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Effects of different cellulases on the release of phenolic acids from rice straw during saccharification

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HIGHLIGHTS

- Phenolic acids released in different forms from rice straw during saccharification.
- Synergistic interaction between FAE and cellulase increased free phenolic acids.
- Synergistic interaction affected ABTS and DPPH capacities of phenolic acids.
- Phenolic acids can be produced as value-added products from rice straw.
- Co-production technology is an attractive process for ethanol and phenolic acids.

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ABSTRACT

Effects of different cellulases on the release of phenolic acids from rice straw during saccharification were investigated in this study. All cellulases tested increased the contents of phenolic acids during saccharification. However, few free phenolic acids were detected, as they were present in conjugated form after saccharification when the cellulases from *Trichoderma reesei*, *Trichoderma viride* and *Aspergillus niger* were used. On the other hand, phenolic acids were present in free form when the *Acremonium cellulolyticus* cellulase was used. Assays of enzyme activity showed that, besides high cellulase activity, the *A. cellulolyticus* cellulase exhibited high feruloyl esterase (FAE) activity. A synergistic interaction between FAE and cellulase led to the increase in free phenolic acids, and thus an increase in antioxidative and antiradical activities of the phenolic acids. Moreover, a cost estimation demonstrated the feasibility of phenolic acids as value-added products to reduce the total production cost of ethanol.

1. Introduction

Lignocellulose, a component of plant cell walls, is one of the most abundant renewable biomass resources that serves as a substrate in the production of biofuels and chemicals (Hideno et al., 2011). In general, lignocellulose is composed of cellulose, hemicellulose, lignin and other carbohydrates (Ahmed et al., 2016). Rice straw is one of the most abundant lignocellulosic biomasses, and as such it has significant potential for the production of second generation biofuels (Abo-State et al., 2014; Binod et al., 2010; Wi et al., 2013). According to the Food and Agriculture Organization of the United Nations points system, 600–900 million tons of rice straw are produced each year globally (Devendra and Pandey, 2016). A major proportion of the rice straw remains unused and is destroyed by burning, which increases air pollution and consequently affects public health (Pu et al., 2013). Rice straw contains 32–47% cellulose, 19–27% hemicellulose and 5–24% lignin (Binod et al., 2010). Cellulose and hemicellulose, the main components of rice straw, can be converted to fermentable sugars by enzymatic hydrolysis (Jung et al., 2015). Therefore, rice straw is considered to be a preferred feedstock for fuel ethanol production due to its high content of cellulose and hemicellulose (Abo-State et al., 2014; Wi et al., 2013).

Cellulases are the key enzymes in biorefineries. They are comprised of three major enzyme classes: endoglucanases (CMCase, *endo*-1,4-β-D-glucanase, EG, E.C 3.2.1.4), which randomly attack β-linked bonds within cellulose molecules; cellobiohydrolases (*exo*-1,4-β-D-glucanase, CBH, EC 3.2.1.91), which produce cellobiose from the ends of the cellulose chain; and β-glucosidases (1,4-β-D-glucosidase, BGL, EC 3.2.1.21), which specifically convert cellobiose and cellooligosaccharides to glucose (Jung et al., 2015;

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Liao et al., 2015). These enzymes are produced by various fungi including *Trichoderma* and *Aspergillus* (Jung et al., 2015). *Trichoderma* sp. have been widely investigated for their high production of cellulase (Kawamori et al., 1986; Morikawa et al., 1985). However, most strains of *Trichoderma* sp. are known to have low β-glucosidase activity, which causes an accumulation of cellobiose (Juhász et al., 2005; Yamanobe et al., 1987). Although many efforts have been made to obtain *T. reesei* mutants by classical mutagenesis, such as RUT-C30, relatively low β-glucosidase activity remains one of the major obstacles to efficient cellulose hydrolysis (Zhang et al., 2012). *Aspergillus* sp. are prominent in producing xylanase (Shi et al., 2011). However, these fungi cannot secrete complete cellulolytic and hemicellulolytic enzymes (Dillon et al., 2006).

Acremonium cellulolyticus, which was first isolated in 1982 from soil in Japan (Yamanobe et al., 1987). The cellulase from *A. cellulolyticus* has been investigated for bioconversion of lignocellulosic biomass into biofuels (Hideno et al., 2011). *A. cellulolyticus* secretes many cellulolytic enzymes and has a high β-glucosidase activity. It was subsequently shown that the cellulase enzymes from *A. cellulolyticus* exhibit high activity in the saccharification of lignocellulosic biomass. Fujii et al., reported that cellulase derived from *A. cellulolyticus* produces glucose from lignocellulosic biomass more rapidly than that from *T. reese*i (Fujii et al., 2009). Gao et al., reported that a commercial enzyme from *A. cellulolyticus* exhibited high pectinase, α-amylase and cellulase activities (Gao et al., 2014). However, there have been no reports of feruloyl esterase (FAE, E.C 3.1.1.73) activity for this enzyme, which is involved in liberating phenolic acids such as ferulic acid (FA) and *p*-coumaric (*p*-CA) acid and their dimers from naturally occurring hemicelluloses and pectins (Mathew and Abraham, 2005).

Phenolic acids in rice straw exist in free acid, conjugated and insoluble-bound forms. *p*-CA and FA are the major hydroxycinnamic acids found in rice straw (Xu et al., 2005). They are valuable compounds with great potential use as antioxidants in the food and nutrition industries. The former is known to be esterified with lignin and the latter is extensively etherified with lignin (Abraham et al., 2016). In particular, FA is associated both with lignin by ether bonding and with hemicellulose by ester linkage in mono- or dimeric form (Xu et al., 2005). In this study, we found that during saccharification phenolic acids are released from rice straw in the free and conjugated forms with different antioxidant capacities. If phenolic acids could be produced as value-added products during saccharification, the cost of saccharification of rice straw would be reduced, leading to more feasible bioconversion of rice straw to useful materials such as ethanol. Therefore, the effects of different enzymes on the release of phenolic acids from rice straw during saccharification were investigated to find the enzymes that are key to this release. Then, the antioxidative and antiradical activities of the released phenolic acids were determined. Lastly, a cost estimation was performed to determine the feasibility of the production of phenolic acids during saccharification of rice straw to reduce the total cost of ethanol production. To the best of our knowledge, this is the first study on the production of phenolic acids from rice straw as added value products during saccharification.

2. Materials and methods

2.1. Materials

Rice straw was harvested from Chongming Island, Shanghai, China. It was ground with a grinder after air-drying, then passed through a 1-mm aperture standard screen. The coarse rice straw was kept at 60 °C and subjected to ball milling. It was milled at 400 rpm with a Desk-Top Planetary Ball Miller (SFM-1, Hefei, Anhui province, China) for 15 cycles at room temperature. The cycling mode was 10 min of milling, followed by a 5-min pause.

2.2. Enzymes and enzyme production

Four different cellulases were investigated in this study. Cellulase from *Acremonium cellulolyticus* was purchased from Meiji Seika Co. (Japan) cellulase from *Trichoderma reesei* ATCC 26921 (CAS: 9012-54-8) and cellulase from *Aspergillus niger* (starch as carrier) (CAS: 9012-54-8) were purchased from Aladdin (Shanghai, China). Cellulase from *Trichoderma viride* (CAS: 9012-54-8) was purchased from Sinopharm Chemical Reagent (Beijing, China).

Aspergillus niger (CICC 41125) was used to produce FAE. Stock cultures of *A. niger* were propagated on potato dextrose agar (PDA) plates at 30 °C for 7 days. For the seed culture, the conidia from sporulating cultures were suspended in 1.5 mL of sterile water, and 1 mL of the suspension (10^6 spores) was transferred to a 100-mL Erlenmeyer flask containing 20 mL of the seed culture medium (A), which was then incubated in an orbital shaker for 1 day at 30 °C and 200 rpm. A total volume of 1 mL of the mycelium suspension (obtained from the seed cultures) was used to initiate cell growth in 100-mL flasks, containing 20 mL of the growth medium (B). The flasks were incubated for 2 days, and the enzyme product was centrifuged at 8000 rpm for 5 min, then filtered through a 0.45-μm filter.

The seed culture and enzyme production medium were as follows: medium A (g/L): 20 glucose, 10 peptone, 0.5 yeast extract (YE), 10 corn steep liquor, 24 KH_2PO_4, 4.7 tartaric acid, 1.2 $MgSO_4{\cdot}7H_2O$, 5 $(NH_4)_2SO_4$, 1 Tween 80, pH 4 medium B (g/L): 30 destarched wheat bran (DSWB), 6 yeast extract, 1.2 $NaNO_3$, 3 KH_2PO_4, 6 K_2HPO_4, 0.2 $MgSO_4{\cdot}7H_2O$, 0.05 $CaCl_2{\cdot}2H_2O$, pH 5 (Gottschalk et al., 2010).

2.3. Enzyme activity and the protein content assay

2.3.1. Enzyme activity assay

The activities of filter paper cellulase (FPase) and endoglucanase (CMCase) were measured as described by Ghose (1987). The methods employed are recommended by the International Union of Pure and Applied Chemistry (IUPAC). The reducing sugars released were analyzed using the dinitrosalicylic acid (DNS) assay (Miller, 1959). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute. FPase activity was represented in filter paper units (FPUs) while CMCase activity was expressed as units of enzyme activity. The activity of β-glucosidase measured as described by Jiajun Hu (Hu et al., 2016).

Beechwood xylan was used as the substrate to measure xylanase activity. Each reaction mixture containing 0.5 mL of 1% xylan (in 50 mM acetic acid buffer, pH 5) and 0.5 mL of enzyme solution was incubated at 50 °C for 30 min. The reducing sugars released were analyzed using the DNS assay (Miller, 1959). One unit of xylanase activity was defined as the amount of enzyme required to produce 1 μmol of xylose per minute (Zhang and Sang, 2015).

FAE activity was assayed by measuring the release of FA in a reaction mixture containing 10 μL of the enzyme solution, 20 μL of 1% ethyl ferulate in dimethylsulfoxide (DMSO), 200 μL of 0.5 M acetate buffer (pH 5) and 770 μL of sterile water. After incubation at 50 °C for 20 min, the reaction was terminated by boiling the mixture for 5 min, followed by the quantification of FA by HPLC. One unit of FAE corresponded to the formation of 1 μmol of FA per minute (Gottschalk et al., 2010).