw was placed in a 250-mL flask with 100 mL water, 70% methanol with 1% HCl or 70% acetone with 1% HCl. The extraction with water was carried out at 60 °C and 150 rpm for 1 h, while the extraction with methanol or acetone was carried out at 30 °C and 150 rpm for 1 h. The resulting supernatant was used to determine the tannins in rice straw. The resulting residue was washed with water. Then, the washed residue was used as the carbon source for cellulase production. No phenolic acids were detected in the supernatants. In addition, the resulting supernatant from the water-based extraction was used as a crude tannin solution for investigating the influence of rice straw-extracted tannins on cellulase production.

The quantification of proanthocyanidins in each fraction was carried out by the modified vanillin assay (39). A 2.5-mL aliquot of a 1:3 v/v sulfuric acid/methanol solution and 2.5 mL of a 1% (w/v) vanillin in methanol solution were mixed with 1 mL of the sample previously obtained by fractionation. The tubes were incubated at 30 °C for 4 h. The absorbance of each tube was measured at 500 nm. A blank was prepared by substituting the vanillin solution in the reaction mix with methanol. The absorbance of the blank was subtracted from the absorbance of the corresponding vanillin-containing sample, and the value was compared with a standard curve. Quantification was performed by means of a standard curve for catechin. The proanthocyanidin concentration was expressed as g catechin equivalents (CE)/L.

Phenolic acid measurement An HPLC system for the measurement of phenolic acids was equipped with a UV detector (EX1600; Exformma, Fairfield, OH, USA). The separation was performed at a flow rate of 1 mL/min at 35 °C with an Eclipse SDB C18 column (250 mm × 4.6 mm; Agilent, Wilmington, DE, USA). The mobile phase was eluted using a linear gradient system, consisting of solvent A (water:acetic acid, 99.5:0.5) and solvent B (methanol:water:acetic acid, 95:4.5:0.5). At 0–5 min, 5% B–5% B; 5–10 min, 5% B–25% B; 10–30 min, 25% B–40% B; 30–45 min, 40% B–50% B; 45–55 min, 50% B–100% B; 55–60 min, 100% B–100% B; and 60–65 min, 100% B–5% B. The injection volume was 10 μL. Phenolic acids were identified by comparing their relative retention times with those of standard compounds at 280 nm.

Sugar composition measurements The sugar composition of the rice straw was determined according to the analytical procedure recommended by the National Renewable Energy Laboratory (40). The materials were acid-hydrolyzed with 72% sulfuric acid at 30 °C for 1 h, followed by acid-hydrolysis with 4% sulfuric acid at 121 °C for 1 h. The sugar contents in the acid-hydrolysates were determined using the HPLC system described below.

The HPLC system for the measurement of the sugar concentrations was equipped with a refraction index detector (Exformma). The separation was performed at a flow rate of 1 mL/min at 80 °C with an Aminex HPX-87H column (7.8 mm × 30 cm; Bio-Rad, Hercules, CA, USA). A solution of water was used as the mobile phase.

Tolerance of phenolic compounds towards *T. reesei* After preculturing, 10 μL broth was transferred on to PDA plates containing phenolic acids or tannins. To investigate the tolerance of phenolic acids to *T. reesei*, commercial FA and CA were used. The concentrations of phenolic acids were set at 0.01 g/L FA + 0.01 g/L CA, 0.02 g/L FA + 0.02 g/L CA, 0.05 g/L FA + 0.05 g/L CA, 0.1 g/L FA + 0.1 g/L CA and 0.2 g/L FA + 0.2 g/L CA. To investigate the tolerance of tannins to *T. reesei*, the crude tannins extracted from 15-cycle milled straw were used. The concentrations of tannins were set at 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.3 g/L and 0.4 g/L. The plates were placed in an incubator at 30 °C for 48 h. The tolerance of *T. reesei* to the phenolic compounds was determined by observing the formation of hyphae.

Electrochemical measurements Electrochemical measurements were carried out on a model 660c Electrochemica Analyzer (CH Instruments USA). A conventional three-electrode system was employed for all of the electrochemical measurements, which consisted of a glass carbon electrode as the working electrode, a saturated calomel electrode as the reference electrode, and a platinum wire electrode as the counter electrode. Before being used, the glass carbon electrode was polished on silk with 0.05 μm alumina slurry, and then ultrasonicated in both ethanol and double-distilled water for 5 min to remove the unspecific adsorbents. We employed an electrochemical technique, cyclic voltammetry (CV) to detect our samples. The broths at 2nd, 4th, 6th and 8th day were centrifuged at 7370 rcf for 10 min. Then, the resulting supernatants diluted 1:4 with 10 mM PBS buffer (pH 7.4) were used to measure the electrolytes. All of the solutions were thoroughly deoxygenated by having high-purity nitrogen bubbled through them for at least 20 min before the measurements were taken. First, we determined whether the buffer influenced the sample determinations. Second, the glass carbon electrode was placed in the solution, and a potential scan from 0 to 0.8 V at a rate of 100 mV/s was employed for the CV experiments.

RESULTS AND DISCUSSION

Chemical composition of rice straw The chemical composition of rice straw was analyzed. The sugar composition of the rice straw was determined according to the analytical procedure recommended by the National Renewable Energy Laboratory (40). The free phenols were obtained from extraction with 0.5 M NaOH. The extracts were identified by HPLC using a UV absorption of 280 nm for the determination of phenolic acids. As shown in Table 1, glucose, xylose and arabinose were the major monosaccharides found in rice straw. In addition to the major monosaccharides, rice straw contained small amounts, 1.38% w/w, of polyphenols. Through the HPLC analysis, gallic acid, epigallocatechin gallate, phthalic acid, vanillic acid, syringic acid, vanillin, CA and FA were determined in rice straw treated with 0.5 M NaOH at 60 °C for 1 h. CA and FA were the predominant phenolic acids at 0.7% and 0.4% w/w, respectively.

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